SYNERGIE SKIN
CLEAN SCIENCE

CLINICAL DATA – ACETYL TETRAPEPTIDE-2

Sagginess inducers:
It is a fact that skin changes when growing older, as a result of a lifetime of exposure to environmental agents, personal habits such as smoking and diet, and modifications that happen in the normal process of cellular ageing. The skin starts appearing less smooth and tight that before, which can lead to visible sagginess, lines and wrinkles among other evidence.

Several external factors are known to exacerbate the natural ageing process like UV exposure or extreme temperatures, but gravity plays a particularly relevant role. As it is a constant force pulling downwards, the skin needs to be firm and elastic to counteract its pressure and stay in place.

Although this attraction force cannot be avoided, the elements that provide skin firmness and elasticity can certainly be enhanced. This is the case with elastin and collagen, both key proteins of the dermal Extracellular Matrix (ECM) that provide the foundation for skin, working in tandem. Collagen cushions and supports the epidermis while elastin allows skin to stretch and flex smoothly. Thus, they confer cohesion and elasticity to the skin, as well as the capacity to recoil after common facial movements like smiling, laughing, drinking and crying. Additionally, several intracellular elements help to reinforce these skin properties and oppose flaccidity.

It is agreed that the functionally active form of both such proteins is reduced with increasing age as well as their correct assembly, diminishing the skins capacity to deal with deforming agents and avoid their visible consequences in facial look [1].

Stimulating the adequate mechanisms that strengthen skin cohesion and tightness would reduce sagginess induced by gravity for instance.

Skin firmness when ageing
Like in other organs of the body, the physiological functions and structures within the skin continuously decline with advancing age. Skin ageing results from the deterioration of its structures and the slowing of its functions, caused by many factors and origins, which may be included into different categories: intrinsic, mechanical and extrinsic ageing.

Biological or intrinsic ageing is often the result of genetically-determined changes that occur naturally within the body from the mid-20's onwards, despite their later evident effects. The chronological age or biological clock determined by genetics also applies to the skin, which gradually loses its ability to function as it once did. This deterioration occurs due to a gradual shift in the balance of certain messenger molecules excreted within the body that lead to natural changes manifesting in outward signs of ageing.

Understood as the consequence of continually repeated muscle movements like smiling or frowning, mechanical ageing also contributes to skin deterioration day by day, exacerbating expression lines.

Environmental or extrinsic ageing takes place due to the daily exposure to external sources in the environment: ultraviolet rays, pollution, smoke, harsh weather, gravity and external stress. These agents limit the ability of cells to function properly and alter the integrity of overall cell composition. Years of accumulated environmental stress on skin cellular structures translates into premature ageing.

UV damage from sun exposure accounts for 90% of premature skin ageing. The damage to skin components caused by both prolonged and incidental sun exposure is called photo-ageing, which is a process that occurs over a period of years (the effects are cumulative) and can lead the skin to lose its repairing ability.

All ageing types cause alterations in the skin which include a slower cellular turnover, reduced collagen production, elastin disturbances and skin thinning [1-2]. Additionally, UV radiation disturbs melanocytes and moisture barrier, and accelerates collagen and elastin loss as well as their fibres breakdown in the skin [1-2]. The alteration of collagen and elastin is controlled by the activity of Matrix Metalloproteinase (MMP) enzymes known as collagenase and elastase, respectively, both of them being activated by UV radiation. Long term elevation of the MMPs results in disorganised and clumped collagen and elastin that is characteristic of photo-damaged skin.

Thus, there are differences between intrinsic and extrinsic ageing effects on the skin, and obviously old versus young skin. If we look at intrinsic or chronological aged skin without environmental influences, it is smooth, generally unblemished and with some exaggerated expression lines, but the skin is well preserved in general, despite an inner flattening of the epidermal-dermal interface and some disruption of the dermal tissues [2].

In direct contrast, extrinsically-aged skin (mainly the face, hands and chest) presents wrinkles, hyper/ hypopigmentation, sallow areas, increased fragility, roughness and a loss of tonicity and elasticity, due to a more fragmented and thick collagen and elastin [2-3].

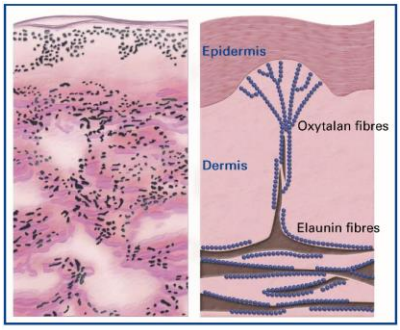


Fig. 1. Elastin presence in young skin.

Elastin fibres are present in adult skin in various stages of maturity, forming a distinctive arrangement within the papillary (outer) and reticular (inner) dermis [2, 4]. The

least mature fibres course perpendicularly from the dermal-epidermal junction to the top of the reticular dermis (oxytalan fibres), whereas more mature fibres containing added deposited elastin are horizontally arranged (elaunin fibres) [2, 4-5]. Both elastin fibres are connected as oxytalan merge from elaunin fibres [2, 4-5]. The most mature are found deeper in the reticular dermis [4].

All ageing types alter elastin fibres, but in different ways. Biologically, our body naturally diminishes elastin production within fibroblasts as we age, so fewer fibres are created and the skin loses resilience. Environmentally, UV rays can penetrate skin layers to damage elastin-producing fibroblasts. Also, as skin cell renewal decreases, the skin thins becoming more susceptible to environmental damage [1]. Finally, mechanical stress can permanently stretch out elastin fibres.

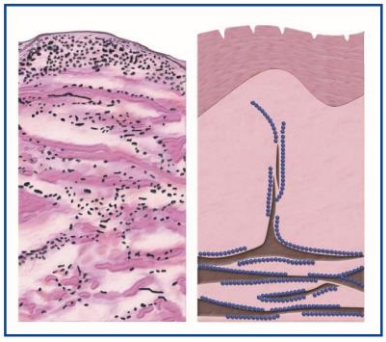


Fig. 2. Elastin in photoprotected aged skin (fewer elastin fibres).

Changes in elastin fibres are so distinctive in photo-aged skin that the condition known as elastosis is considered one of its hallmarks [1, 4]. This is characterised by an accumulation of amorphous elastin protein and a breakdown in the typical structural layout, which resists in decreased skin elasticity and tensile strength. This phenomenon accounts for why more photo-exposed skin takes longer to assume its original position when extended or pulled.

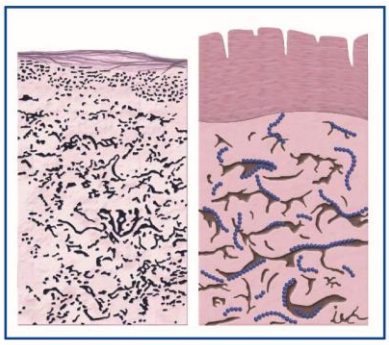


Fig. 3. Elastin in photoexposed aged skin (thicker and disorganised elastin fibres).

