Cosmetic Active Ingredients

MATRIXYL®Morphomics™

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ENGLISH

MATRIXYL® Morphomics™

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IT'S TIME TO SHAPE THE SKIN'S FUTURE

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SYNOPSIS

Description	Lipopeptide Prolyl-Palmitoyl-Lysyl-Histidyl-Glycine (Pal-K(Pro)HG), in a water soluble excipient. This matrikine activates cellular pathways that influence cell and dermis morphology.

SUBSTANTIATED ACTIVITY

in vitro

All the tests have been performed with an equivalent of 1, 1.4 or 2% **MATRIXYL® Morphomics™** on dermal human fibroblasts, skin equivalents or explants. Refer to the following pages for detailed protocols and results.

MECHANISM OF ACTION VIA THE FOXO-AMPK PATHWAY

- FOXO1 gene .... Expression (x1.65, $p<0.01$)
- FOXO3 gene .... Expression (x1.73, $p<0.01$)
- SESN2 gene .... Expression (x2.22, $p<0.01$)
- SESN3 gene .... Expression (x2.15, $p<0.01$)
- AMPK (phosphoproteomics) .... +189% ($p<0.01$)

RECONNECTING THE NUCLEUS TO THE EXTRACELLULAR MATRIX: THE CYTOSKELETON

- Cofilin (LC-MS/MS) .... +95% ($p<0.05$)
- α-actinin (LC-MS/MS) .... +82% ($p<0.05$)
- Actin (Immunocytology) .... +187% ($p<0.01$)
- Talin (Immunohistology) .... +65.8% ($p<0.01$)
- Integrin α2/β1 (Immunohistology) .... +55.3% ($p<0.01$)
- Fibronectin (Elisa) .... +61% ($p<0.01$)

DERMAL MORPHOLOGY

- Fibrillar collagen production and maturation:
  - Collagen I (LC-MS/MS) .... +42% ($p<0.01$)
  - Collagen III (LC-MS/MS) .... +58% ($p<0.01$)
  - Fibrillar collagen maturation enzymes (LC-MS/MS) .... +43% ($p<0.05$) to +161% ($p<0.01$)
  - MMP2 (Enzymatic activity) .... -71% ($p<0.01$)
  - PAI (LC-MS/MS) .... +54% ($p<0.01$)
- Collagen IV (Immunohistology) .... +12.1% ($p<0.01$)
- Collagen VI (Immunocytology) .... +45% ($p<0.01$)
- Hyaluronic acid (Immunohistology) .... +25% ($p<0.01$)

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in vivo

Clinical study evaluating the effect of **MATRIXYL® Morphomics™** on the vertical lines and crow's feet wrinkles of two female panels and one male panel. The volunteers applied a cream containing 2% of **MATRIXYL® Morphomics™** twice daily for 6 weeks against placebo.

PERCEPTION OF WRINKLES BY EXPERT JUDGES

- Cases with reduced frown lines ....+21.3% *vs* placebo ($p<0.01$)
- Cases with reduced marionette lines ....+14.1% *vs* placebo ($p<0.01$)
- Cases with reduced nasogenian fold ....+12.3% *vs* placebo ($p<0.01$)
- Cases with reduced crow's feet wrinkles ....+10% *vs* placebo ($p<0.05$)

INSTRUMENTAL EVALUATION OF VERTICAL LINES

- Frown line volume .... -9.2% *vs* placebo ($p<0.05$)
- Frown line perimeter .... -7.8% *vs* placebo ($p<0.07$)
- Recovery after frowning .... +82.3% *vs* 69.7% placebo ($p<0.09$)
- Marionette line volume .... -21.7% *vs* placebo ($p<0.08$)
- Marionette line perimeter .... -9.6% *vs* placebo ($p<0.06$)

INSTRUMENTAL EVALUATION OF CROW'S FEET

- Surface occupied by deep wrinkles (female panel) .... -14.3% *vs* placebo ($p<0.06$)
- Wrinkle surface (male panel) .... -13.2% *vs* placebo ($p<0.05$)

RECOMMANDED USE

GENERAL INFORMATION

- Recommended pH: 4.00 to 6.00.
- Add **MATRIXYL® Morphomics™** to the emulsion preferably at the end of the process, at a temperature lower than 50°C.
- Solubility: water soluble

TOXICOLOGY / SAFETY

SkinEthic test
Patch test
HETCAM
Neutral Red Releasing Method
Reconstructed human Cornea-like Epithelium (RhCE) test
Skin Sensitisation: human Cell Line Activation Test (h-CLAT)
Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)
HRIPT (100 volunteers)
Ames test
Micronucleus test on cultured human lymphocytes
Phototoxicity: non-significant absorption in UVA and B
Expert Toxicologist Certificate

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1. INTRODUCTION

Generaly speaking, the median age in the population of a region is increasing due to declining fertility rates and/or rising life expectancy. Most countries have rising life expectancy and an ageing population (trends that emerged first in More Economically Developed Countries, but which are seen now in Less Economically Developed Countries). This is the case for every country in the world except the 18 countries designated as "demographic outliers" by the United Nations.
The aged population is currently at its highest level in human history. It is predicted that the rate of population ageing in the 21st century will exceed that of the previous century. The **number of people aged 60 years and over** has tripled since 1950, reaching 600 million in 2000 and surpassing 700 million in 2006.
It is projected that the combined senior and geriatric population will reach 2.1 billion by 2050. Countries vary significantly in terms of the degree and pace of ageing. Among the countries currently classified by the United Nations as more developed (with a total population of 1.2 billion in 2005), the **overall median age** rose from 28 in 1950 to 40 in 2010, and is forecast to rise to 44 by 2050. The corresponding figures for the world as a whole are 24 in 1950, 29 in 2010, and 36 in 2050. For the less developed regions, the median age will go from 26 years in 2010 to 35 years in 2050. The UN expects populations that began ageing later will have less time to adapt to its implications.

With an ageing population around the globe, the demand for anti-ageing skincare is not decreasing. Moreover, rise in urbanisation and *per capita* expenditure in developing countries is also driving the growth of the anti-ageing product market. The global anti-ageing skincare market is projected to grow to US$ 30 billion in 2020, with a CAGR 2016-2020 of 4.1%. The highest total spend will come from a mix of Millennials and Gen-Xers. Millennials, ages 15-29, constitute the **dynamic drivers** of the premium skincare growth. They are the early adopters interested in new trends and products. Millennials are excited to share new discoveries with others, strive to be the first to discover and the first to share. 57% of Millennials use anti-ageing products daily, while 28% use anti-ageing products at least weekly. Gen-Xers, ages 30-59, are the **continual drivers** of the premium skincare growth. They have an entrepreneurial spirit, are well-educated yet can be sceptical. 68% of Gen-Xers use anti-aging products daily, while 20% use anti-ageing products at least weekly. Anti-ageing products have shifted to not just target wrinkles and fine lines but also to target issues such as dry skin, skin firmness, uneven skin tone, hyper-pigmentation, and under-eye dark circles. Nevertheless, wrinkles are still the number one concern among the ageing signs after 25 years old, as being the first noticeable sign.

The face reflects the different facets of the personality, its different muscles allowing a multitude of expressions intended for a silent communication in society. This great facial mobility is paid at a high price, since the repeated folds lead progressively to the disorganisation of the underlying dermis and permanently print the skin giving the frown lines, marionette lines, the nasogenian fold and the crow's feet at the corner of the eye. These wrinkles, linked to the expressions, form more or less marked furrows that become difficult to resorb with ageing.
When young, the skin instantly resumes its appearance as it was before the stress but with age the skin tends to keep the "fold" in memory. Moreover, the successive foldings favor the production of matrix proteases intended to promote the renewal of the matrices. Unfortunately, this incessant repeated stress generate too many proteases which then gradually disorganise the support tissue of the cells, whereas in parallel, with age, the cells produce less matrix (collagen in particular). Wrinkles then settle and then widen gradually.

The cellular process of matrix protein production must first be revived and improved, but also the impact of the protease-related disorganisation must be reduced by better controlling them. Recently, it has been

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observed that providing the cells with a "young" matrix environment dynamically favors new syntheses, which allows the development of a better architecture and organisation of a denser dermis.

In addition to its matrix, the cell must have a relay at its surface to be connected to its environment, which allows the transfer of mechanical signals from this environment to the nucleus and induces mechano-sensitive genes. It is the role of the actin cytoskeleton and its anchor plate which, by constantly remodelling, allow the cells to adapt effectively to external stresses by activating genes and forming proteins of the matrix. The decrease of this dynamism, linked to a less dense matrix, gradually disconnects the cell from its environment. Being less stimulated, the cell gradually extinguishes, leading to its premature ageing. Certain longevity-beneficial genes, which can be called “longevigenes™”, stimulate the production of the proteins of the matrix and are of great interest for delaying the effects of ageing.

2. THE FIBROBLAST CONNECTIONS

All tissues in the body are composed of variable amounts of cells and extracellular matrices (ECM). Tissue homeostasis is controlled in particular by interactions between these elements (ECKES *et al.*, 2006). The cells produce the surrounding ECM, which plays a fundamental role since the ECM connects the cells of the same tissue to each other, allowing them to locate themselves in space and receive signals of attachment, multiplication or favouring certain syntheses. Moreover, the ECM relays to the cells a part of the mechanical environmental signals received by the organism and allows it to adapt to it.

2.1. The cytoskeleton: actin and tension

Actin is a protein which, polymerised, serves as a cytoskeleton inside the cell. It exists in an isolated (globular) and polymerised (fibrillary) form; cofiline being one of the necessary actors for this dynamism. Actin gives shape to the cell, allows it to orient itself, is essential during divisions and allows the transfer of molecules in the cell.
It also serves as a relay in the cell for the transmission of external mechanical signals, which allows the induction of mechano-sensitive genes as are the genes of the ECM proteins (ROLIN *et al.*, 2014). However, it has been shown that, in parallel with the reduction of the ECM syntheses linked to the age of the cell, there is a reduction in mechanical stimulations of the cells, which also induces a decrease in the amount of intact collagen fibres (VARANI *et al.*, 2006).

Actin is linked to ECM by integrins, in particular integrin α2/β1, and to a set of proteins whose combined talin and α-actinin allow this binding (Figure 1). This great complexity is essential for the cell which needs systems allowing it to withstand mechanical stresses. A mammalian cell is essentially composed of an aqueous gel delimited by a bubble of lipids which is less resistant to stretching continuously undergone by the skin. These anchoring points made of proteins allow the tensions to be exerted and the cells to resist them.

2.2. The anchoring complex

The strong interactions between cells and matrix are illustrated in particular by the following experiment: an isolated mouse heart was prepared so as to keep only the ECM skeleton while being totally devoid of cells. Human pluripotent stem cells, seeded on this skeleton, colonised it and in contact with it, in 20 days, reprogrammed to beat the heart again (LU *et al.*, 2013). Other trials showed that young cells included in an

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aged and damaged matrix had decreased syntheses, whereas cells from a relatively old person had their matrix syntheses reintroduced when they were included in a young matrix (FISHER *et al.*, 2009).
In the dermis, as elsewhere, ECM is a complex three-dimensional network involving many molecules and proteins that interact with each other and are associated with cells. Of all the existing collagens, type I fibrillar collagen, by its quantity and function, is one of the fundamental pillars of dermal ECM. It is found in association with a very important glycoprotein, fibronectin, which reinforces the adhesion of fibroblast to collagen thanks to a complex protein peg made of integrins, α-actinin and talin (Figure 1). In addition, fibronectin can be used to organise the coherence of ECM protein deposition and the establishment of a good quality dense tissue (SEVILLA *et al.*, 2013).

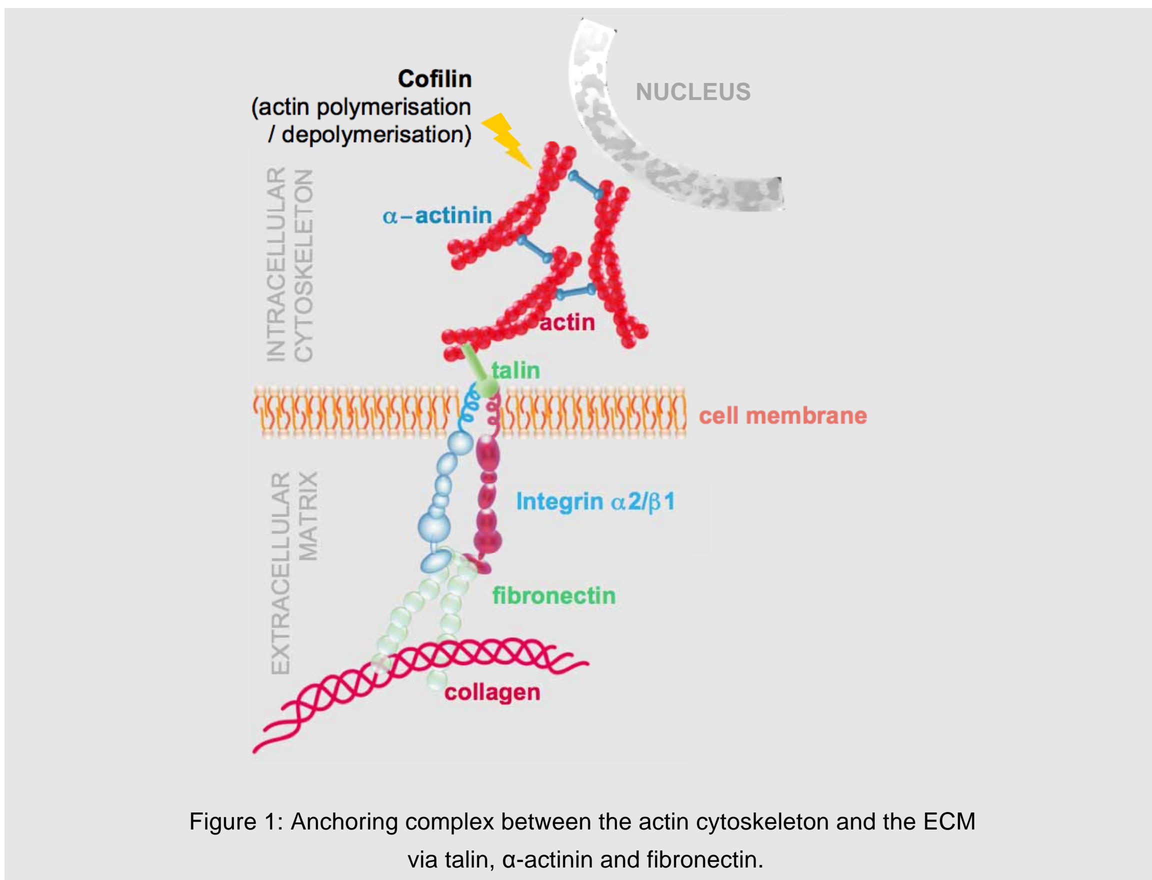

Figure 1: Anchoring complex between the actin cytoskeleton and the ECM via talin, α-actinin and fibronectin.