Ageing alters skin structure and inner components like elastin and collagen, which need to be enhanced to reduce skin flaccidity.

Key elements combating skin flaccidity

In order to protect skin from external and internal agents that reduce its capacity to stay firm, it is first important to know the current mechanisms and elements that manage to do so, preserving skin elasticity and firmness until passing years reduce this intrinsic capacity. Although elastin and collagen are the most well-known proteins that provide firmness, other elements located in the ECM and inside skin cells that contribute to such important task. All together, they capacitate the skin to be firm and elastic at the same time, avoiding sagginess.

Elastin fibres

With an estimated molecular mass of 64-66kDa, elastin is a protein found in any elastic connective tissue and, in the skin, it is mainly located in the dermis, being responsible for cutaneous critical properties [2, 5-7]. The unique gene responsible for elastin production is mainly expressed before birth and in the first years of life, but it is substantially turned down with the passing years, so most of elastin found in adults comes from this initial production [6, 8]. Although elastin has proved to be the longest lasting protein in the body with a half-life of 70-74 years approximately, its slower adult

production may lead to a non-complete repair when the passing years damage it, a fact that implies a reduction of skin elasticity [2, 6-7].

Elastin and microfibrils are the two components of the elastic fibres, which represent the largest structure of the ECM [2, 5, 8-9]. The major and core component is elastin, which is formed in the process of elastogenesis through the assembly of cross-linking of its precursor protein known as tropoelastin [2, 6-8]. In fibroblasts, the expression of elastin gene results in the intra-cellular formation of soluble tropoelastin monomers of 60-70kDa, that may be different in length due to undergoing alternative splicing, and their secretion to the cell surface [2, 6-9].

Mature tropoelastin monomers are able to self-assemble and aggregate by coacervation, which implies tropoelastin being more concentrated and aligned for subsequent cross-linking [2, 6-9]. Coacervated tropoelastin (microassembly) is then deposited onto long linear microfibrils (10-15nm) of the ECM (macroassembly), which serve as a scaffold to guide cross-linking [2, 6-9]. In tissues, microfibrils form packed parallel bundles close to cell surface and their main structural elements are fibrillins, which are large glycoproteins (by 350kDa) that form their backbone [2, 5-7]. Being a product of fibroblasts and keratinocytes, fibrillin-1 appears as the principal components these microfibrils in adults [2, 6-8].

It is agreed that specific elements are needed for this last step of the elastin fibre formation to occur properly, fibulin 5 (FBLN5/DANCE) and lysyl oxidase-like 1 (LOXL1) being key players for a proper assembly.

FBLN5 and LOXL1 role

Fibulins are a family of ECM glycoproteins between 50-200 kDa that are associated with the stabilisation of structures like elastic fibres, binding to tropoelastin with different affinities [8, 10-12]. FBLN5 (66kDa) is one of the five members of this family and it is thought to be essential in elastin fibre organisation as it is colocalised with such fibres, its overexpression increases their assembly and its decrease and absence causes their defective development and disorganisation, as it happens when ageing [8, 10-17]. This glycoprotein is recognised as a bridge molecule because it binds not only to tropoelastin, but also to LOXL1, fibrillins-1 and integrins, all of them necessary components for the adequate assembly of elastin fibres [5, 8, 10, 12-13].

LOXL1 is one of the members of the lysyl oxidase family (LOX), which compromises LOX and LOX-like proteins from 1-4 [5, 8, 10, 12-13]. Secreted to the ECM, this enzyme catalyses the formation of covalent cross-links between two adjacent tropoelastin molecules, ensuring spatially defined deposition of elastin and originating the insoluble elastin polymer [5, 8, 10, 12-13, 16, 18-19]. Thus, it is found in site of elastogenesis, which FBLN5 could be responsible for its binding and activation as well [8, 16, 19]. As it occurs with FBLN5, LOXL1 levels decrease with advanced age [18].

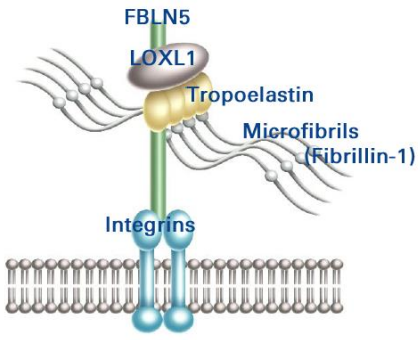


Fig. 4. Mature elastin fibre formation.

Apart from binding to FBLN5, fibrillins-1 from the microfibrils also binds to integrins which are transmembrane receptors that externally bind to the ECM and internally to the contractile cytoskeleton [8]. Therefore, the complex forming the mature elastin fibres get linked to cells due FBLN5 and to fibrillins-1 as both elements connect with this family of receptors, which in turn join other important elements for skin firmness like type I, IV and VI collagen [20-22].

Collagen cohesive function

When the term collagen appears, it is usually applied to type I collagen, which is the most abundant protein in the ECM and in the human body. Actually, this type is the principal collagen in the skin, first found as procollagen before it is cleaved and assembled into collagen fibril polymers first and then aggregated into larger bundles of collagen fibres. It offers the major platform for cell attachment and anchorage to macromolecules, providing cutaneous structural support.

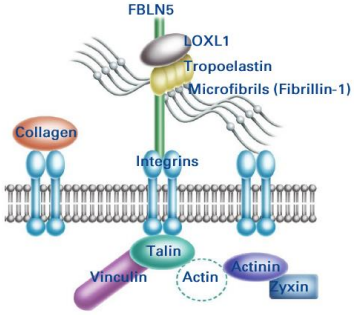
Conversely, type IV collagen is a specific non-fibrillar type found in the basement membrane, which serve as structural barrier and substrate for cellular interactions [23]. Additionally, type IV collagen is able to join type I and IV collagen, among other compounds and form supramolecular networks that bind to collagen fibrils and elastic fibres as well, providing cohesion to the fibrillar components of the dermis and influencing cellular adhesion and migration, all of them fundamental for the integrity and function of membrane basements under mechanical demands [20, 22, 24-26].

Furthermore, it seems that type IV collagen is assembled into microfibrils distributed in elastic and non-elastic tissues, where the microfibrils function as essential structural elements [20, 23].

Type XIV collagen is localised near the surface of collagen fibrils, regulating the fibrillogenesis process in sites with high mechanical demand, like the skin, its alteration leading to skin laxity and flaccidity [20, 22-24, 27-30]. Moreover, it is thought that this collagen type is associated with type I collagen fibrils and also with cellular adhesion mechanisms [3].

Focal adhesions

Cellular adhesion to the ECM can occur due to cell surface integrins that get to link intra- and extra-cellular components via multiprotein complexes, called Focal Adhesions (FAs). This bond needs the coordinated binding of integrins receptors to adhesive domains in ECM ligands but also FAs assembly and their interactions with the cytoskeleton of actin (a key protein in cellular movement, contraction and shape maintenance) [31]. Thus, FAs act as an interface between the actin cytoskeleton and the ECM compounds [32].



Upon cellular adhesion, one of the numerous structural elements of the FAs, known as talin, rapidly accumulates in focal contacts and is able to directly bind to integrins [33].

Talin is a high-molecular weight protein with binding sites for actin but also for vinculin, another protein that stabilises cell-cell and cell-matrix junctions [33]. Tensile and mechanical forces acting on talin activate its union to vinculin, enhance FA assembly and increase the strength of the linkage between integrins and the actin cytoskeleton [30-31, 33]. Mechanical forces also induce the recruitment of zyxin, a protein that facilitates actin filament assembly and may be involved in adhesion-stimulated changes in gene expression and the cytoskeletal organisation of actin bundles, which translates into reinforcement of the final binding function of the FAs [31]. Actinin is a necessary protein as it links the actin filaments to zyxin associating these filaments to the membrane.

FBLN5 and LOXL1 are key components to maintain elastin fibres properly assembled and functioning. Together with collagen molecules and elements of the FAs like talin and zyxin, they are responsible for the firmness and resistance of the skin.

Acetyl tetrapeptide-2, counteracting the force of gravity

Acetyl tetrapeptide-2 is a peptide designed to fight the undesired effects of a lack of skin firmness and cohesion by enhancing the natural elements that help to maintain collagen levels and elastin fibres correctly assembled, and facilitating the union between cells and the ECM.

This ingredient not only serves to activate the FBLN5 and LOXL1 promoters but also increased both protein levels *in vitro*, that as we previously mentioned are necessary elements for a correct assembly of elastin fibres. Moreover, it highly induced the synthesis of elastin.

It also showed to upregulate genes related with FAs and collagen synthesis, highly inducing the synthesis of type I collagen as well.

In vivo, the peptide showed to clearly reduce specific parameters linked to skin flaccidity and dermal disorganisation, which translates into a better inner restructure of the dermis and skin cohesion.

Acetyl tetrapeptide-2 helps the skin to remain firm and fight the effects of external and internal agents that damage its cohesion and key firming elements, like elastin and collagen.

***In vitro* efficacy**

Activation of FBLN5 and LOXL1 Promoters

This study wanted to confirm the activation of the human FBLN5 and LOXL1 promoters on a double-transfected stable human epithelial FBLN5/LOXL1-reported cell line expressing Firefly and Renilla Luciferase genes upon the activation of FBLN5 and LOXL1 promoters respectively, induced by Acetyl tetrapeptide-2.

Transfected cells were seeded for luciferase activity detection and, after 24h, they were washed and incubated with medium for 6h. Then, they were treated with IL-1β (10ng/mL, positive control) or Acetyl tetrapeptide-2 (0.1 or 1.0 mg/mL), and incubated for 16-24h. Cells cultured with just medium were used as the negative control (basal).

At the end of the treatment, Firefly and Renilla luciferase substrates were added to quantify the reactions with their respective luciferases using a multiplate luminometer (relative light units per second).

In parallel, a different set of plates with transfected cells were washed and stained with crystal violet to quantify number of cells per well and normalise luciferase units.

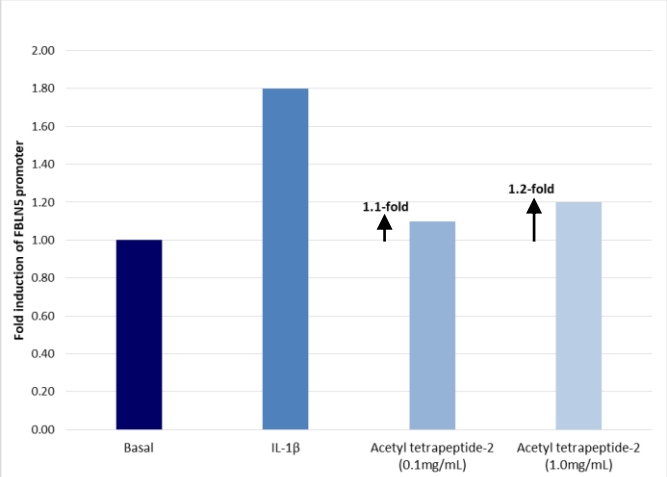


Fig. 5. Fold induction of FBLN5 promoter in transfected cells after the active treatment (*p<0.05, **p<0.01).

Results showed that the Tetrapeptide increased the activity of FBLN5 promoter up to 1.2 times compared to cells only cultured with medium.

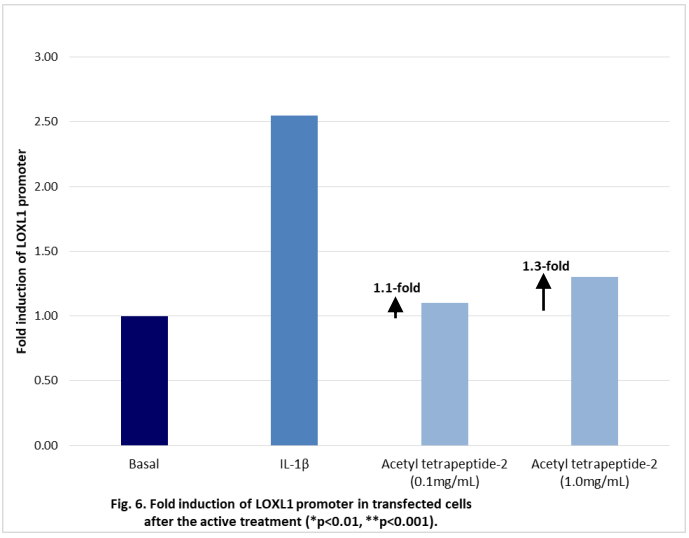


Fig. 6. Fold induction of LOXL1 promoter in transfected cells after the active treatment (*p<0.01, **p<0.001).

As the obtained values confirmed, the active ingredient raised the activity of the LOXL1 promoter up to 1.3 times versus the cells only cultured with medium.

Increase of FBLN5 and LOXL1 protein levels

The aim of this assay was to evaluate the efficacy of Acetyl tetrapeptide-2 as an elastic fibre inducer due to its action of FBLN5 and LOXL1 proteins through and immunocytochemistry assay (with fluorescent antibodies) in Human Dermal Fibroblasts (HDFa).

After the HDFa were seeded into well plates with supplemented medium for 72h, cells were incubated for 48h with medium alone (basal control), with TGF-β1 (5ng/mL, positive control), with IL-1β (20ng/mL, positive control) or with Acetyl tetrapeptide-2 (0.5mg/mL).

After this period, an immunofluorescence staining protocol was followed to detect FBLN5 and LOXL1 proteins, consisting in washing, fixing and finally staining cells. From each taken image of these proteins values of Integrated Optical Density (IOD) were quantified and normalised.