2.3. Fibronectin

Fibronectin is a dimeric adhesive glycoprotein. It has numerous sites of attachment to proteins of the ECM (collagen, proteoglycans, fibronectin, etc.) and, thanks to the tripeptide RGD, to the integrins of the cell. This also means that fibronectin by connecting the cells and some components of the ECM, allows the creation of an interconnected network where forces of external tensions are exerted. The latter trigger reactions in the cell via phosphorylation cascades which result in the remodelling of the actin cytoskeleton.
Fibronectin exists in a compacted inactive form and in an elongated, so-called fibrillar form. Binding to the fibroblast makes it possible to pass from one form to another by stretching up to 4 times its length (GAO *et al.*, 2006). Fibronectin limits tumor proliferation and promotes haemostasis. It is also described as the central actor of the organisation of the ECM by orchestrating more specifically the deposition and the end maturation of collagen fibres (SEVILLA *et al.*, 2013). In fact, fibronectin allows the collagen I to form an optimal architecture in the ECM in the form of long mature fibres.
On the contrary, reducing or preventing the deposition of fibronectin in ECM reduces in parallel the deposition and maintenance of collagens I and III on the same matrix (McDONALD *et al.*, 1982, SOTTILE and HOCKING, 2002). Moreover, the collagen deposited does not exhibit an optimal architecture and its

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fibres are then finer and sensitive to proteases such as MMPs (SEVILLA *et al.*, 2013). The *in vivo* turnover of this protein is as fast as it is *in vitro*: it has been found that about 40% of the deposited fibronectin was lost within 24 hours (REBRES *et al.*, 1995).

Thus, it is established that fibronectin, when produced in parallel with collagens, makes it possible to provide a high quality compacted ECM and that it connects the cell to the outside world.

2.4. The collagen I factory

Type-I fibrillar collagen is essential to have dense and firm skin as it provides the main source of tension in the tissues. This large and abundant protein, which represents 80% of the weight of the dermis, is produced in the fibroblast thanks to an extremely complex and orchestrated metabolism. Almost each step, if faulty, can result in significant defects (HULMES, 2002; MYLLYHARJU and KIVIRIKKO, 2004).

Recently, it has been shown that some unexpected genes were involved in the synthesis stimulations of collagen. These genes called FOXOs (Forkhead Box O) were identified a few years ago as longevity genes in a primitive organism and have since been the subject of much research. FOXO1 and FOXO3A have been positively linked to the longevity of Japanese, German, Italian and Chinese populations. Moreover, FOXO1 is very clearly in favor of the longevity of Chinese centenarians (LI *et al.*, 2009).
FOXOs indirectly coordinate the energy activities of the cell and promote mechanisms delaying premature ageing by promoting biogenesis. According to the Vadlakonda model, the activation of FOXOs leads to activate the recycling of the amino acids in the cell by autophagy and to promote the mobilisation of glucose. The FOXOs also promote the formation of SESTRIN3 (SESN3). Sestrines 2 and 3 are natural antioxidants that inhibit senescence, in particular by controlling the surplus of radical species produced by the mitochondria (NOGUEIRA *et al.*, 2008). SESN3 also activates AMPK, the cell's energy sensor of the cells. FOXOs can be transferred into the mitochondria by AMPK and favor the production of ATP necessary for the synthesis of new proteins (VADLAKONDA *et al.*, 2013). Then, this system being homeostatic, it returns to equilibrium before a new stimulation.

Concerning more specifically the skin, mutants which exhibit reduced FOXO activity synthesise less the collagen I; and it is noted that in parallel the density of the dermis is less good which would affect its quality. The involvement of FOXO is also underlined by the fact that mouse fibroblasts, with a FOXO deficiency, synthesise less collagen I after UV irradiation (MORI *et al.*, 2014).

The messenger RNAs of the collagen, once produced, are read by the ribosomes which then produce the elementary chains of pro-collagen (2 chains pro-α1 and a pro-α2). These elementary units of collagen type I are modified in the endoplasmic reticulum where enzymes and chaperone molecules will give it a form and stabilise it, avoiding it thus to fold on itself and become inactive (HENDERSHOT and BULLEID, 2000; LAMANDÉ and BATEMAN, 1999, Figure 2).

These pro-collagen chains produced are first modified by PPI (Peptidylproline cis-trans Isomerase) which isomerises the cis-proline residues, the main amino acid of the collagen in its trans form. This allows P4H (Prolyl-4-hydroxylase) to convert them into hydroxyproline, stabilising the nascent pro-collagen molecule, making it more resistant and creating a dense network of water around it (BELLA *et al.*, 1994-1995, KIVIRIKKO and MYLLYHARJU, 1998; PRIVALOV, 1982). The deficiency of vitamin C, the essential cofactor of P4H, reduces the hydroxylation of the pro-collagen which then remains in the cell, which blocks future neo-syntheses, it is scurvy.

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The lysine residues of these same chains are also hydroxylated by PLOD1 (Lysyl Hydroxylase), which then allows the addition of galactoses and glucoses by COLGALT and GGT (Collagen Galactosyl Hydroxylysyl Transferase and Galactosylhydroxylysyl-glucosyl Transferase, HARWOOD *et al.*, 1974-1975). To these additions, a role for the control of fibrillogenesis, remodeling, interactions with the cells has been shown but many questions about their exact role need further investigations (JÜRGENSEN *et al.*, 2011; SRICHOLPECH *et al.*, 2011; YANG *et al.*, 1993).
The procollagen chains are assembled by their C-propeptide end (BULLEID *et al.*, 1997) and the addition of disulphite bridges by the PDI enzyme (Protein Disulfite Isomerase) is observed in these three chains (KOIVU, 1987).
The three chains are wrapped on themselves, forming the pro-collagen on which the chaperone protein HSP47 binds. The latter is specific to collagen and is indispensable for the protection of this nascent fibre during its travel in the internal structures of the cell (SATOH *et al.*, 1996). The pro-collagen is excreted from the cell after its passage through a vacuole, its two ends are then cleaved by two different enzymes, N-proteinase (or ADAMTS), and PCOLCE-associated C-proteinase (Type I Procollagen C-proteinase Enhancer protein) which increases its activity. Thus released, the tropocollagen self-assembles with the existing fibrils (HULMES *et al.*, 1989; KADLER *et al.*, 1987). LOX (Lysyl Oxydase) will then bind the tropocollagens to each other via their lysine and hydroxylysine residues. The fibril is then mature and can bind directly to cells via integrins α2/β1 or be managed by fibronectin (ECKES *et al.*, 2006).

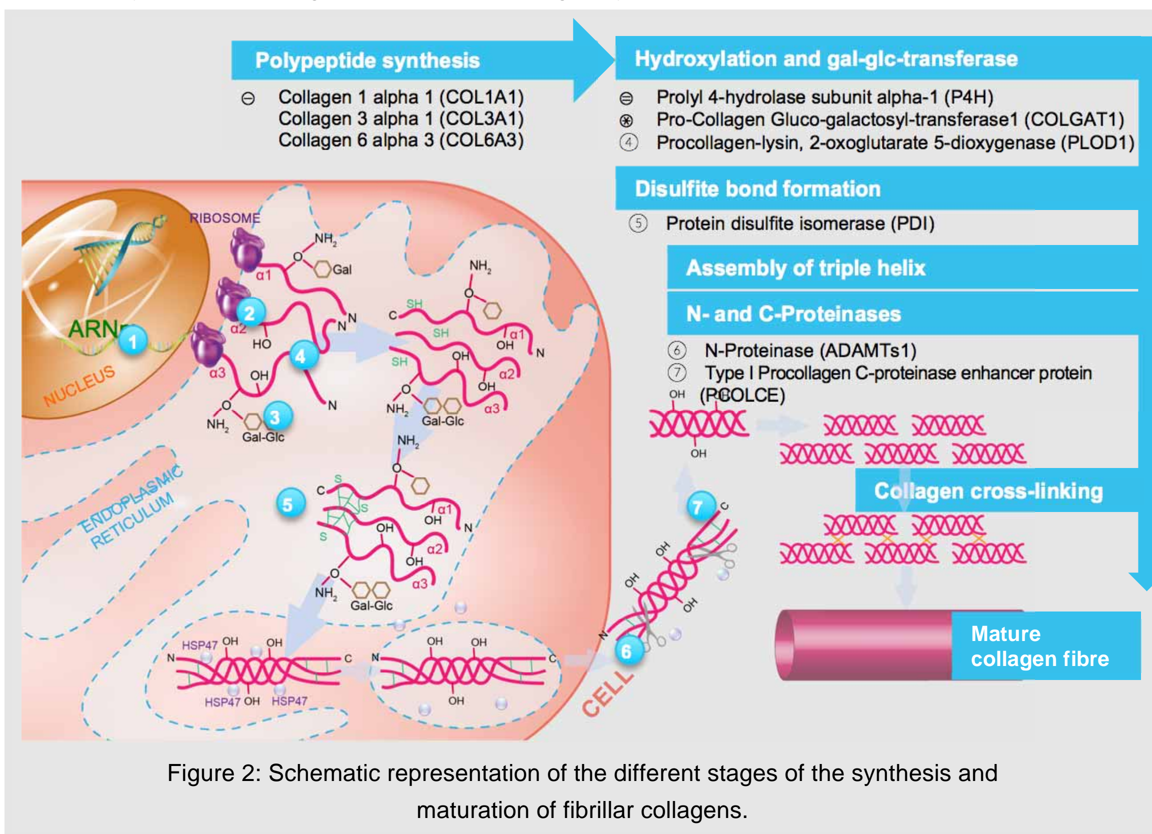

Figure 2: Schematic representation of the different stages of the synthesis and maturation of fibrillar collagens.

This shows the great complexity of the metabolism of collagen production and maturation, each step potentially adversely affecting the final result. Conversely, the improvement of each step would make it possible to increase the yields of production and maturation of this protein.

2.5. The extracellular matrix

Two other collagens, but non-fibrillar, the -VI and the -IV, are also of great importance to the skin. The collagen VI, little known, possesses, like fibronectin, a role of organiser of the ECM. Its impairment induces

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a finer dermis, less supplied in collagen and proteoglycans (WATSON *et al.*, 2001, THEOCHARIDIS *et al.*, 2016). This collagen is bound to collagen I and forms a mesh around it, but it also interacts with collagen IV and fibronectin. The deposition and the organisation of the latter are altered in the absence of collagen VI.
Collagen IV is one of the essential components of the basal membrane, its quantity and organisation are modified with age and photo-exposures. It forms a fine felting and serves to tie strongly epidermis and dermis (FERU *et al.*, 2016).

This assembly allows the cell, once hooked, to be in communication with the outside world, to perceive the tensions inside the ECM and the external mechanical forces. Hyaluronic acid by ensuring the hydration of the tissues, also helps in the transmission of signals. This set ensures a continuum with the intracellular skeleton, made of actin (Figure 1), and activates the gene regulation through epigenetic mechanisms of the cell to adapt to its environment (NOGUERA *et al.*, 2012) . It is known that fibroblasts do not behave in the same way according to their state of tension. The higher and better the production of collagens and hyaluronic acid, the more the synthesis of matrix proteases (MMPs) is disadvantaged directly or through the production of inhibitors such as PAI-1 (Plasminogen Activator Inhibitor-1). Conversely, a matrix too loose, causes the formation of proinflammatory mediators and MMPs that fragment the matrix (PITTAYAPRUEK *et al.*, 2016). In parallel collagen synthesis decreases contributing to skin ageing (ECKES *et al.*, 2006).

3. THE SEDERMA CONCEPT

SEDERMA offers a totally innovative peptide thanks to its three-dimensional structure and its activities. By promoting the pathway of "longevigenes™", it promotes the synthesis of proteins like collagen and it improves the yields in their chain of production. It also promotes the architecture of the dermis and the cell via the productions of organisers of the dermis that are fibronectin and collagen VI. The ECM, more dense and better organised, is thus source of positive tensions that promote in the cell the syntheses of matrix molecules of interest as much in quality as in quantity.

MATRIXYL® Morphomics™

is a novel matrikine™ which mechanism of action was elucidated using the powerful “Proteomics of Youth™” technology. It reboots the connection between the nucleus and the ECM by ensuring the cytoskeleton integrity, and stimulates the collagen fibre production and maturation to rebuild a functional matrix network. **MATRIXYL® Morphomics™** constitutes an unprecedented way of combating the appearance of wrinkles by influencing dermal morphology.

4. PRESENTATION OF MATRIXYL® MORPHOMICS™

MATRIXYL® Morphomics™ is a cosmetic active based on a peptide created by the Laboratory of Prospective and Applied Chemistry of SEDERMA after a long process of selection among more than one hundred other candidates.
Its amino acid sequence consists of the binding of a Lysine with Histidine and Glycine, the anagram sequence of the well-known GHK matrikine. This peptide is original in that it is substituted at the level of Lysine by the cyclic amino acid Proline, which is key in the helical morphology of collagen- I (Figure 3). In addition, this tetrameric sequence is linked to a palmitic acid. Its abbreviation is therefore Pal-K(Pro)-HG (Figure 4).

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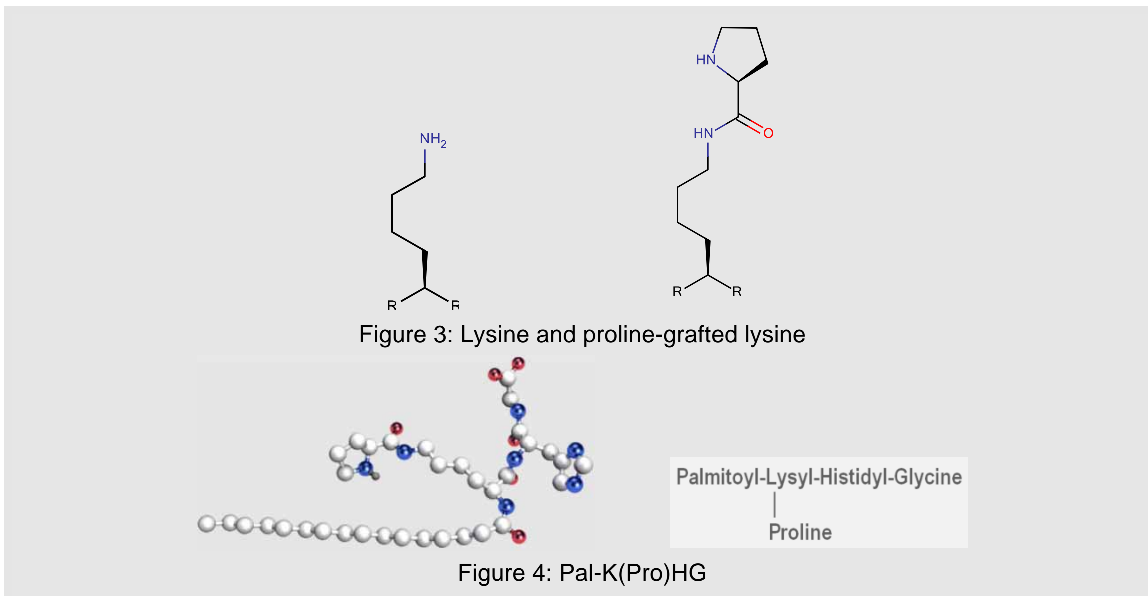

Figure 3: Lysine and proline-grafted lysine

Figure 4: Pal-K(Pro)HG

The original idea of designing a ramified matrikine was inspired by the cross-link structure of various collagens found in the human body: they include a cyclic amino acid.