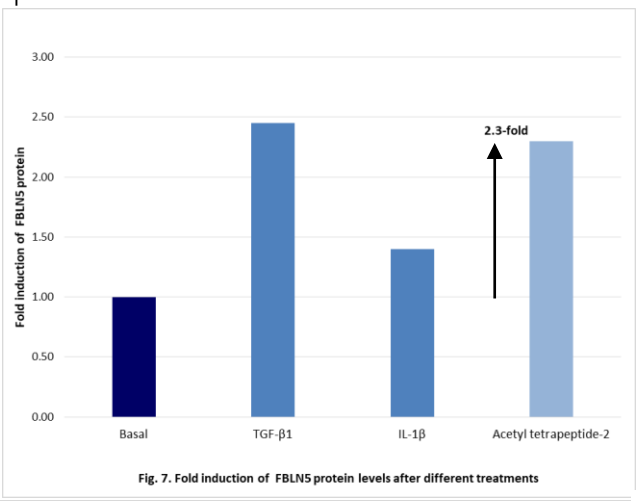
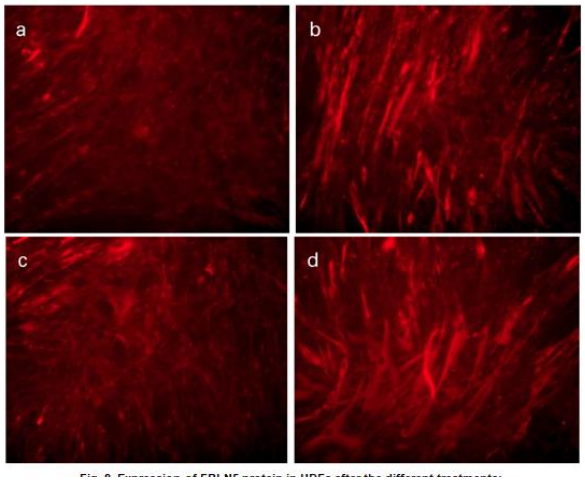


Fig. 7. Fold induction of FBLN5 protein levels after different treatments



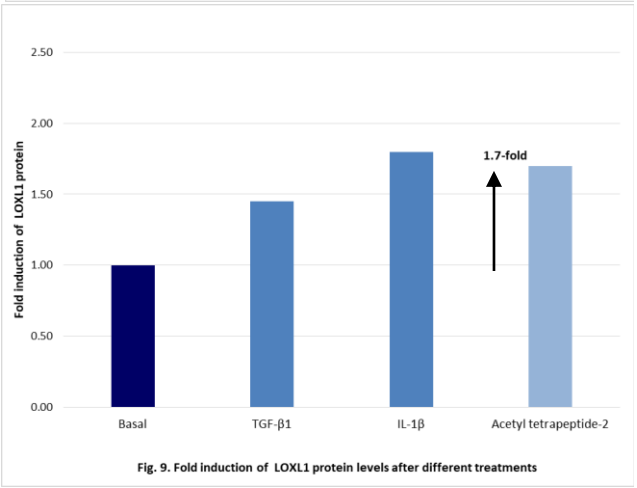


Fig. 9. Fold induction of LOXL1 protein levels after different treatments

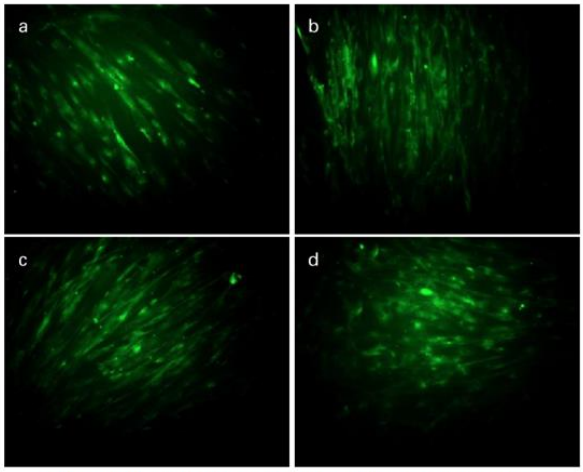


Fig. 10. Expression of LOXL1 protein in HDFa after the different treatments: a) Basal, b) TGF-β1, c) IL-1β, d) Acetyl tetrapeptide-2

As the images demonstrated, the Tetrapeptide augmented the expression of LOXL1 protein by 1.7-fold versus the cells only cultured with medium.

Microarray analysis

This assay was developed to detect the genes that were upregulated in HDFa in presence of Acetyl tetrapeptide-2 using an ASurePrint G3 Human Gene Microarray v2.

HDFa were seeded and after 7 days cells were ready to be incubated with Acetyl tetrapeptide-2 (0.05mg/mL) in supplemented medium for 24h. Afterwards, cells were lysed directly in the cell-culture flasks following the kits protocol. Then, RNA samples were obtained and their quality was verified before microarray processing.

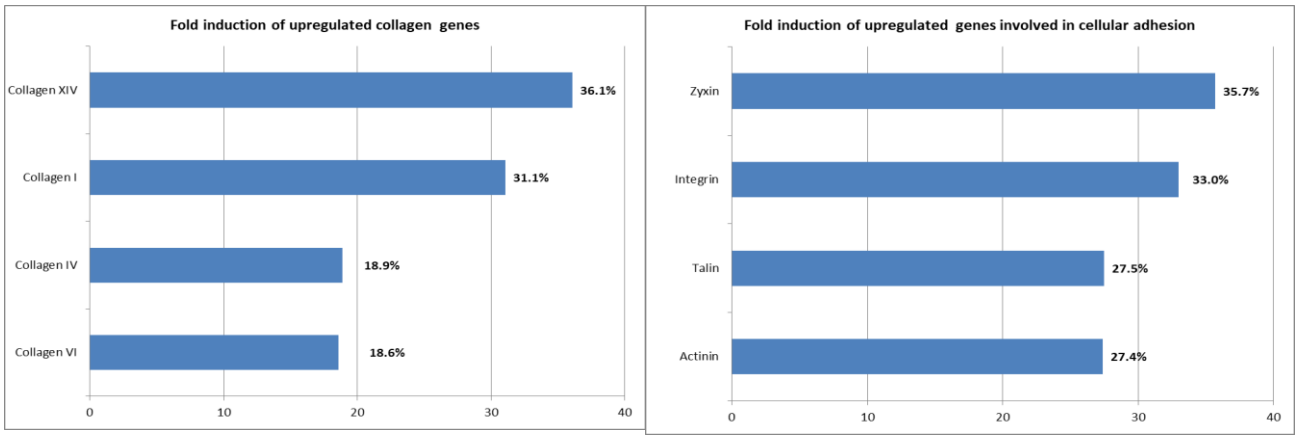


Fig.11. Percentage of fold induction of significantly overexpressed Collagen genes and genes involved in cellular adhesion.

After analysing and normalising the arrays, microarray data were used to obtain the genes were different and more altered expression.

Acetyl tetrapeptide-2 upregulates the expression of genes involved in collagen synthesis and focal adhesions, all of them implied in skin firmness.

Elastin induction

The aim of this study was to analyse the effect of Acetyl tetrapeptide-2 on the induction of elastin synthesis on HDFa by the Fastin Elastin Assay, a quantitative dye-binding method for the analysis of soluble elastin based on the release of this bond-specific dye upon treatment with a destaining reagent and measurement of its absorbance.

HDFa were grown until confluence in medium with specific growth factors. After cells were seeded into well plates and incubated for 72h, fresh medium containing TGF-1β (10 ng/mL, positive control) or Acetyl tetrapeptide-2 (0.1 mg/mL) was added and plates were incubated for 48h more. Non-treated cells were used as negative control. Afterwards, elastin was extracted from cells.

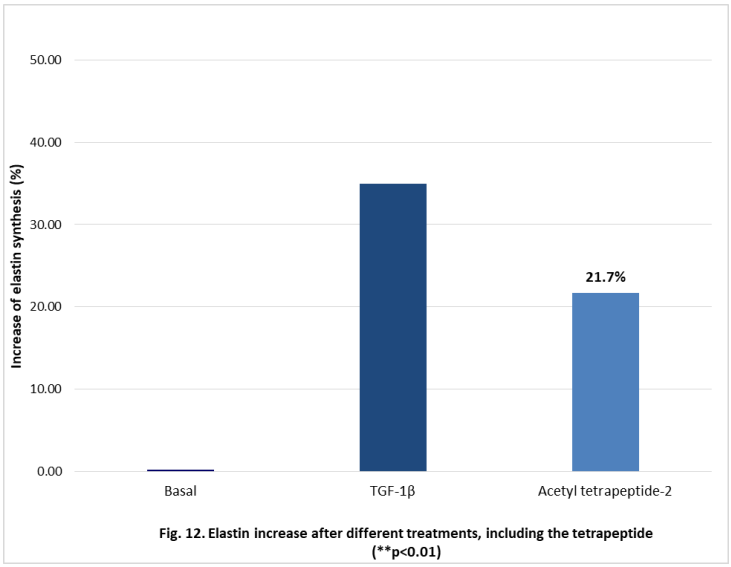


Fig. 12. Elastin increase after different treatments, including the tetrapeptide (p<0.01)**

Absorbance was read at 540nm in a microtiter plate ready, determining elastin concentration using a linear regression of the elastin standard curve.

The active peptide increased elastin synthesis by 21.7% compared to non-treated cells.

TYPE I Collagen induction

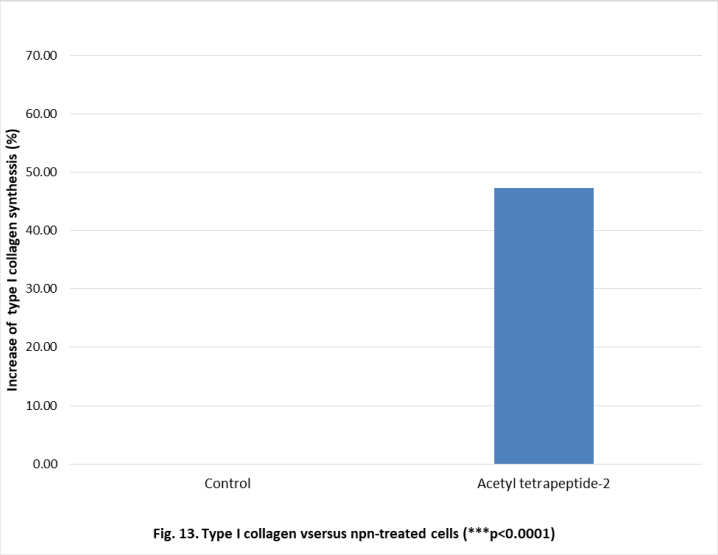


Fig. 13. Type I collagen vsersus npn-treated cells (***p<0.0001)

To analysis the effect of Acetyl tetrapeptide-2 on the induction of type I collagen synthesis, an Enzyme-Linked Immunosorbent Assay (ELISA) was carried out on HDFa.

HDFa were grown until confluence in medium with specific growth factors. After cells were seeded into well plates and incubated for 24h, fresh medium containing Acetyl tetrapeptide-2 (0.01µg/mL) was added and plates were incubated for 48h more. Non-treated cells were used as control. Then, well medium was collected and 50µL was analysed by an ELISA.

Absorbance values were read at 490 nm in a microtiter plate reader and collagen concentrations were determined using a linear regression of type I collagen standard curve.

The ingredient showed to raise type I collagen synthesis by 47.3% versus non-treated cells.

***In vivo* efficacy**

Increase of firmness

The objective was to examine the efficacy of Acetyl tetrapeptide-2 in ameliorating skin firmness by ballistometry. For this purpose, 19 female volunteers between 50-60 years old with a stable weight and presenting saggy facial skin applied an emulsion containing 2% Acetyl tetrapeptide-2 solution on the face twice a day for 55 days.

The principle of ballistometry is based on the use of an impacting mass (ballistometer hammer) on the skin surface to measure the mechanical properties of the skin through their interactions. Thus, a vibrational movement is imposed on the skin and the rebounds are transduced into quantifiable electrical signals.

Two parameters were selected: the indentation (mm), which is the peak penetration depth of the probe tip beneath the skin on its first impact, and the area between the bounce profile and skin zero datum (mm^2). The higher the indentation or area value, the higher the skin flaccidity.

Measurements were taken from the cheek at the initial time and after 55 days in order to compare the efficacy of the active emulsion versus the initial time.

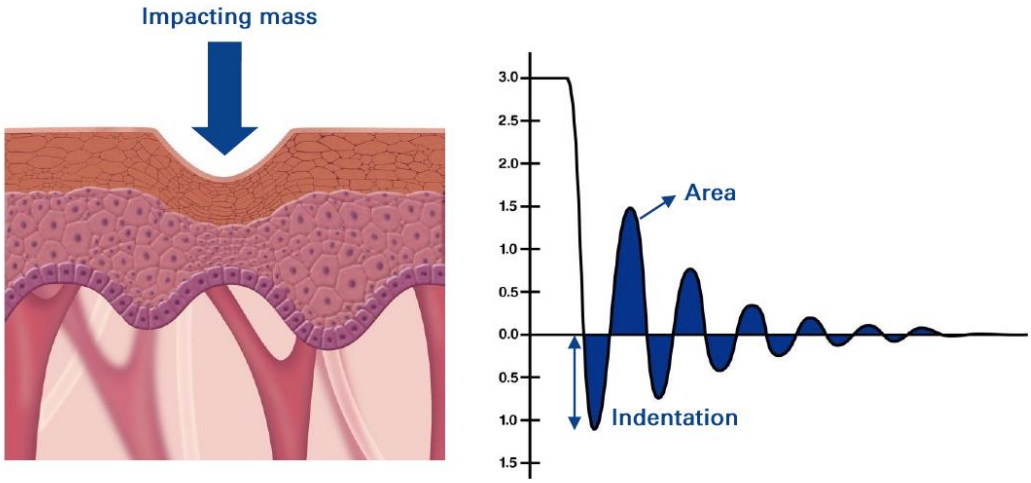


Fig. 14. The indentation and area parameters considering the direct effect of an impacting mass and the induced rebounds respectively.