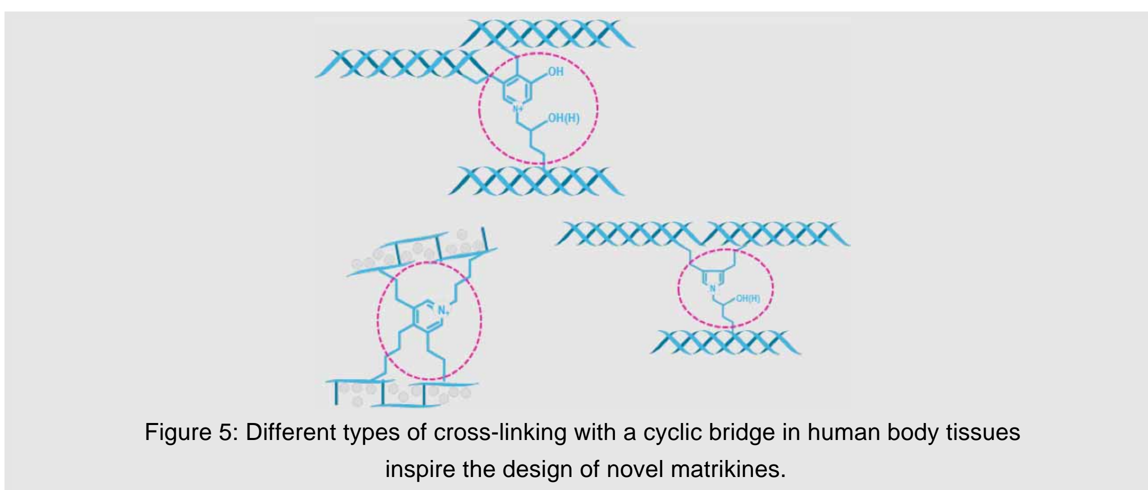

Figure 5: Different types of cross-linking with a cyclic bridge in human body tissues inspire the design of novel matrikines.

The various candidates were evaluated on cultures of human dermal fibroblasts, which made it possible to select the most versatile peptide in terms of biological activities. Some of these candidates had varied groupings found in different types of collagens, cyclic or non-cyclic, attached to Lysine and patented.

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5. *IN VITRO* TESTING

The following *in vitro* tests were carried out on several complementary biological models, all of human origin: fibroblasts of the dermis, equivalent skin and skin explants resulting from cosmetic surgery. **MATRIXYL® Morphomics™** was tested either in the form of a peptide in solution in its solvent (ethanol) or in its commercial form included at 2% in a skincare formulation (see formula in 9. ANNEX).

GLOSSARY AND REMINDERS ON THE TECHNIQUES USED:

1. Elisa (or enzyme-linked immunosorbent assay)

In order to survive, cells need a large number of proteins that support cell structures (collagen, fibronectin, elastin, actin ...), defend them or activate biological reactions (enzymes). These proteins can be quantified using antibodies that stick very specifically to them. Indeed, all human proteins cause a rejection reaction once they are in contact with immune cells of another animal species, these cells then produce antibodies which, by sticking to the proteins, neutralise them. Biologists use these antibodies for testing. In the ELISA, the culture media are brought into contact with antibodies fixed on a plastic support, the latter will capture the protein to be assayed, a second antibody, equipped with fluorescent molecules, is then added and once bonded, allows to reveal and quantify the amount of protein in the culture medium.

2. Immunocytology and immunohistology

In immunocytology or immunohistology, cell layers or thin sections of skins are immersed in a solution of antibodies which bind strongly and specifically to the proteins studied, a second type of antibody carrying a fluorescent molecule is then applied on the section to reveal and quantify the protein of interest.

3. Proteomics by LC-MS/MS

This very powerful technique makes it possible to evaluate at the same time several hundred proteins present in the same sample and to compare the result with control cases.
Fibroblast cultures are brought into contact with an active ingredient in a suitable culture medium. Then, a cell homogenate is prepared with a suitable buffer, the proteins are recovered and cut out specifically using a protease which creates longer or shorter peptide fragments. Liquid Chromatography Coupled Tandem Mass Spectrometry analysis consists in separating the peptides by liquid chromatography (Ultimate 3000, Dionex) according to their hydrophobicity, ionising them and then separating them in a detector (Q- Exactive HF, Thermo) which classifies them according to their mass and their charge (m/z). A software (Mascot, v 2.4.) allows to analyse the masses and the charge of each element and to deduce the presence and the quantity of the pre-existing proteins above the detection threshold. These data can then be processed through a bioinformatics/biostatistical analysis that identifies biological processes and metabolic pathways modulated by an active ingredient.

4. qRT-PCR

In order to produce proteins, it is necessary that more or less numerous messages (mRNA) go from the genes to production organelles called ribosomes. Each mRNA is specific to a single protein, the amount of mRNA produced indicates the potential amount of protein to be produced by the cell. After applying a stress and / or a product to the cell, the mRNAs produced are extracted from the cells and their relative amount is evaluated by the qRT-PCR (Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction) method.

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5.1. Preliminary study: ECM production

PRINCIPLE

Our first experiments were carried out on cells aged experimentally by a protocol of replicative senescence (13 replication culture). The cells thus obtained (Figure 6) have a physiology and an aspect similar to that observed with cells from an elderly person: strong dispersion, less multiplication, specific markers (SA β-galactosidase, etc.). These pre-senescence cells were then brought into contact with **MATRIXYL® Morphomics™**.

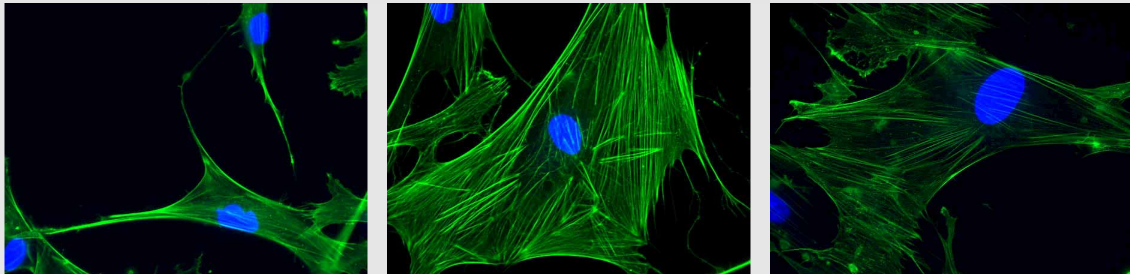

Figure 6: Aspect of fibroblasts of the dermis of a young person (left), Aged (middle) and obtained by replicative senescence (right).

RESULTS

Tested aged cells showed that the equivalent of 0.6% and 1% of **MATRIXYL® Morphomics™** significantly and dose-dependently activated production of collagen I (+41%* and +137%** respectively, n=5). Similarly, we observed an improvement in collagen IV production (+35%*, n=3, for the equivalent of 1.4% of **MATRIXYL® Morphomics™**).

* *p<0.05; ** p<0.01.*

This model, of great experimental interest, however, can't be used for an exhaustive study in view of the slow growth of these old cells which prevented us from having sufficient cellular material for our tests. We verified that young cells responded appropriately to **MATRIXYL® Morphomics™** and therefore continued the tests with the latter on four main topics:

1. study of FOXO-AMPK pathway proteins
2. proteomics of the cytoskeleton proteins
3. genomics and proteomics of the collagen I metabolism
4. analysis of other extracellular matrix proteins

5.2. Synthesis and activation of FOXO-AMPK pathway proteins

Two types of tests have been carried out to follow this path. In the first case, we looked at the expression of the genes by qRT-PCR. In the second case, we followed the phosphorylation variations (presence of phosphorus on an amino acid) of certain proteins whose activity is totally dependent on these phosphorylations.

PRINCIPLE

1) Fibroblasts were brought into contact with the equivalent of 1% of **MATRIXYL® Morphomics™** in a suitable culture medium, the control being the solvent (ethanol 0.1%). At the end of this, the cells were homogenised, the mRNAs extracted and after conversion to cDNA, the variation in their expression was evaluated by qRT-PCR with respect to negative controls.

2) Fibroblasts were brought into contact with the equivalent of 1% **MATRIXYL® Morphomics™** in a suitable culture medium, the control being the solvent (ethanol 0.1%). At the end of this, the cells were

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homogenised. The quantity of homogenised proteins and the extracts were deposited on membranes pre-labeled with antibodies specific for the phosphorylated forms of the studied proteins. After addition of an antibody coupled to the peroxidase, the membranes were revealed by chemiluminescence using a CCD camera.

Results

We observed that several genes were significantly expressed in relation to the control: FOXO1, FOXO3, SESN2 and SESN3. In addition, AMPK is more phosphorylated than in control cases (Table 1).

Table 1: Impact of **MATRIXYL® Morphomics™** on the expression of genes (pink) or phosphorylation variation (blue) of proteins bound to the FOXO-AMPK pathway;

(qRT-PCR n=3-4: results are a ratio of the control; phosphorylation n=4: results in % of the control).

	FOXO1	FOXO3	SESN2	SESN3	AMPK
MATRIXYL® Morphomics™ eq. **1%**	**1.65; *p<0.01***	**1.73; *p<0.01***	**2.22; *p<0.01***	**2.15; *p<0.01***	**+189%; *p<0.01***

The results indicate a strong activation of the longevigenes™ involved in the FOXO-AMPK pathway by **MATRIXYL® Morphomics™**, meaning a potential benefit on protein metabolism, DNA repair, ROS detoxification, energetic homeostasis and cell viability.

5.3. Cross-talking cell nucleus/ECM: the cytoskeleton

Principle

Cells are not just embedded in a matrix, they receive signals either in the form of molecules or in the form of tensions that will trigger protein production. To function, these tensions require strong anchor points, binding collagens and fibronectin to intracellular actin. Talin, α-actinin and integrins are part of these anchor structures of actin (see Figure 1).

We observed an increase of two proteins related to this theme in the proteomics LC-MS/MS study (n=3): α-actinin (+82%, *p<0.05*) and cofilin (+95%, *p<0.05*). Then, with an equivalent cellular model, we performed a western-blot on actin and observed its increase by 24.2% for a 1.4% equivalent of **MATRIXYL® Morphomics™** *versus* its solvent. This indicated the potential interest of exploring this aspect further with three additional tests.

1. Abdominal skin explants (from 2 Caucasian women, 33-44 years old) received a cream containing 2% **MATRIXYL® Morphomics™** daily for 6 days or placebo. Skin sections were then made to search by immunohistology the talin protein (Table 2).
2. In a second series of tests, the peptide of **MATRIXYL® Morphomics™**, or its solvent as a control, was inserted into the culture medium and then skin equivalent models were sectioned to visualise integrin α2/β1 by immunohistology (Table 3).
3. In the third series of tests, the peptide of **MATRIXYL® Morphomics™**, or its solvent as control, was placed in a confluent normal human fibroblast culture medium. We then performed a labeling of the actin and cofilin proteins by immunocytology (Table 4, Figure 7).

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RESULTS

Table 2: Variation of talin synthesis in skin explants
after application of 2% **MATRIXYL® Morphomics™** or placebo (n=5, 59-60 photos/trial).

	Talin (AFU*)	**Variation (%)**
Placebo	2.30 ± 0.30	*Reference*
MATRIXYL® Morphomics™ 2%	3.80 ± 1.20	**+65.8%; *p<0.01***

Table 3: Variation of integrin α2/β1 synthesis in skin equivalents
after contact with **MATRIXYL® Morphomics™** (n=3-4; 34-46 photos/trial).

	Integrin α2/β1 (AFU*)	**Variation (%)**
Control	2.87 ± 1.11	*Reference*
MATRIXYL® Morphomics™ eq. 1.4%	4.46 ± 1.13	**+55.3%; *p<0.01***

Table 4: Variation of actin and cofilin synthesis by NHF
in contact with **MATRIXYL® Morphomics™** (n=3, 15 photos/trial).

	Actin (AFU*/10^5 cell.)	**Variation (%)**	**Cofilin** (AFU*/10^5 cell.)	**Variation (%)**
Control	5892 ± 3428	*Reference*	456 ± 376	*Reference*
MATRIXYL® Morphomics™ eq. **1%**	16939 ± 11428	**+187%; *p<0.01***	2494 ± 498	**+446%; *p<0.01***

No toxic effect was observed at these concentrations.

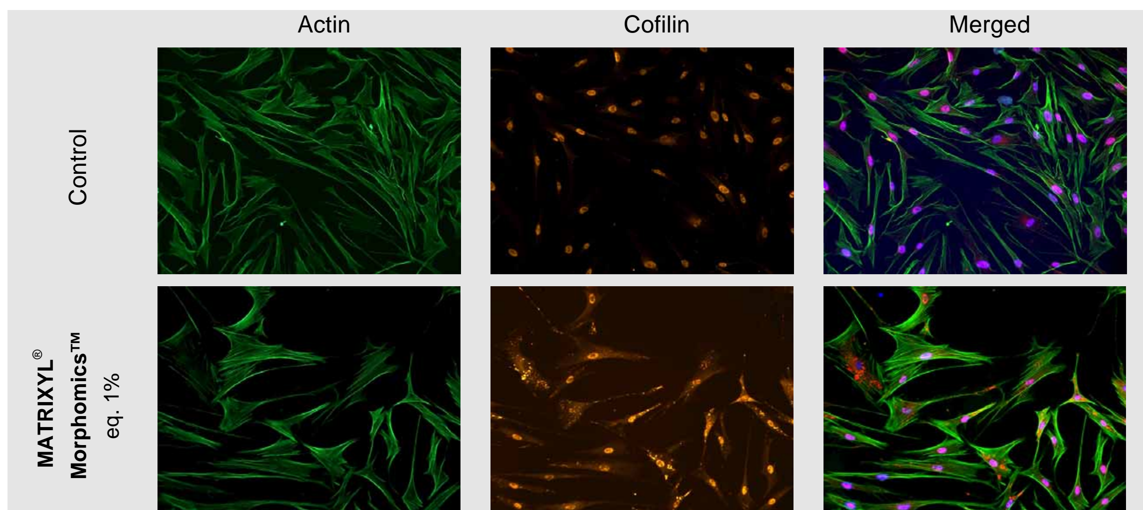

Figure 7: Visualisation of the variation of actin and cofilin synthesis by NHF in contact with **MATRIXYL® Morphomics™** or the control.