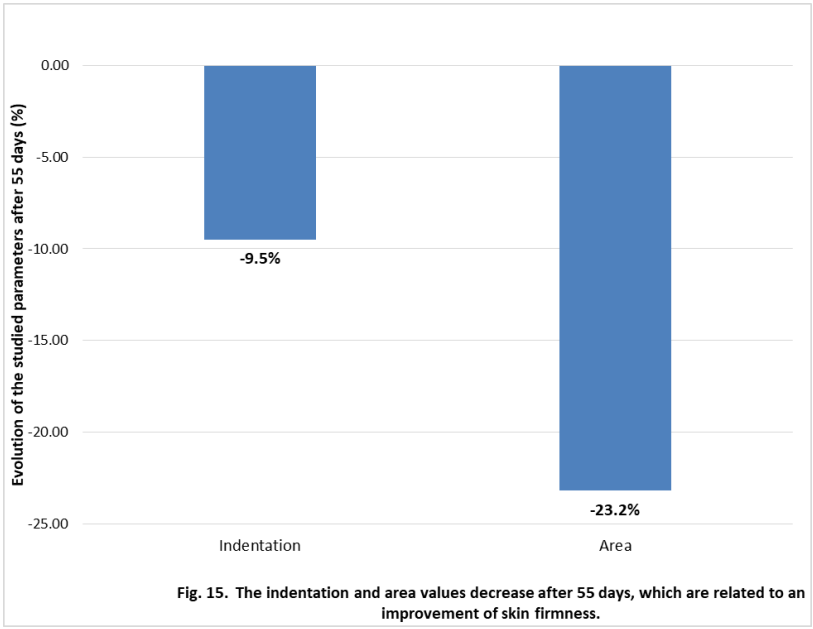


Fig. 15. The indentation and area values decrease after 55 days, which are related to an improvement of skin firmness.

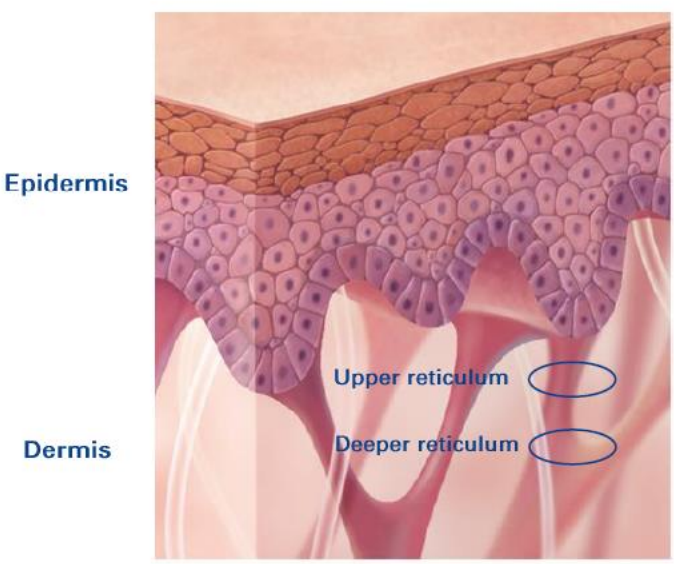
The ingredient offered a statistically significant diminution ($p \leq 0.05$) of the indentation and the area (-9.5% and -23.2% respectively), characteristic of an amelioration of skin firmness at the end of the treatment versus the initial time.

Restructuration of the dermis

In order to observe the efficacy of Acetyl tetrapeptide-2 in the dermal organisation, 19 female volunteers (50-60 years old) with a stable weight and saggy skin applied a placebo emulsion on half of the face and an emulsion containing 2% Acetyl tetrapeptide-2 solution on the other half, twice a day for 55 days. The *in vivo* confocal microscopy was used to quantify the tissular structure of the superficial reticular dermis at two levels, facilitating the measurement of the fragmentation rates at different depths.

Two stacks of each check were acquired at the beginning and at the end of the treatment. Two images were selected of each stack: one from the most superficial part of the reticular dermis and another on from 18µm deeper. A region was defined in each image and two parameters were studied: the fragmentation rate of the upper reticulum and the deeper reticulum. The decreased of these parameters demonstrates an improvement of the reticular dermis.

A VivaScope was used to carry out the acquisitions and specific software to analyse the digital images, permitting to characterise and quantify the fibres network. The more and small "objects" (in red) represent a high fragmentation of such fibres.



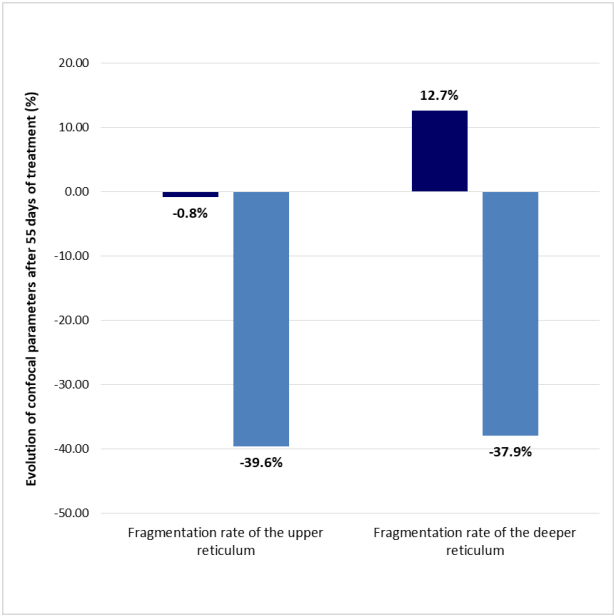


Fig. 16. Changes in the studied parameters comparing their values before and after the different treatments.

The active treatment caused a statistically significant reduction of both parameters (-39.6% and -37.9%). Additionally, the difference between the effect of the placebo and the active treatment was also found to be statistically significant for both parameters ($p \leq 0.05$).

Acetyl tetrapeptide-2 restructured the dermis at the studied depths, improving skin inner cohesion.

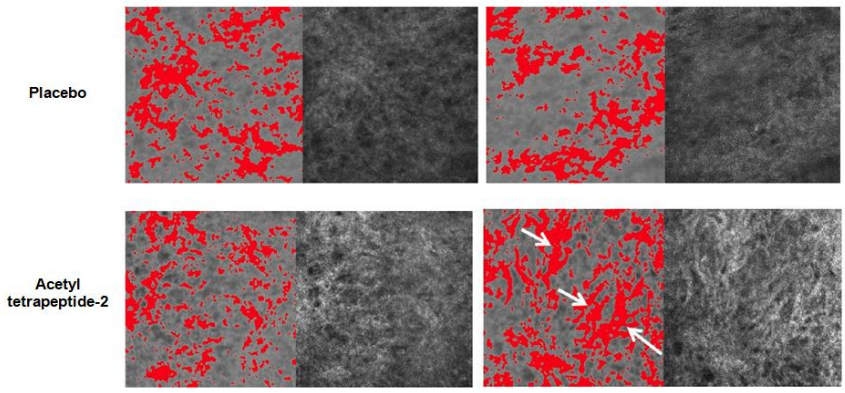


Fig. 17. Processed and non-processed images (from the upper reticulum) of a volunteer before (2 left images) and after the treatments (2 right images), highlighting the fibres network.