These results confirm the effect of **MATRIXYL® Morphomics™** in ensuring a viable cytoskeleton: We saw that the level of the main proteins involved in the structure of the cytoskeleton was restored. The cytoskeleton dynamism is also maintained through the stimulation of cofilin production. The cross-talking between the intracellular cytoskeleton and the extracellular matrix is fully operational, and the cell can answer to the external information and produce effectively matrix molecules.

* Arbitrary fluorescence units

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5.4. Connecting the cytoskeleton to the ECM: Fibronectin production

PRINCIPLE

We have seen that fibronectin, along with collagen I and -VI, played a major role in the quality of the ECM and in that the cell was connected to the extracellular environment (see §2.3). Fibroblasts received **MATRIXYL® Morphomics™** in a suitable culture medium. At the end of this, the culture media were assayed by ELISA to evaluate the amount of fibronectin excreted, in comparison with the solvent control. In parallel, a quantification of the number of cells was carried out using a marking of the cell nucleus.

Table 5: Variation of fibronectin production by NHF
in the presence of **MATRIXYL® Morphomics™**, (n=3).

ELISA	Fibronectin (ng/10^4 cell.)	Variation (%)
Control	1231 ± 149	*Reference*
MATRIXYL® Morphomics™ eq. **1%**	1806 ± 123	**+47%; *p<0.01***
MATRIXYL® Morphomics™ eq. **1.4%**	1983 ± 172	**+61%; *p<0.01***

There is thus a clear increase in the production of fibronectin which echoes those of collagens.

5.5. Dermal morphology

5.5.1. Collagen I

PRINCIPLE

Collagen I was assessed using several complementary techniques described below.

a. Proteomics: Fibroblasts received the equivalent of 1% **MATRIXYL® Morphomics™** in a suitable culture medium, the control being the solvent (ethanol 0.1%). At the end of this, the cells were homogenised, prepared as indicated above and analysed by LC-MS/MS (see page 14) in collaboration with Phylogene (France).
b. qRT-PCR: Following information collected by LC-MS/MS, qRT-PCR was used to check whether some of the proteins listed above were also increased at the gene level. Equivalent of 1% **MATRIXYL® Morphomics™** in a suitable culture medium was applied on fibroblasts, the control being the solvent (ethanol 0.1%). At the end of this, the cells were homogenised, the mRNAs extracted and after conversion to cDNA, the variation in their expression was evaluated by qRT-PCR with respect to negative controls.
c. ELISA: Fibroblasts received **MATRIXYL® Morphomics™** in a suitable culture medium. At the end of this, the culture media were assayed by ELISA to evaluate the amount of collagen I excreted, but still in solution, in comparison with the solvent control (0.1% ethanol). In parallel, quantification of the number of cells was carried out using a labelling of the nuclei (HOECHST 33258, PAPADIMITRIOU and LELKES, 1993).
d. Immunocytology: Fibroblasts received **MATRIXYL® Morphomics™** in a suitable medium. Then, once rinsed, the cell layers were labeled with an antibody to visualise and evaluate by immunocytology the amount of mature collagen I, deposited as fibres. The solvent was used as a control. A quantification of the number of cells was carried out on these same cells by marking their nuclei as before.
e. Immunohistology: In addition to the previous tests on cells, a cream containing 2% **MATRIXYL® Morphomics™** was applied to skin explants (from abdominal surgery of a 33-years-old Caucasian woman) once a day for 6 days. In parallel, other explants were similarly given the placebo cream (see formula in 9. ANNEX). At the end of the contact, 7 µm frozen sections were

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made with the microtome (Leica) and collagen I (Figure 9), collagen IV (Figure 11) and hyaluronic acid (Figure 12) were labelled. Pictures were taken and the images analysed.

RESULTS

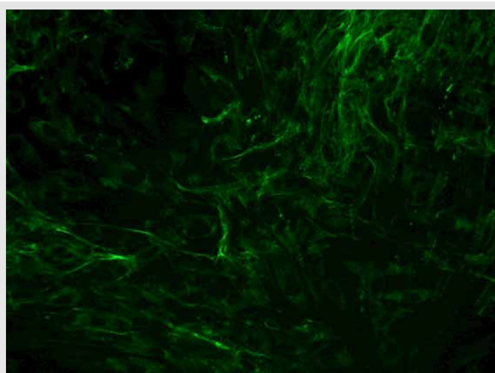
Table 6: Variation of collagen I production in the presence of **MATRIXYL® Morphomics™** according to various techniques.

a. Proteomics LC-MS/MS (n=3)	**Chromatography peak surface ($x10^8$)**		
	Control	**MATRIXYL® Morphomics™** 1%	Variation *vs* control (%)
Collagen 1 alpha-1 (COL1A1)	433.1 ± 60.6	615.7 ± 75.3	**+42%; *p<0.01***

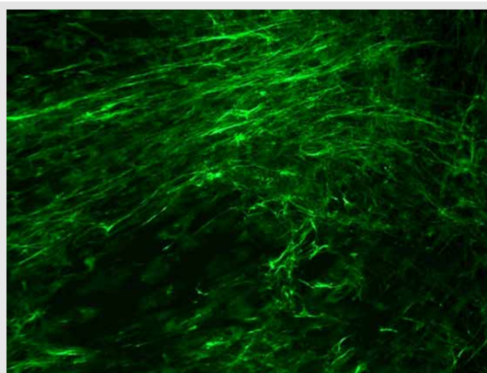
b. qRT-PCR (n=3-4)	**COL1A1 gene**
MATRIXYL® Morphomics™ eq. **1%**	1.81; *p<0.01*

c. Elisa (n=4-5)	**Collagen I (ng/10^6 cell.)**	**Variation (%)**
Control	6189 ± 1308	*Reference*
MATRIXYL® Morphomics™ eq. **0.6%**	8765 ± 1666	**+42%; *p<0.05***
MATRIXYL® Morphomics™ eq. **1.4%**	9542 ± 1300	**+54%; *p<0.01***
MATRIXYL® Morphomics™ eq. **2%**	11919 ± 1427	**+93%; *p<0.01***

d. Immunocytology (n=4-5; 43-48 photos/trial)	**Collagen I (AFU*/10^5 cell.)**	**Variation (%)**
Control	504 ± 296	*Reference*
MATRIXYL® Morphomics™ eq. **0.6%**	693 ± 388	**+38%; *p<0.02***
MATRIXYL® Morphomics™ eq. **1.4%**	1971 ± 1016	**+291%; *p<0.01***
MATRIXYL® Morphomics™ eq. **2%**	1030 ± 495	**+104%; *p<0.01***



Placebo



MATRIXYL® Morphomics™

Figure 8: Variation of mature collagen I fibre production by dermal fibroblasts in the presence of **MATRIXYL® Morphomics™** (eq. 1.4%) (x 200). Immunocytology.

e. Immunohistology (n=5; 57 photos/trial)	**Collagen I (AFU*)**	**Variation (%)**
Placebo	21.3 ± 5.7	*Reference*
MATRIXYL® Morphomics™ 2%	24.4 ± 8.0	**+14.3%; *p<0.05***

* Arbitrary fluorescence units

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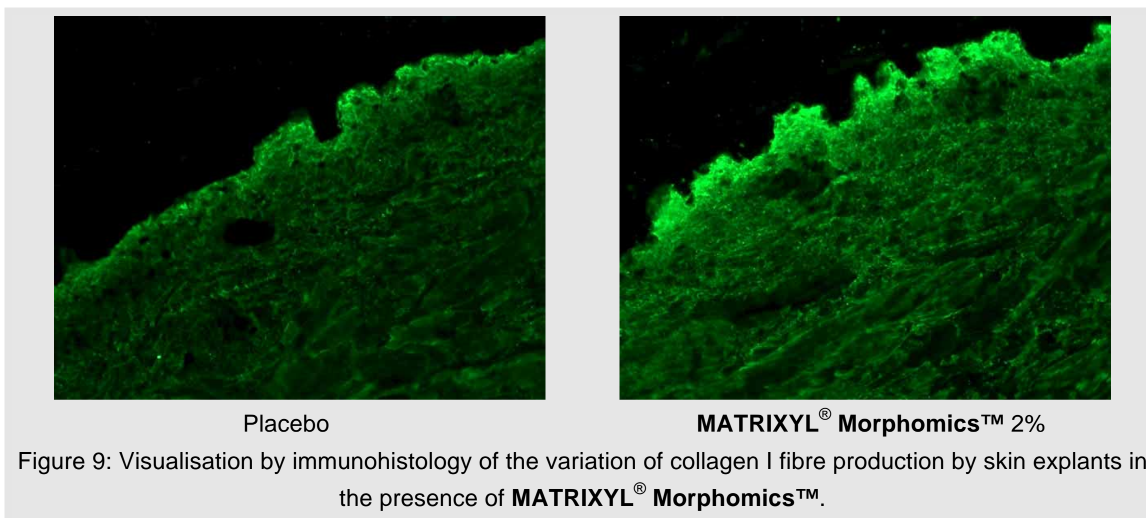

Placebo | **MATRIXYL® Morphomics™** 2%

Figure 9: Visualisation by immunohistology of the variation of collagen I fibre production by skin explants in the presence of **MATRIXYL® Morphomics™**.

All these complementary techniques and results confirm the efficiency of **MATRIXYL® Morphomics™** to stimulate the production of the main macromolecule in the extracellular matrix: collagen I.

5.5.2. Other collagens

PRINCIPLE

The same techniques as described above (see §5.5.1.) were used to determine the effect of **MATRIXYL® Morphomics™** on the production of collagen III, IV and VI.

RESULTS

Table 7: Variation of collagen III, IV and VI production in the presence of **MATRIXYL® Morphomics™** according to various techniques.

a. Proteomics LC-MS/MS (n=3)	**Chromatography peak surface (x10⁸)**		
	Control	**MATRIXYL® Morphomics™ 1%**	Variation *vs* control (%)
Collagen 3 alpha-1 (COL3A1)	**17.3 ± 3.3**	**27.3 ± 2.7**	**+58%; *p<0.01***
Collagen 6 alpha-3 (COL6A3)	**6.9 ± 2.2**	**14.6 ± 8.6**	**+113%; *p<0.05***

c. ELISA (n=5)	**Collagen IV ($ng/10^7$ cell.)**	**Variation (%)**
Control	46.7 ± 5.7	***Reference***
MATRIXYL® Morphomics™ eq. **0.6%**	63.7 ± 7.8	**+36%; *p<0.01***
MATRIXYL® Morphomics™ eq. **1.4%**	83.4 ± 7.9	**+79%; *p<0.01***
MATRIXYL® Morphomics™ eq. **2%**	131.6 ± 10.6	**+182%; *p<0.01***

d. Immunocytology (n=4-5; 45-48 photos/trial)	**Collagen VI (AFU*/10^5 cell.)**	**Variation (%)**
Control	4988 ± 1827	***Reference***
MATRIXYL® Morphomics™ eq. **0.6%**	5809 ± 2024	**+16%; *p<0.05***
MATRIXYL® Morphomics™ eq. **1.4%**	6331 ± 2426	**+27%; *p<0.01***
MATRIXYL® Morphomics™ eq. **2%**	7245 ± 1729	**+45%; *p<0.01***

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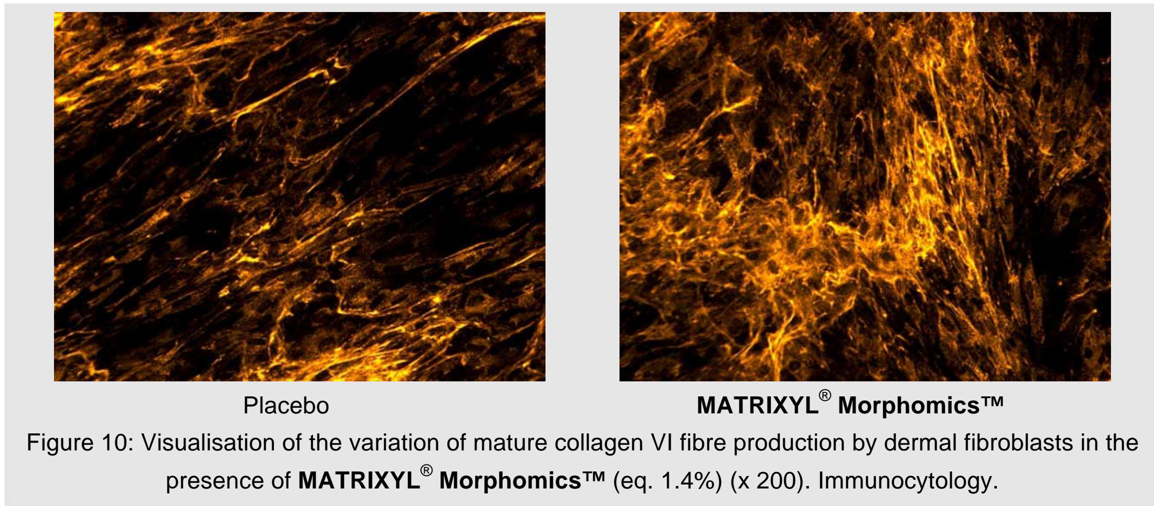
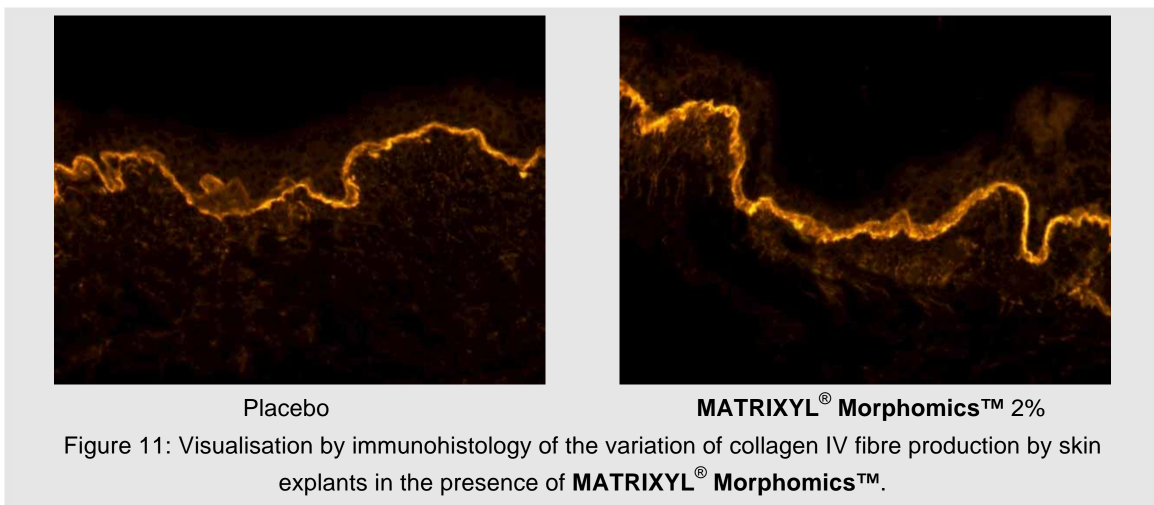


Placebo | **MATRIXYL® Morphomics™**

Figure 10: Visualisation of the variation of mature collagen VI fibre production by dermal fibroblasts in the presence of **MATRIXYL® Morphomics™** (eq. 1.4%) (x 200). Immunocytology.

e. Immunohistology (n=4-5; 48-59 photos/trial)	**Collagen IV (AFU*)**	**Variation (%)**
Placebo	**49.8 ± 4.5**	***Reference***
MATRIXYL® Morphomics™ 2%	**55.8 ± 10.6**	**+12.1%; *p<0.01***



Placebo | **MATRIXYL® Morphomics™** 2%

Figure 11: Visualisation by immunohistology of the variation of collagen IV fibre production by skin explants in the presence of **MATRIXYL® Morphomics™**.

Complementary studies including “Proteomics of Youth™” by LC-MS/MS show **MATRIXYL® Morphomics™** versatility to stimulate the production of different types of collagens involved at various levels in the skin. Indeed, it is noted that in addition to a greater quantity of collagens of type I, **MATRIXYL® Morphomics™** also activates the production of collagen III, IV and VI (but also V, not shown).

* Arbitrary fluorescence units

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5.5.3. Fibrillar collagen enzymes

PRINCIPLE

When the collagen fibres are produced by the fibroblast, a series of modifications is necessary in order to obtain a fully operational network in the dermis. This maturation process can take place thanks to several enzymes that were studied by qRT-PCR and proteomics with protocols described above (see §5.5.1.).

RESULTS

Table 8: Effect of **MATRIXYL® Morphomics™** on enzymes envolved in the fibrillar collagen maturation process.

a) Proteomics LC-MS/MS (n=3)	Chromatography peak surface ($x10^8$)		Variation *vs* control (%)
	Control	MATRIXYL® Morphomics™ 1%	
Prolyl 4-hydrolase subunit alpha-1 (P4HA1)	20.2 ± 5.8	35.1 ± 4.5	**+74%; *p<0.01***
Procollagen-lysin, 2oxoglutarate 5-dioxygenase (PLOD1)	19.5 ± 5.2	27.9 ± 8.6	**+43%; *p<0.05***
Pro-Collagen Gluco-galactosyltransferase-1 (COLGALT1)	4.9 ± 2.7	12.8 ± 4.4	**+161% ; *p<0.01***
Protein disulfite isomerase (PDI)	6.1 ± 0.6	8.8 ± 1.8	**+45%; *p<0.01***
Procollagen C-endopeptidase enhancer-1 (PCOLCE)	2.6 ± 0.6	4.2 ± 0.8	**+62%; *p<0.01***
N-Proteinase (ADAMTs1)	2.3 ± 0.9	3.9 ± 1.6	**+72%; *p<0.02***
Plasminogen Activator Inhibitor-1 (PAI-1)	1.1 ± 0.2	1.7 ± 0.3	**+54%; *p<0.01***
Matrix metalloproteinase-2 (MMP2)	2.8 ± 0.7	1.3 ± 0.8	**-54%; *p<0.01***

b) qRT-PCR (n=3-4)	P4HA1	PLOD1	COLGALT	PCOLCE
MATRIXYL® Morphomics™ eq. **1%**	**1.53; *p<0.01***	**2.03; *p<0.01***	**1.94; *p<0.01***	**1.61; *p<0.01***

The inhibitory effect of **MATRIXYL® Morphomics™** on the Matrix metalloproteinase-2 was confirmed with a cell-free assay that makes it possible to evaluate the activity of the MMP2 protease in the presence of increasing concentrations of the **MATRIXYL® Morphomics™** peptide and the substrate of the enzyme coupled to a fluorescent molecule. MMP2 further degrades the substrate as it is active, this is visualised by an increase in the fluorophore released in the medium.

Table 9: Evaluation of the effect of **MATRIXYL® Morphomics™** on MMP2 activity (n=2).

	MMP2 (m units)	Variation (%)
Control	221 ± 5.4	***Reference***
MATRIXYL® Morphomics™ eq. **1%**	112 ± 4.2	**-49%; *p<0.01***
MATRIXYL® Morphomics™ eq. **1.4%**	91 ± 2.5	**-59%; *p<0.01***
MATRIXYL® Morphomics™ eq. **2%**	65 ± 0.9	**-71%; *p<0.01***

The process of manufacture and maturation of the collagens is strongly favored in the presence of **MATRIXYL® Morphomics™**. Next to structural proteins, the enzymes involved in the early and terminal maturation of collagen, and proteins related to its protection or degradation (PAI and MMP) were favourably modulated in order to influence the maintenance of a dermis with characteristics of a young skin.

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5.5.4. Hyaluronic acid

PRINCIPLE

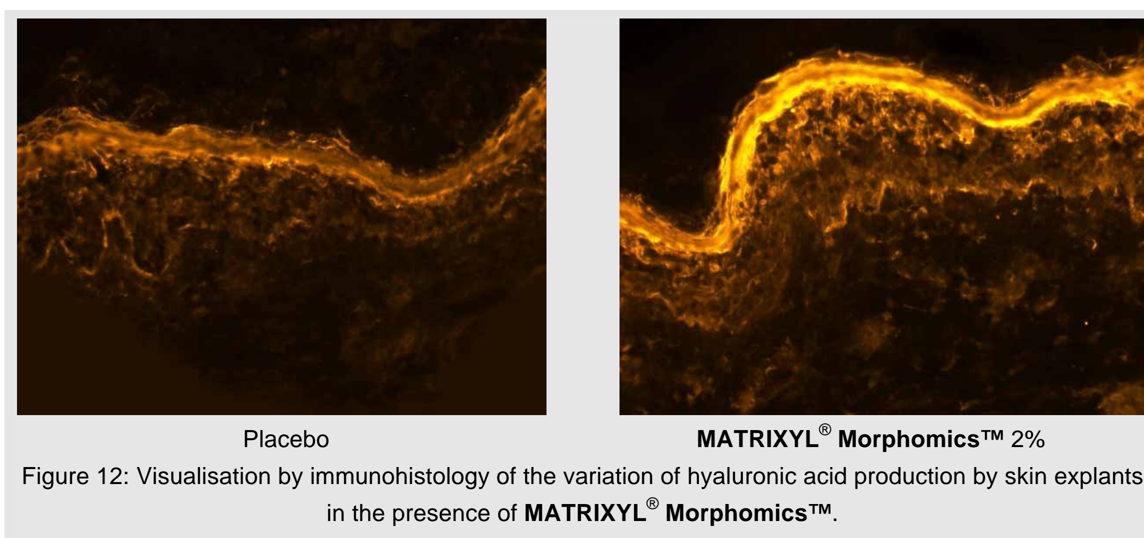
Hyaluronic acid in the dermis is an important macromolecule that ensures a properly hydrated environment for enzyme activity.

The stimulation effect of **MATRIXYL® Morphomics™** on its production by fibroblasts was evaluated by immunohistology as decribed above (see §5.5.1.e).

RESULTS

Table 10 : Variation of hyaluronic acid production by skin explants in the presence of **MATRIXYL® Morphomics™**.

e) Immunohistology (n=5; 57 photos/trial)	Hyaluronic acid	Variation (%)
Placebo	**40.0 ± 7.0**	***Reference***
MATRIXYL® Morphomics™ 2%	**50.0 ± 5.5**	**+25.0%; *p<0.01***



Placebo | **MATRIXYL® Morphomics™** 2%

Figure 12: Visualisation by immunohistology of the variation of hyaluronic acid production by skin explants in the presence of **MATRIXYL® Morphomics™**.

CONCLUSION

The results of this series of tests, which are complementary to one another, show that **MATRIXYL® Morphomics™** stimulates the production of collagen by cells in solution and to observe a greater quantity of mature collagens around the cells. In addition, the formation of fibronectin and hyaluronic acid is promoted while the activity and amount of MMP2 are reduced.

Studies of the proteome by LC-MS/MS and then the organisation of these data into functional groups show that **MATRIXYL® Morphomics™** acts on dermal matrices. Collagen formation is highly favoured by **MATRIXYL® Morphomics™**. Indeed, it is noted that in addition to a greater quantity of collagens of type I, III and VI in particular (but also V, not shown), it is observed an increase in the enzymes involved in the protection, production and maturation collagen fibres.

PDI and P4H1 are known to support pro-collagen in the cell and to transform it so that it has the indispensable hydroxyprolines. PLOD1, COLGALT enzymes are known to allow the addition of carbohydrate residues used for the future recognition and addressing of collagen. PCOLCE and ADAMTs1 enzymes prepare the final deposit of the fibre once outside the cell. The partial inhibition of the activity of the MMP2, either by reducing its production or by increasing the PAI, limits the destruction of the matrix fibres and better preserve their integrity.

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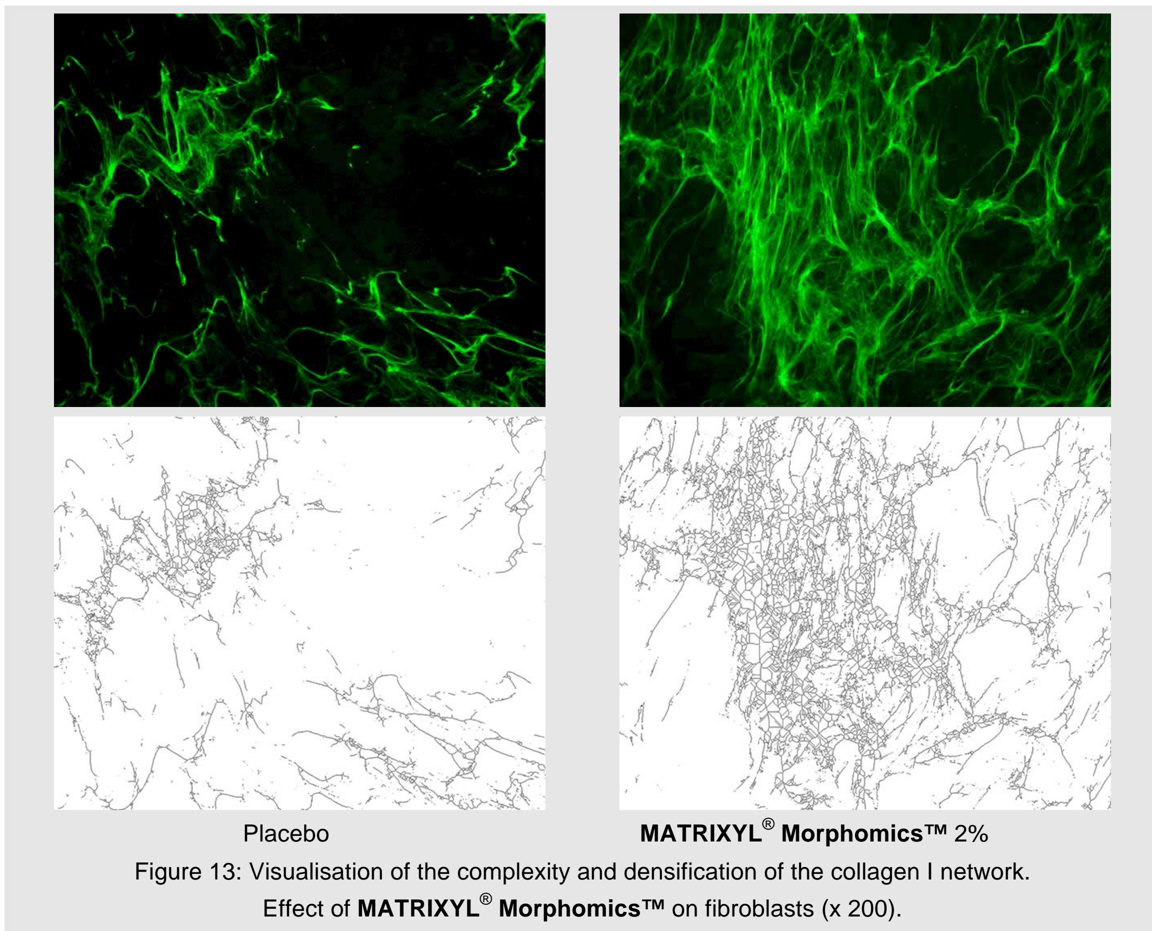
Thus, there is an improvement, point by point, in a set of agents essential to the formation and the coordinated deposition of the mature collagen chains. Although the production of mRNAs of collagen chains is observed, in particular by FOXO stimulation, it also appears that the collagen production yields are increased at the various stages of the manufacturing process. There would then be a better "delivery" of mature proteins.

Interestingly, in addition to the increase in the quantity of collagens and their quality, there is an increase in the production of fibronectin and collagen VI, two known proteins for architecting the collagen network. Indeed, without one or the other, the dermis is less well organised and the formation of mature collagen highly compromised.

An original way of visualising the quality of the matrix network architecture (both in terms of quantity and complexity) is to look for the main vectors of the mature fibres in study photos (Figure 13). With this method, one can see at a glance, the complexity of the network, which, as for a large city, is denser and rich in interconnections than a village.



Placebo | **MATRIXYL® Morphomics™** 2%

Figure 13: Visualisation of the complexity and densification of the collagen I network.
Effect of **MATRIXYL® Morphomics™** on fibroblasts (x 200).

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6. *IN VIVO* CLAIM SUBSTANTIATION

SEDERMA: FEBRUARY - APRIL 2016; SPINCONTROL MARCH - APRIL 2016.

The evaluation of the effectiveness of **MATRIXYL® Morphomics™** was carried out on a total of 84 volunteers in three independent studies:

- A placebo-controlled study of vertical lines: frown lines, marionette lines and nasogenian fold, and crow's feet wrinkle analysis using fringe projection and fingerprint analysis (SEDERMA).
- A totally original study where the effect of **MATRIXYL® Morphomics™** on the skin smoothness recovery after frowning is evaluated, against placebo, by projection of fringes (SEDERMA).
- A placebo-controlled study of the crow's feet wrinkles of male volunteers (SPINCONTROL).

6.1. Study of the effect of MATRIXYL® Morphomics™ on vertical wrinkles

PROTOCOL

SPECIFIC INCLUSION CRITERIA

Note: A study with two creams to be applied at the same time without mixing on the forehead is impossible. So we were forced to use two panels for this area (see diagram below).