The images of the actively treated areas showed larger red objects at the end of the treatment, which implied minor fragmentation rate compared to the initial time but also to the placebo treatment.

Acetyl tetrapeptide-2 reduced the fragmentation of the fibres network.

Age correlation

On the basis of the results obtained above, the correlation between the fragmentation rate of the reticular dermis and the equivalent age of the subject's skin was studied. This calculation was made on a linear statistical correlation between the real age of the volunteers and the level of their fragmentation rate. The estimated difference in age after 55 days was calculated for the placebo and for the 2% Acetyl tetrapeptide-2 solution treatment.

Acetyl tetrapeptide-2 rejuvenated the estimated age of the skin by almost 9 years.

Tensor efficacy

The firming and anti-sagging efficacy of Acetyl tetrapeptide-2 was evaluated with a group of 40 female volunteers (40-60 years old) with apparent signs of sagging skin and loss of elasticity on the face. The panel was divided into two different groups and, using a randomisation scheme, the concentration of Acetyl tetrapeptide-2 solution to be used by each group was stated. The first one applied a cream with 1% Acetyl tetrapeptide-2 solution, while the second group used 2% Acetyl tetrapeptide-2 solution, twice a day for 56 days.

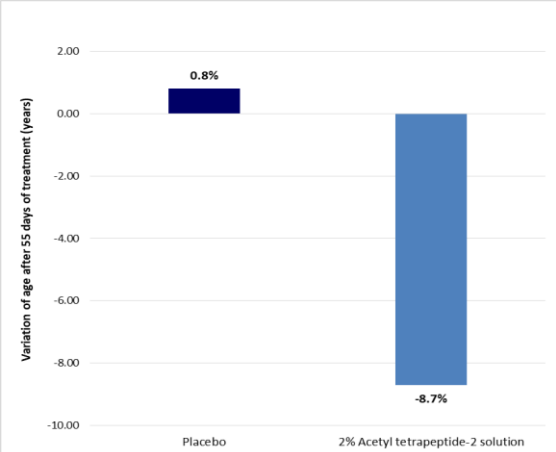


Fig. 18. Effect of the active ingredient on the skin age of the subjects.

The elasticity of the skin was measured by means of a cutometer and the parameters were evaluated were R0 and R2, corresponding to the maximal deformation and overall elasticity of the skin respectively.

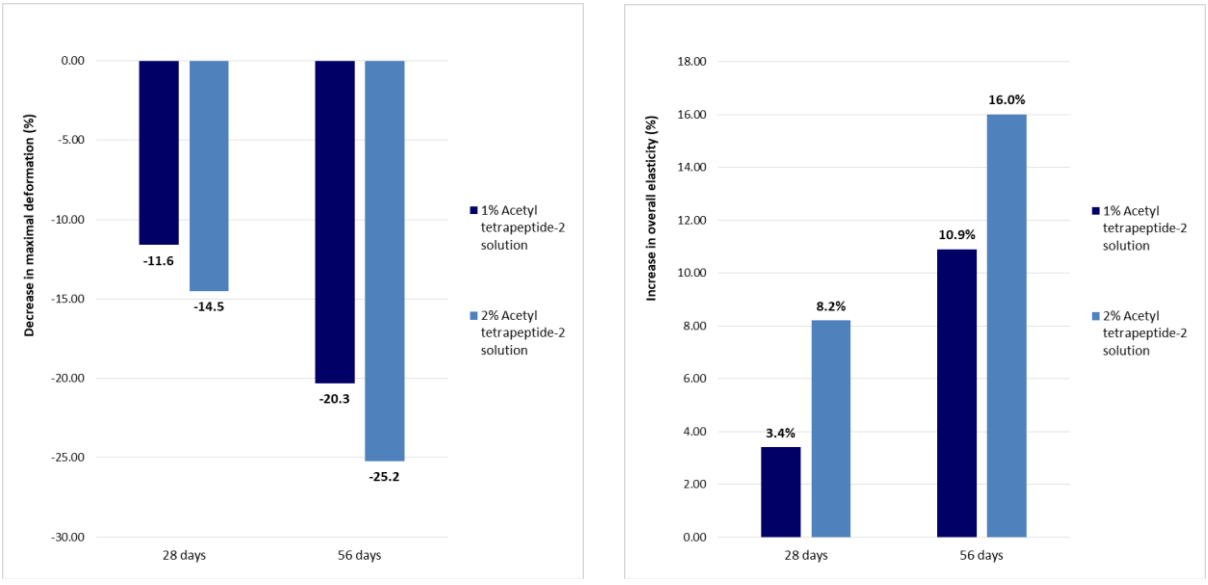


Fig. 19. Variations of the R0 and R2 parameters comparing two different concentrations of the active ingredient at two different times.

The decrease in the maximal deformation and the increase in the overall elasticity corroborate the firming efficacy of the active ingredient.

The treatment at 1% showed a decrease in the maximal deformation of 20.3% and an increase in the overall elasticity of 10.9% after 56 days. On the other hand, the treatment with 2% Acetyl tetrapeptide-2 solution presented a reduction in the maximal deformation of 25.2% and an improvement in the overall elasticity of 16.0% after 56 days.

Acetyl tetrapeptide-2 improves skin firmness and enhances the general elasticity of the skin.

In order to evaluate the tensor and reshaping effect, morphometric image analysis on frontal digital pictures of the volunteers were used. This effect was measured by taking three different positions of the face into account.

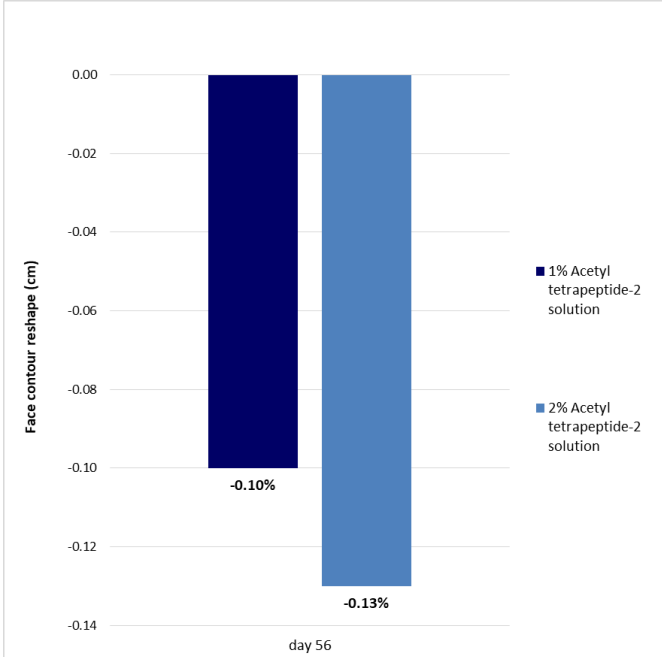


Fig. 20. Comparison of the reshape in the face contour among two different concentrations of the active ingredient.