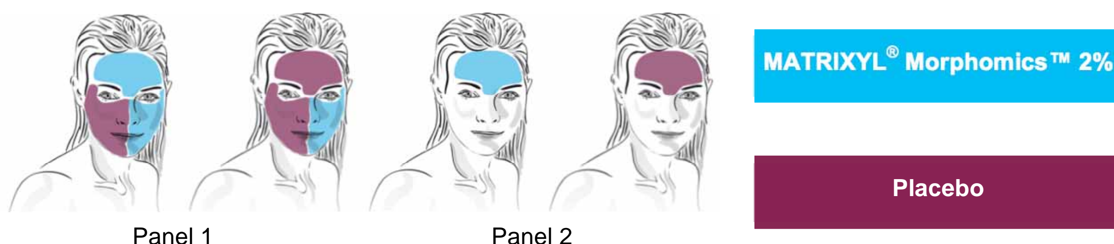
The first panel consisted of 34 women (mean age: 64 years, 45-76 years), presenting visible marionette lines, frown lines, nasogenain fold and crow's feet wrinkles.

The second panel consisted of 30 women (mean age: 58 years, 45-75 years), with visible frown lines.

Applications of treatment creams were banned 15 days before the first appointment and throughout the study period. In addition, exposures to the sun or UV were forbidden one month before the first appointment and during the study.

TYPE OF STUDY, DURATION AND PRODUCT APPLICATIONS

This study was conducted as a single blind on the face. The diagram and table below summarise the application protocol. The creams (see formula in 9. ANNEX) were applied in twice-daily massage for six weeks.



Expert evaluation	MATRIXYL® Morphomics™			Placebo		
	Panel 1	Panel 2	Total	Panel 1	Panel 2	Total
Frown lines	17	15	32	17	15	32
Smoothness recovery	11	11	22	11	10	21
Marionette lines	26*	-	26	26*	-	26
Crow's feet winkles	32*	-	32	32*	-	32

** ½ half face application for each volunteer*

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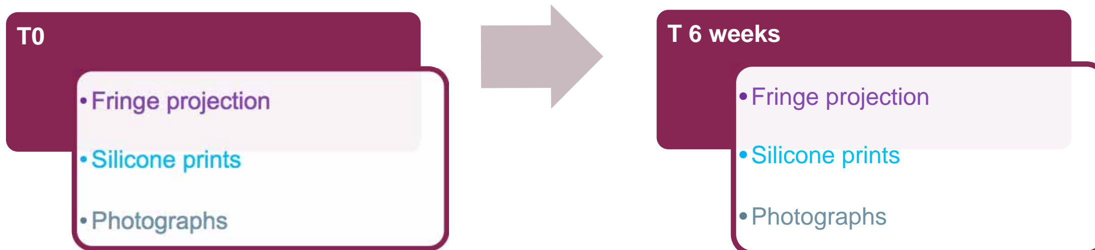
The synopsis of the study can be summarised in the diagram below.



STATISTICS

Statistical studies were performed using the Student's t-test or, if necessary, with a non-parametric Wilcoxon or Mann-Whitney test. For the questionnaire evaluations, a Khi² test was used.

6.1.1. Evaluation by experts of the perceived effects

The standardised photographs obtained for each type of wrinkles were subjected to the quotation of 5 experts. For each volunteer, the expert was faced with a series of photos before and after the applications and had to indicate whether he saw a decrease in wrinkles or not.

The percentage of positive responses for each type of wrinkle is shown in the graph below.

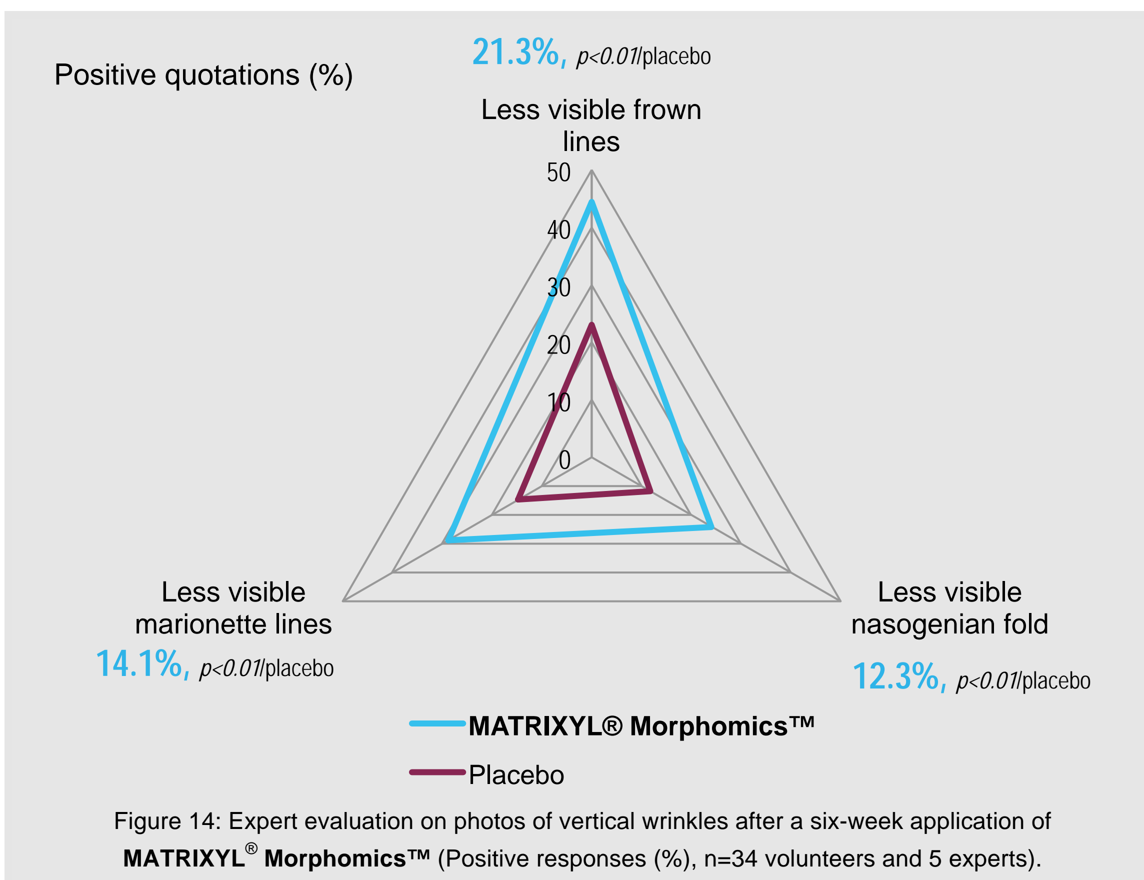


Figure 14: Expert evaluation on photos of vertical wrinkles after a six-week application of **MATRIXYL® Morphomics™** (Positive responses (%), n=34 volunteers and 5 experts).

The analysis of the graph shows that the application of **MATRIXYL® Morphomics™** for 6 weeks produces a noticeably better improvement than that produced by the placebo. This difference in appreciation is significant for the three different types of wrinkles of the face: marionette lines (+14.1%, *p<0.01*), frown lines (+21.3%, *p<0.01*), as well as the nasogenian fold (+12.3%, *p<0.01*).

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T0 T6 weeks

Figure 15: Example of the perceivable improvement of vertical lines after use of 2% **MATRIXYL® Morphomics™** for 6 weeks.

6.1.2. Frown lines

a. Direct measurement of the frown wrinkle parameters

The Fast Optical *In vivo* Topometry System (FOITS) was used to acquire the relief of the frown lines. In this technique, shadows in the form of fringes are projected onto the zone to be analysed at T0 and at the end of the test. The deformations of these shadows by the relief are captured and allow a 3D reconstruction of the relief. An analysis can then extract at each time the volume of the frown lines, their circumference as well as the overall roughness of the surface (Sa). The joint reduction of these three parameters demonstrate a smoothing effect of the surface associated with a specific decrease in the frown lines.
Standardised photos were also produced at both times using a high definition digital photographic bench. The position of the face, the photo and lighting parameters were standardised and controlled to be reproduced over time.

To compare the effect with respect to T0, bilateral tests were performed on matched series. To compare the products among themselves, bilateral tests were carried out on unpaired series.

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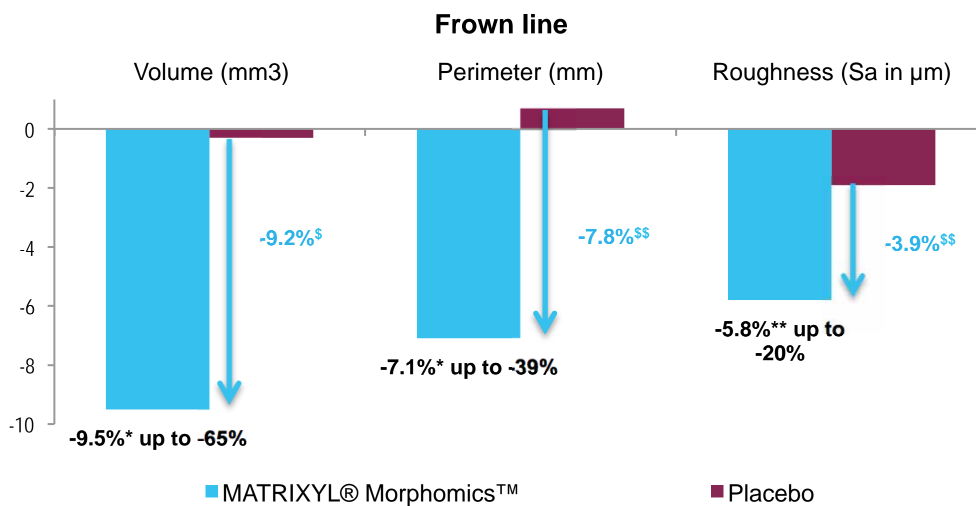
Table 11: Variation of the frown line relief after application of 2% **MATRIXYL® Morphomics™** or a placebo for six weeks (n=32).

FOITS	Volume (mm^3)				Perimeter (mm)				Roughness (Sa in µm)			
	MATRIXYL® Morphomics™		Placebo		MATRIXYL® Morphomics™		Placebo		MATRIXYL® Morphomics™		Placebo	
	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w
Mean	4.75	4.30	3.59	3.58	122.1	113.4	108.8	109.6	70.9	66.8	58.5	57.4
SD	*2.48*	*2.27*	*2.55*	*2.72*	*24.2*	*28.7*	*22.0*	*27.0*	*15.3*	*14.5*	*11.5*	*12.7*
Variation *vs* T0 (%)		**-9.5%**		**-0.3%**		**-7.1%**		**+0.7%**		**-5.8%**		**-1.9%**
Significance Maximum Respondents		*p<0.05* **-65%** 66%		nsd		*p<0.05* **-39%** 66%		nsd		*p<0.01* **-20%** 75%		nsd
Variation *vs* placebo (%) Significance	**-9.2%** *p<0.05*				**-7.8%** *p<0.07*				**-3.9%** *p<0.07*			



* or ** Significant variation compared to T0: *p<0.05* or *0.01* / $ or $$ Significant variation compared to placebo: *p<0.05* or *0.07.*

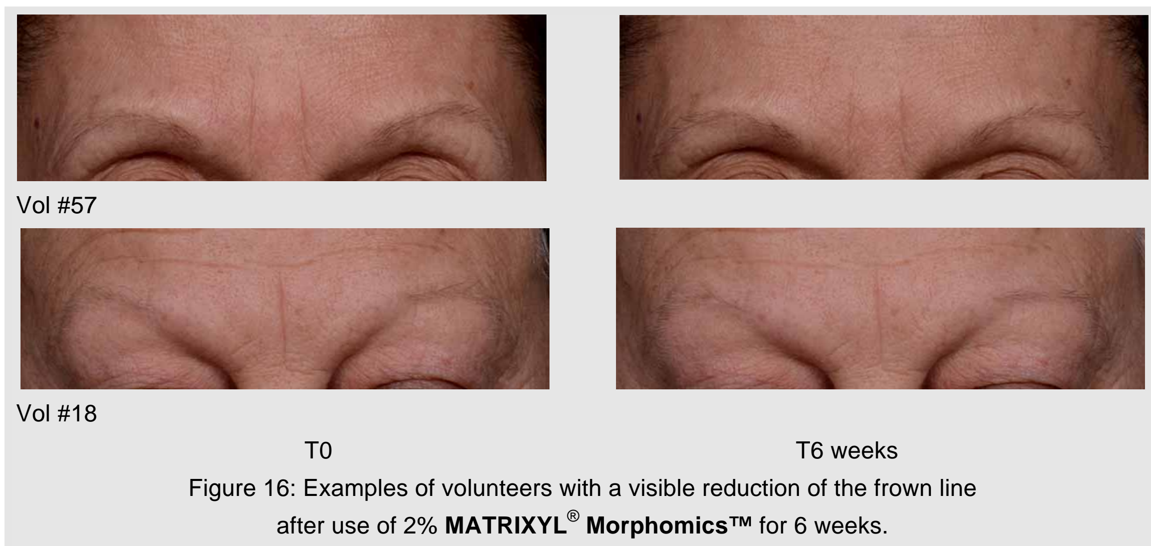


Figure 16: Examples of volunteers with a visible reduction of the frown line after use of 2% **MATRIXYL® Morphomics™** for 6 weeks.

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b. Skin smoothness recovery after frowning

In order to evaluate **MATRIXYL® Morphomics™** benefit on this type of expression lines that are difficult to treat, we have tried an innovative approach.
It is known that with age the skin marks more easily and keeps the imprint of the deformations that are applied to it. By pulling on the skin of the hands, it is observed that the return to normal is more or less long according to the age. We have asked volunteers to frown in a reproducible manner to mark their frown lines, and then, after measuring the volume thus formed, we looked at the return to normal as a function of time (Figure 17). The FOITS technique was used; The return to the initial state is a 100% recovery. Statistical studies to compare products between them used unilateral tests on unpaired series.

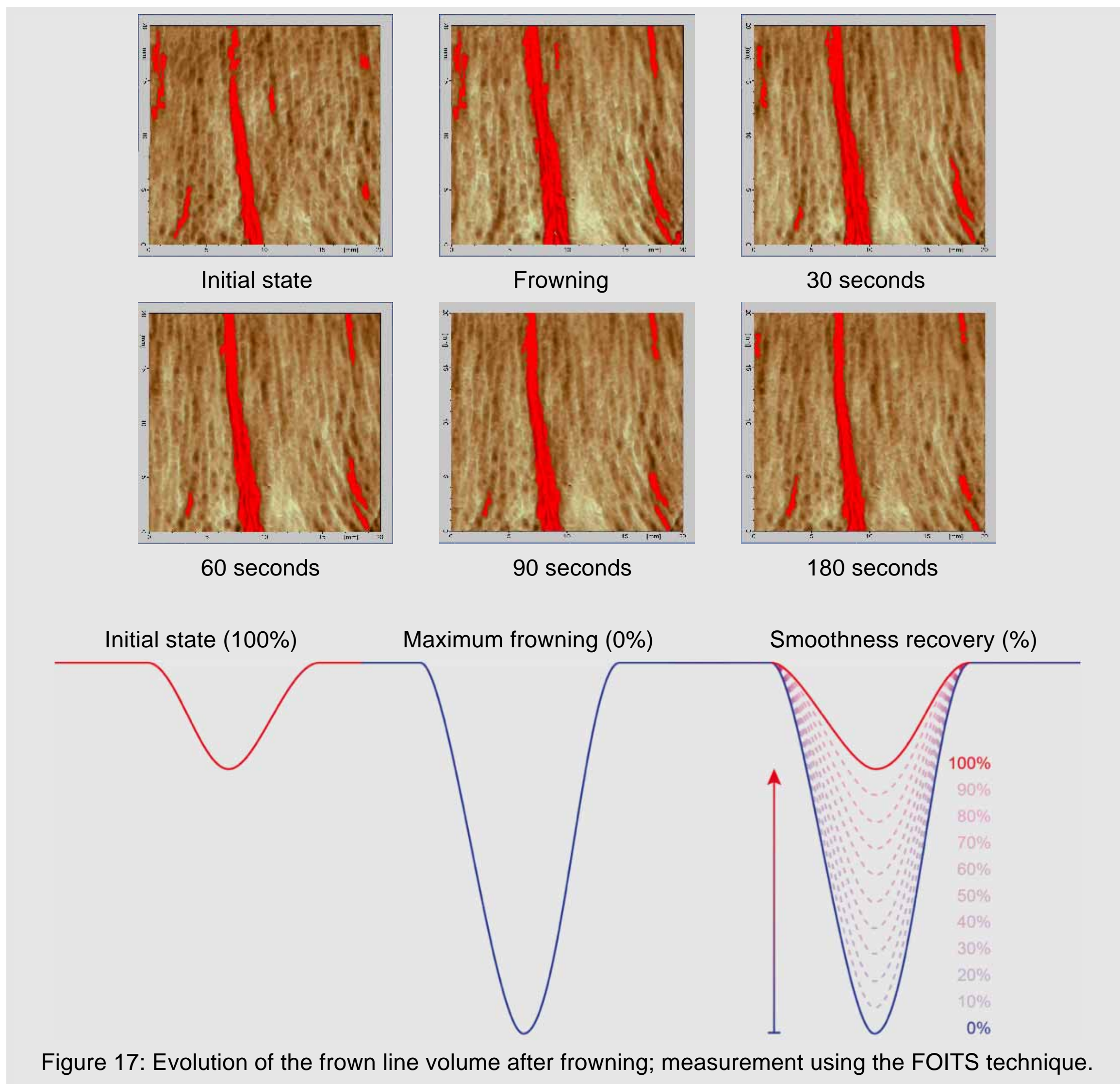

Figure 17: Evolution of the frown line volume after frowning; measurement using the FOITS technique.

RESULTS

This test was performed after use of the 2% **MATRIXYL® Morphomics™** cream or its placebo on the forehead. Only a part of the volunteers from each panel (22 for **MATRIXYL® Morphomics™** and 21 for placebo) were selected on the basis of their ability to follow the recommendations of the experimenters (able to maintain the tensing time, the head position...). The results (Table 12 next page) show that there is a significant difference of approximately 12-13% between the effect of **MATRIXYL® Morphomics™** and the effect of the placebo. This difference in favor of **MATRIXYL® Morphomics™** demonstrates the improvement of the post-tension recovery.

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Table 12: Variation of the recovery after frowning, after use of **MATRIXYL® Morphomics™** or the placebo.

Measurements Dermatop	Volume (mm^3)					
	MATRIXYL® Morphomics™ (n=22)			Placebo (n=21)		
	Time			Time		
	30 sec	60 sec	180 sec	30 sec	60 sec	180 sec
Mean	2.52	3.14	3.94	1.82	2.46	3.20
SD	*1.53*	*1.74*	*1.92*	*1.17*	*1.62*	*1.87*
Smoothness recovery	**52.7%**	**65.6%**	**82.3%**	**39.6%**	**53.5%**	**69.7%**
Significance *vs* placebo	*p=0.07*	*p=0.07*	*p=0.08*			

Conclusion

This original test allowed us to highlight the gain brought by **MATRIXYL® Morphomics™** for the return to normal of the skin of the forehead after a constraint. It is noted that the dynamism of the skin at this level is significantly better for the panel who applied **MATRIXYL® Morphomics™** compared to the placebo. This makes the wrinkle less visible for a longer time.

6.1.3. Marionette lines

Principle

The non-contact technique FOITS was used to acquire the relief of the marionette lines on the volunteers who exhibited a clear one (26 volunteers). For this application another bench than that used to evaluate the frown line above, was used in order to capture a large area of the face. An analysis then allows to extract the volume of the marionette lines and their circumference, before and after treatment. The joint reduction of these two parameters demonstrates the smoothing effect of the surface associated with a specific decrease in bitterness wrinkles.

Standardised photos were also made at both times using the same bench as above (see §6.1.2.a).

Statistical studies were carried out as before (see §6.1.2.a).

Results

T0 T6 weeks

Figure 18: Example of improvement of the marionette lines after use of **MATRIXYL® Morphomics™** for 6 weeks.

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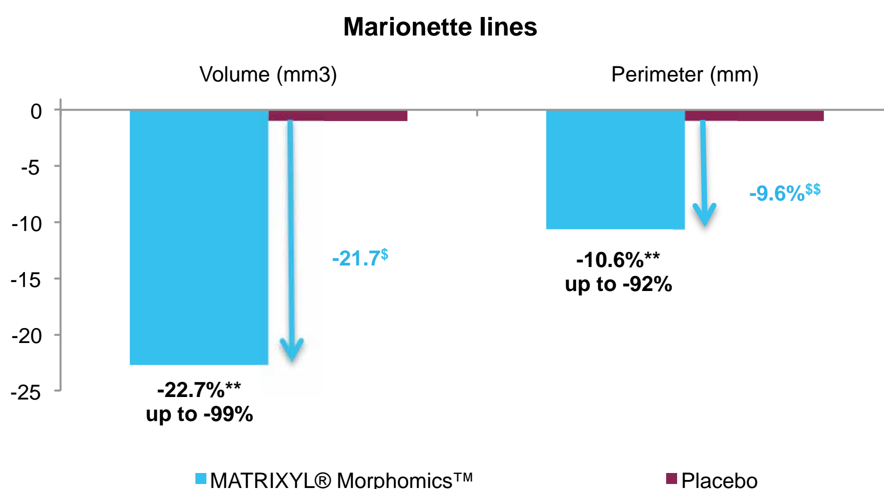
Table 13: Variation of the size of the marionette lines
after use of **MATRIXYL® Morphomics™** or the placebo, n=26.

Measurements AEVA	Volume (mm^3)				Perimeter (mm)			
	MATRIXYL® Morphomics™		Placebo		MATRIXYL® Morphomics™		Placebo	
	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w
Mean	968	748	1185	1173	35.8	32	38.2	37.8
SD	*647*	*497*	*777*	*849*	*11.8*	*15.5*	*15.7*	*16.9*
Variation *vs* T0 (%)		**-22.7%**		**-1%**		**-10.6%**		**-1%**
Significance Maximum Respondents		*p<0.01* -99% 73%		nsd		*p<0.01* -92% 65%		nsd
Variation *vs* placebo (%) Significance	**-21.7%** *p<0.08*				**-9.6%** *p<0.06*			



** significant variation compared to T0: *p<0.01* / $ or $$ significant variation compared to placebo: *p<0.08* or *p<0.06.*

Conclusion

These results show that **MATRIXYL® Morphomics™** used at 2% for six weeks exerts a significant reduction in the volume and the circumference of marionettes lines responsible for a sad expression on the face (reduction by 22.7% and 10.6%, *p<0.01* compared to T0). These two parameters were also significantly lower compared to the placebo (*p<0.08* and *p<0.06*).

6.2. Crow's feet wrinkles

6.2.1. Female panel

Principle

At T0 and six weeks later, we made a negative footprint of the volunteers of panel 1 using a silicone polymer (photo below). The relief of each impression was then analysed by the shadowing technique obtained by grazing LEDs at an angle of 35°. The importance of the shadows is proportional to the height of the wrinkles. The acquisition of this image is carried out by means of a high resolution digital camera and the quantification of the reliefs carried out by a specific Mountains Map software (Digital Surf).

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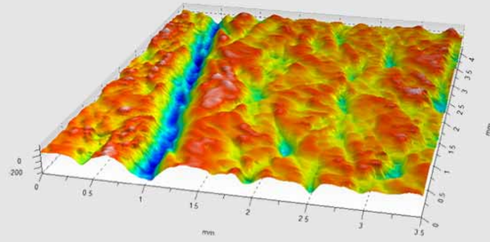
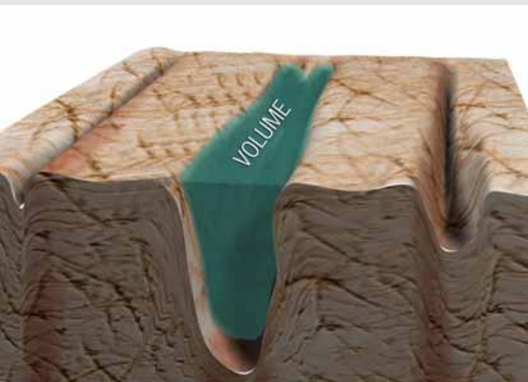


Crow's feet silicone print

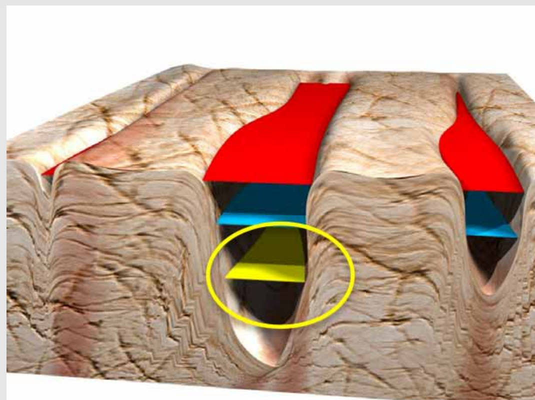
3D representation of the skin surface

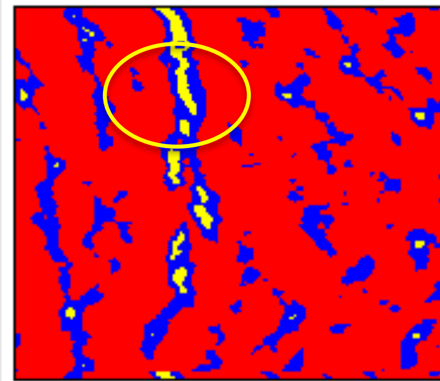
Five parameters representing various aspects of the cutaneous relief were analysed:

- Volume and mean depth of one of the main wrinkles.



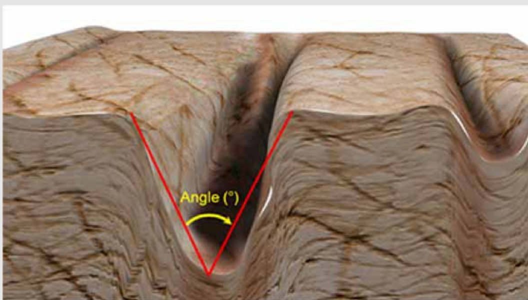
- Surface occupied by medium wrinkles which corresponds to the projected surface of wrinkles greater than 150 µm in depth.

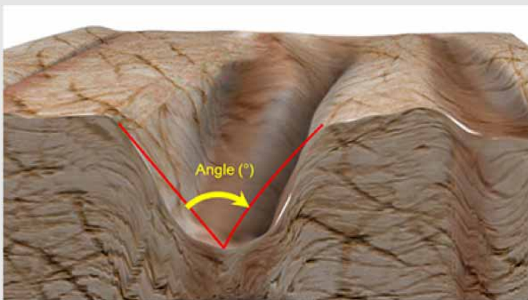




- Angle width of a major wrinkle

This angle can be calculated to quantify the perception of the wrinkle, in fact, a wrinkle with a narrow groove will allow less light to enter than a wide (more open) furrow. The first will be more easily perceived than the second (Figure below).





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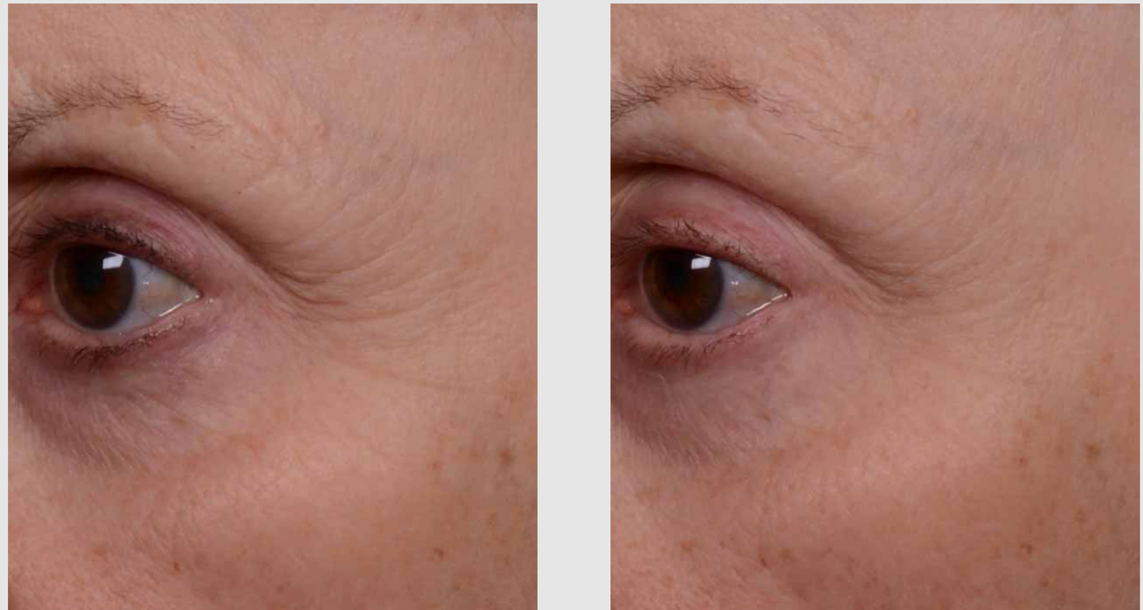
- Mean roughness

Standardised photos were also produced at both times using the same bench as previously (see §6.1.2.a). Statistical studies were carried out as before (see §6.1.2.a).