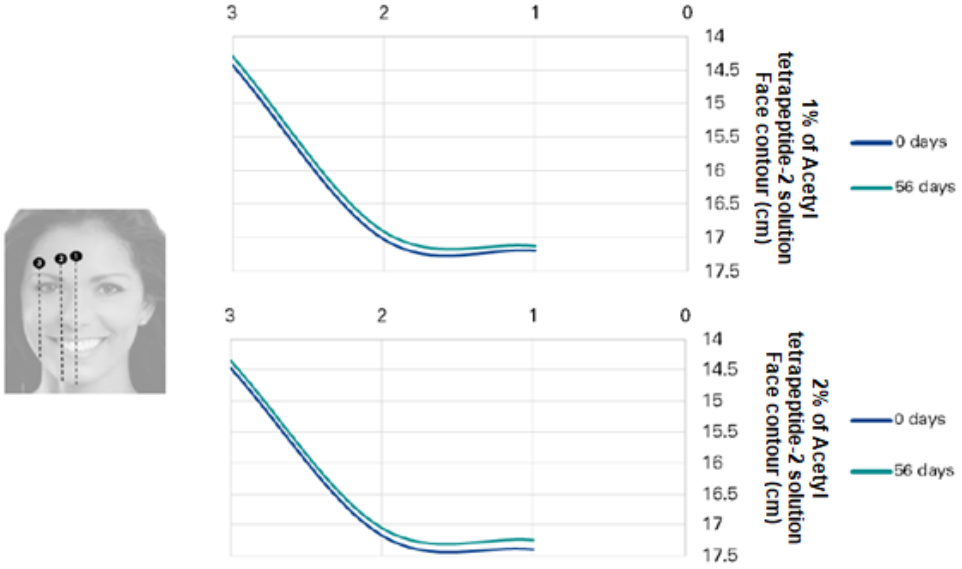


Fig. 21. Face contour measurements for two different concentrations at positions 1) middle of the face, 2) inner side of the eye and 3) outer side of the eye.

A decrease of 0.10cm and 0.13cm was observed after 56 days for the 1% and 2% Acetyl tetrapeptide-2 peptide solution respectively.

Acetyl tetrapeptide-2 reshapes the face contour, providing a lifting effect.

The face contour reshape was visible by means of digital images of the volunteers.

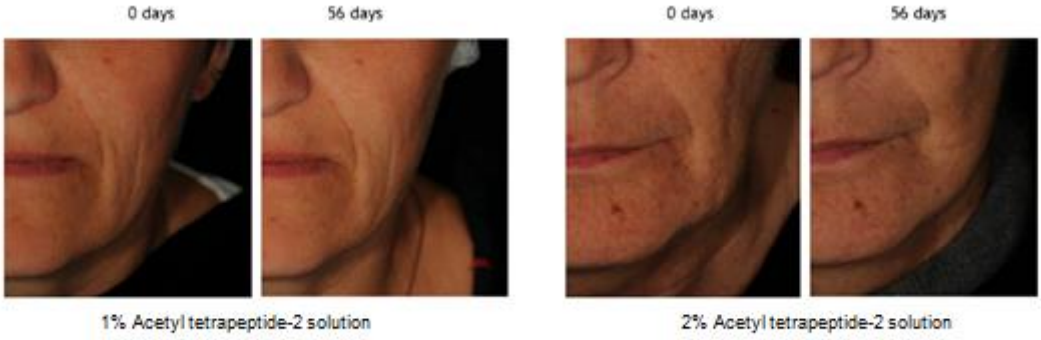


Fig. 22. Digital pictures of two different female volunteers at different concentrations of the active ingredient.

The analysis was finally completed with a self-evaluation of the volunteers regarding the efficacy of the product there were assigned.

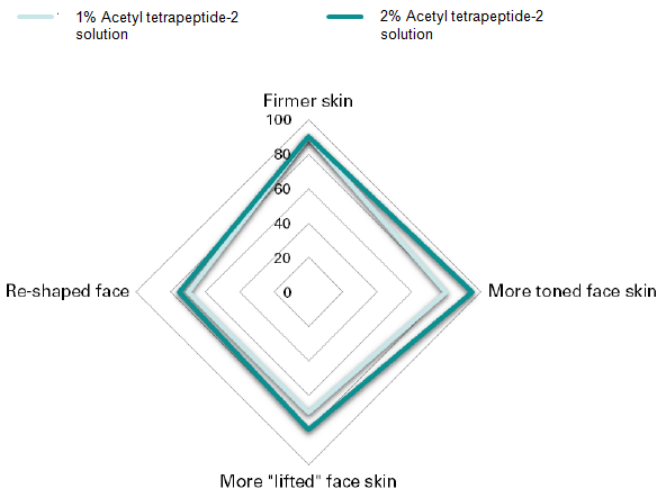


Fig. 23. Self-assessment of the volunteers for both concentrations of the active ingredient.

95% of volunteers noticed a more toned skin after using the cream with 2% Acetyl tetrapeptide-2 solution. 90% believed that the 2% treatment offered a firming effect.

Volunteers claimed to have a firmer and more toned skin after the treatment with Acetyl tetrapeptide-2.

Cosmetic properties

Acetyl tetrapeptide-2:

- Innovative Tetrapeptide to increase skin firmness, avoiding sagginess and flaccidity.
- Raised the activity of FBLN5 and LOXL1 promoters by 1.2-fold and 1.3-fold respectively (at 1mg/mL), promoting the expression of these key elements and the correct assembly of elastin fibres.

- Augmented FBLN5 and LOXL1 protein levels by 2.3-fold and 1.7-fold respectively, which help elastin fibres to be assembled accurately.
- Upregulated the expression of genes involved in collagen synthesis and FAs (talin and zyxin among others), all of them beneficial elements that improve skin cohesion
- Provided a statistically significant induction of elastin synthesis (21.7%), protein directly linked to skin elasticity and recoil.
- Increased type I collagen synthesis by 47.3% (statistically significant value), helping to improve skin firmness.
- Improved skin cohesion and firmness, as *in vivo* it reduced the indentation and area parameters and it decreased the fragmentation of the fibres network. Calculations extrapolate a decrease in the apparent age of the volunteers by almost 9 years.
- Enhanced the general elasticity of the skin and improved skin firmness, as at 1% decreased maximal deformation (-20.3%) and increased overall elasticity (10.9%), and regarding the 2% treatment it reduced maximal deformation (-25.2%) and increased overall elasticity (16%), after 56 days.
- Assisted in visibly reshaping the face contour, providing a lifting effect.

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