RESULTS



Vol #13

Vol #88

T0 T6 weeks

Figure 19: Examples of volunteers with a visible reduction of the crow's feet wrinkles after use of 2% **MATRIXYL® Morphomics™** for 6 weeks.

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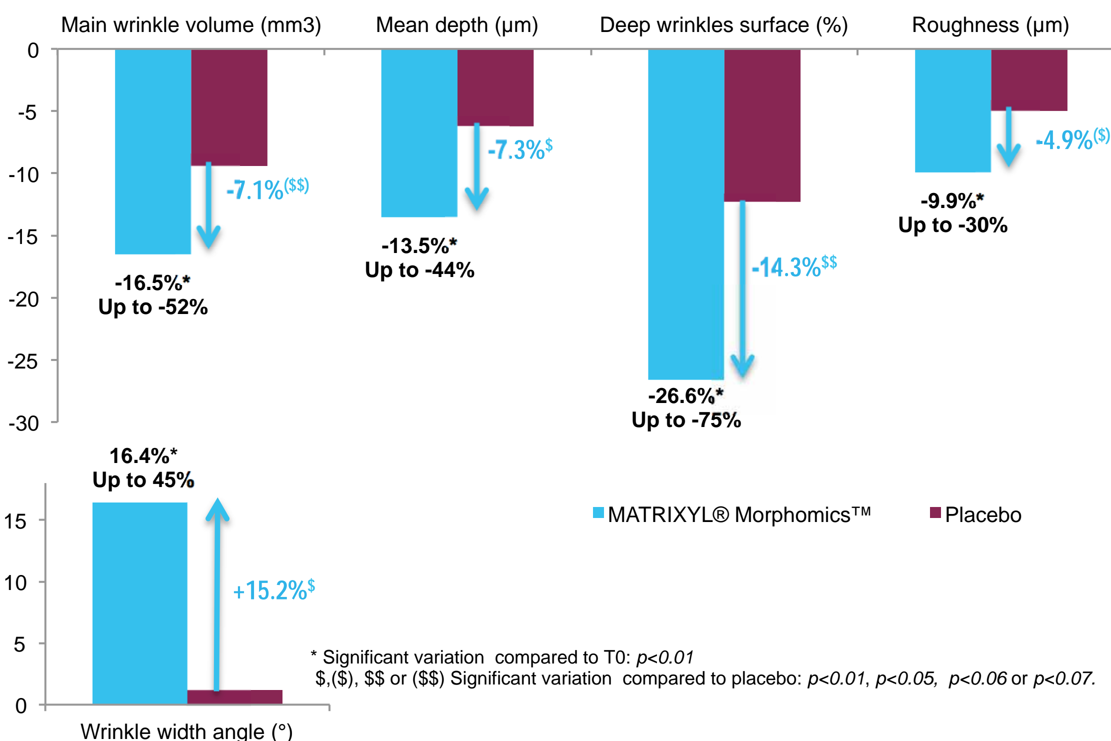
Table 14: Variation of the crow's feet parameters after application of **MATRIXYL® Morphomics™** 2% or its placebo; (n=32).

PRINT measurement	Main wrinkle volume (mm³)				Main wrinkle mean depth (µm)				Deep wrinkle surface (%)			
	MATRIXYL® Morphomics™		Placebo		MATRIXYL® Morphomics™		Placebo		MATRIXYL® Morphomics™		Placebo	
	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w
Mean	697	582	724	656	114.1	98.7	115.4	108.2	3.05	2.24	3.26	2.86
SD	*282*	*286*	*264*	*272*	*23.7*	*25.8*	*24.9*	*28.1*	*1.51*	*1.43*	*1.74*	*1.59*
Variation *vs* T0 (%)		**-16.5%**		**-9.4%**		**-13.5%**		**-6.2%**		**-26.6%**		**-12.3%**
Significance Maximum Respondents		*p<0.01* **-52%** 88%		*p<0.01*		*p<0.01* **-44%** 91%		*p<0.01*		*p<0.01* **-75%** 91%		*p<0.01*
Variation *vs* placebo (%) Significance	**-7.1%** *p<0.07*				**-7.3%** *p<0.01*				**-14.3%** *p<0.06*			

PRINT measurement	Main wrinkle angle width (°)				Roughness (µm)			
	MATRIXYL® Morphomics™		Placebo		MATRIXYL® Morphomics™		Placebo	
	T0	T6 w	T0	T6 w	T0	T6 w	T0	T6 w
Mean	72.5	84.4	81.2	82.2	62.9	56.7	64.5	61.3
SD	*13.5*	*17.2*	*11.8*	*16.0*	*10.5*	*10.7*	*11.9*	*11.8*
Variation *vs* T0 (%)		**+16.4%**		**+1.2%**		**-9.9%**		**-5%**
Significance Maximum Respondents		*p<0.01* **45%** 78%		nsd		*p<0.01* **-30%** 84%		*p<0.01*
Variation *vs* placebo (%) Significance	**15.2%** *p<0.01*				**-4.9%** *p<0.05*			

Crow's feet wrinkles



* Significant variation compared to T0: *p<0.01*
$,($), $$ or ($$) Significant variation compared to placebo: *p<0.01*, *p<0.05*, *p<0.06* or *p<0.07*.

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CONCLUSION

The analysis of the results shows that the application of **MATRIXYL® Morphomics™** leads to a significant improvement of various crow's feet winkle size parameters after six weeks. All these parameters were also significantly improved compared to the placebo ($p<0.01$ to $p<0.07$).

Additionally, the crow's feet wrinkles were also evaluated by a panel of expert on standardised pictures (see 6.1.1). It was obtained that the cream containing **MATRIXYL® Morphomics™** gave significantly better results on the crow's feet wrinkle perception by 10%, $p<0.05$ compared to the placebo.

6.2.2. Male panel

PROTOCOL

SPECIFIC INCLUSION CRITERIA

This study was carried out on a panel composed of 20 men of mean age 59 years (46-68 years), with wrinkles that are clearly visible at the level of the crow's feet. The same conditions as those demanded of the women were required. Moreover they had to shave beard and mustache the morning of the appointments.

TYPE OF STUDY, DURATION AND APPLICATIONS

This study was conducted as a single blind on the face. The volunteers applied a 2% **MATRIXYL® Morphomics™** cream and a contra-lateral placebo cream to the face (see formula in 9. ANNEX). The creams were applied in bi-daily massage for 1.5 months.

The synopsis of the study was identical to that of the women (but without prints).

STATISTICS

Statistical studies were performed using Student's t-test or, if necessary, with a nonparametric Wilcoxon test. Bilateral tests were carried out on matched series.

RESULTS

For this study, the FOITS technique was used.

T0 T6 weeks

Figure 20: Example of a visible reduction of the crow's feet wrinkles for a male volunteer after use of 2% **MATRIXYL® Morphomics™** for 6 weeks.

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Table 15: Variation of the crow's feet surface after application of
MATRIXYL® Morphomics™ 2% or its placebo; (n=20).

FOITS	Wrinkle surface (mm^2)			
	MATRIXYL® Morphomics™		Placebo	
	T0	T 6 weeks	T0	T 6 weeks
Mean	10.11	8.92	9.86	10
SD	*3.88*	*4.80*	*3.33*	*4.04*
Variation *vs* T0 (%)		**-11.8%**		**+1.4%**
Significance **Maximum** **Respondents**		*p<0.05* **-49%** 75%		nsd
Variation *vs* placebo (%) **Significance**	**-13.2%** *p<0.05*			

These results show that **MATRIXYL® Morphomics™** used at 2% for six weeks can significantly help to reduce the relief of the crow's feet in a male panel. The decrease in the surface occupied by wrinkles was -11.8% (*p<0.05*), whereas the change due to placebo was non significant. The performance effect was significantly better compared to placebo with *p<0.05*.

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7. CONCLUSION

With an increasing number of Millennials and Gen-Xers, the potential of the anti-ageing skincare market is predicted to grow by 4.1% per annum in the next few years. Anti-ageing products have shifted to not just target wrinkles and fine lines but also to target issues such as dry skin, skin firmness, uneven skin tone, hyper-pigmentation, and under-eye dark circles. Nevertheless, wrinkles are still the number one concern among the ageing signs after 25 years old, as being the first noticeable sign.

Lipopeptides based on matrikines have demonstrated their efficacy in fighting against the effects of ageing on the dermis that lead to wrinkles and skin sagging. Some lipopeptides can protect the fibroblasts from reaching a senescent state too early (**MATRIXYL® 3000**), other lipopeptides can help maintain the dermal-epidermal junction (**MATRIXYL® synthe'6™**). The SEDERMA R&D teams constantly search for new matrikines that will answer the consumer needs in novel ways of fighting skin ageing either by preventing or treating. **MATRIXYL® Morphomics™** is based on a novel lipopetide, created after a long process of selection among more than one hundred other candidates. The original idea of designing a ramified matrikine was inspired by the cross-link structure of various collagens found in the human body: they include a cyclic amino acid.

The mechanism of action of **MATRIXYL® Morphomics™** was elucidated using the powerful "Proteomics of Youth™" technology. It reboots the connection between the nucleus and the ECM by ensuring the cytoskeleton integrity, and stimulates the collagen fibre production and maturation to rebuild a functional matrix network.
Firstly, we saw a strong activation of the longevigenes™ involved in the FOXO-AMPK pathway by **MATRIXYL® Morphomics™**, meaning a potential benefit on protein metabolism, DNA repair, ROS detoxification, energetic homeostasis and cell viability.
Secondly, using several complementary techniques, we have identified the benefits of **MATRIXYL® Morphomics™** at the intracellular level, on the cytoskeleton viability and at the extracelular level on the matrix network morphology:
The effect of **MATRIXYL® Morphomics™,** showing a strong stimulation of the production of α-actinin, actin, talin, integrin α2/β1, and fibronectin, ensure a viable cytoskeleton: The level of these proteins involved in the structure of the cytoskeleton was restored similarly to young cells. The cytoskeleton dynamism is also maintained through the stimulation of cofilin production. The cross-talking between the intracellular cytoskeleton and the extracellular matrix is fully operational, and the cell can answer to the external information and produce effectively matrix molecules.
Collagen-I is the most abundant macromolecule in the extracellular matrix. **MATRIXYL® Morphomics™** is able to significantly stimulate its synthesis (+42% collagen I alpha 1 chain) but also its maturation process. Indeed, using Proteomics of Youth™ and qRT-PCR, it was demonstrated that **MATRIXYL® Morphomics™** increased the level of a series of enzymes involved in this process.
Finally, collagen III, collagen IV, collagen VI and hyaluronic acid production were also significantly increased by the effect of **MATRIXYL® Morphomics™.**

The benefits of **MATRIXYL® Morphomics™** were studied on a male and a female panel. The vertical lines (frown and marionette lines, and nasogenian fold) and the crow's feet wrinkles were assessed either by picture scoring by a panel of experts, by FOITS or print analysis, before and after use of a cream containing 2% **MATRIXYL® Morphomics™** for six weeks.
The evaluation by a panel of experts on female volunteers concluded that the application of **MATRIXYL® Morphomics™** for 6 weeks produces a noticeably better improvement than that produced by the placebo. This difference in appreciation was significant for the three different types of wrinkles of the

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face: marionette lines (+14.1%, $p<0.01$), frown lines (+21.3%, $p<0.01$), as well as the nasogenian fold (+12.3%, $p<0.01$).

The instrumental evaluations confirmed those results:

- A significant reduction by -9.2% in the frown line volume and -7.8% in the frown line perimeter,
- A improved capacity of the skin to recover its smoothness after frowning,
- A strong and very significant reduction in the marionette line volume (-22.7% up to -99% / T0) and perimeter (-10.6% up to -92% / T0),
- A reduction in the appearance of crow's feet wrinkles for the majority of the male and female volunteers.

By re-establishing and rebooting the skin connections between the fibroblast nucleus and the extracellular matrix, and influencing cellular and dermal morphology**, MATRIXYL® Morphomics™** constitutes an unprecedented way of combating the appearance of wrinkles and especially vertical lines.

It is recommended to incorporate MATRIXYL® Morphomics™ at 2% in anti-ageing skincare.

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9. ANNEX

IN VIVO TEST FORMULATIONS

Raw material	INCI name	Supplier	Placebo	Active product
Part A				
H_2O	Water		qsp 100	qsp 100
Carbopol Ultrez 10	Carbomer		0.30	0.30
Part B				
Brij S2-SS-(RB)	Steareth-2	CRODA	0.40	0.40
Brij S10-SO-(RB)	Steareth-10	CRODA	1.20	1.20
Crodafos CES-PA-(RB)	Cetearyl Alcohol (and) Dicetyl Phosphate (and) Ceteth-10 Phosphate	CRODA	4.00	4.00
Crodacol CS90-PA-(RB)	Cetearyl Alcohol	CRODA	1.50	1.50
Crodamol AB-LQ-(RB)	C12-15 Alkyl Benzoate	CRODA	2.00	2.00
Crodamol OSU-LQ-(JP)	Diethylhexyl Succinate	CRODA	7.00	7.00
Part C				
Glycerin	Glycerin		4.00	4.00
Octanediol	Caprylyl Glycol		0.50	0.50
Part D				
Phenoxyethanol	Phenoxyethanol		qs	qs
Part E				
Potassium sorbate	Potassium Sorbate		qs	qs
Part F				
H_2O	Water		4.00	4.00
NaOH 30%	Sodium Hydroxide		0.40	0.40
Part G				
Excipient	-	-	2.00	0.00
MATRIXYL® Morphomics™	-	SEDERMA	0.00	2.00
Part H				
Fragrance		MLW	0.10	0.10

PROCEDURE:

Part A: sprinkle the Carbomer on the water and allow swelling for 30 minutes. Heat Part A to 75°C in bain-marie. Weigh and heat Part B to 75°C in bain-marie. Mix well. Weigh and melt Part C to 45°C. Mix well. Allow Part C to cool down. Add Part D to Part C. Pour Part C+D into Part A with rotor stator stirring (s=1000 rpm). Homogenise well. Pour Part B into the previous part with quick rotor stator stirring (s=2500 rpm). Homogenise well. Add Part E extemporaneously with rotor stator stirring (s=1000 rpm). Neutralise with Part F and mix well. Add Part G and stir well. Add Part H and mix well.

APPEARANCE: White emulsion; pH: 5.80± 0.5; Viscosity: 70.000 cps ± 10% Sp.93, 2.5 rpm, 1 min , 25°C, Brookfield DV-I Prime.

STABILITY: 3 months at 4°C, 25°C, 40°C and 1 month at 50°C; challenge test validated norm ISO 11930.

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