Innovation you can build on™

Matrixyl®synthe'6®

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MATRIXYL® synthe'6®

Patents and publication: FR 2 941 231 ; WO 2010/082175 ; WO 2010/082177 ;
EP 2 382 230 ; EP 2 382 231 ; US 8,697,656 ; JP 5643769

WRINKLE FILLING BY STIMULATION OF THE SYNTHESIS OF 6 MAJOR STRUCTURAL COMPONENTS OF THE SKIN

SYNOPSIS

Description: Palmitoyl-Lysyl-Dioxymethionyl-Lysine, a matrikine-mimetic peptide, which stimulates the synthesis of matrix molecules and helps fill wrinkles.

INCI Name: Glycerin (and) Water (Aqua) and Hydroxypropyl Cyclodextrin (and) Palmitoyl Tripeptide-38

Demonstrated cosmetic activity

In vitro (Tests performed with 5 ppm Pal-KMO$_2$K equiv. to 2% MATRIXYL® synthe'6®)

➢ ***Increase in the synthesis of matrix molecules:***

- HSP47: .....+123%; (*p<0.01*)
- Collagen I:.....+111% on HDF; (*p<0.01*)
  .....+37% on explants; (*p<0.01*)
- Collagen III:.....+104%; (*p<0.01*)
- Fibronectin: .....+59%; (*p<0.01*)
- Hyaluronic acid: .....+174%; (*p<0.01*)

➢ ***Boosting the effect of luminotherapy on UV-aged skins:***

- Collagen: ..... +12%; (p<0.01)

➢ ***Increase in the synthesis of molecules at the dermal-epidermal junction:***

- Collagen IV: ..... +42%; (p<0.01)
- Laminins:..... +75%; (p<0.01)

In vivo

Clinical study to assess the smoothing and filling of forehead lines and crow's feet. Panel: 25 women who applied 2% **MATRIXYL® synthe'6®** cream twice a day for two months:

➢ ***Anti-wrinkle effect on the forehead determined by fringe projection (FOITS):***

- Reduction in the volume of a main wrinkle: .....-31%; (p=0.05)
- Reduction in visible cutaneous roughness (lifting effect):.....-28%; (p<0.05)
- Reduction in the maximum depth of a main wrinkle: .....-16.3%; (p<0.05)

➢ ***Anti-wrinkle effect on crow's feet determined by fringe projection or imprinting:***

- Reduction in the surface occupied by deep wrinkles:.....-28.5%; (p<0.05)
- Reduction in the volume of a main wrinkle: .....-21%; (p<0.05)
- Reduction in the mean depth:.....-15%; (p<0.05)
- Reduction in visible cutaneous roughness (lifting effect):.....-12.6%; (p<0.05)
- Opening of the wrinkle: .....+8.4%; (p<0.05)

Recommendations for use:

- ***Overview:***

  - Recommended pH: 3 - 9
  - Add **MATRIXYL® synthe'6®** to the emulsion preferably between 25 and 50°C; depending on the type of formulation, temperatures up to 80°C can be acceptable for maximum 2 hours.
  - Solubility: Water soluble

- ***Recommended concentration for use:*** ***2%***

Toxicology:

Patch-test on humans (10 volunteers)
HET CAM + Neutral red test
HRIPT (100 volunteers)
Ames' test
Expert certification

CONTENT

06/2015/V2

1. INTRODUCTION

Skin ageing causes a decrease in the condition of the skin and an increase in the appearance of imperfections, such as wrinkles. These furrows appear prematurely in stressed areas of the skin and are called expression wrinkles. Women sometimes find these furrows charming in men, but they never find them attractive on themselves.

Over time, daily constraints build up which progressively create forehead lines and crow's feet. The skin ends up with deep, permanent marks where tiny folds were almost undetectable in the past. Moreover, overexposure to ultraviolet rays or other external factors, such as pollution, smoke, dehydration…, tends to deepen these lines. They become furrowed, which first makes them more visible only under certain lighting, and then makes them permanently visible.

The properties of the dermal macromolecules which support the epidermis, such as collagen or hyaluronic acid, provide much of the mechanical or physiological properties of the skin. During the ageing process, whether simply chronological or due to external factors, these macromolecules decrease in quantity; the complexity of the dermal network that joins them diminishes, and the intermolecular links become less effective.

Even though ultraviolet luminotherapy, which was the subject of a Nobel Prize in 1903 due to its immunostimulating qualities, was quickly abandoned, visible-light luminotherapy has been used for thirty years to re-synchronise body rhythms. Some recent data, still rare but more and more numerous, inform that certain visible lights exert the power to activate the synthesis of dermal macromolecules.

Therefore, it seems worthwhile to have an active ingredient capable of stimulating, in a coordinated fashion, the synthesis of macromolecules of the dermis and the dermal-epidermal junction. Using luminotherapy to reinforce this stimulation may provide additional benefits to such a product.

WRINKLES: PRESENTATION

Wrinkles appear gradually; they are the result of the multiple, daily stresses that our face undergoes. Each frown, and unfortunately, each smile and laugh, contributes to gradually deepening these lines in the skin.

Slow but cumulative destruction of the macromolecules of the dermis and the dermal-epidermal junction progressively diminish the youthful qualities of the skin. During the ageing process, the synthesis of filling macromolecules diminishes, and destruction is no longer offset as it should be. Synthesis is no longer integrated, and molecular associations become unbalanced. The dermal-interdermal junction, which is highly complex and rich in macromolecules, becomes less dense and therefore less able to adapt to stress.

In addition to the mechanical effects of our facial expressions, daily stresses, such as oxidising and micro-inflammatory stresses (smoke, pollution, food), and especially ultraviolet light (natural or artificial) lead to skin damage. They aggravate the mechanical stress caused by our expressions.

Wrinkles can be of different degrees, depending on their location, their number and how bad they are. Crow's feet are well-known and have been the subject of many studies. Forehead lines are also unwelcome, since they mark the face with highly visible, horizontal furrows.

SIX CONSTITUTIVE DERMAL/EPIDERMAL MACROMOLECULES AND HSP47

The macromolecular composition and the three-dimensional arrangement of proteins, proteoglycans and GAGs in the skin are very complex (PUGLIESE, 2006). Amongst the most important entities,from the quantitative point of view, one can find the collagen I, collagen III, fibronectine and hyaluronic acid (hyaluronan) in the dermis. When collagen IV and laminin-5 in the dermal/epidermal junction are important to ensure the cohesive function. Here is a brief abstract describing these six macromolecules and their role in the skin:

Collagen I: Along with collagen III, it constitutes by far the most abundant collagen in the tissues (approx. 30%), and overall in the dermis (80%). The collagen molecules, secreted by the fibroblasts, auto assemble to each other in order to form the characteristic fibres of the tissue. In that way they constitute the cross-linked structure able to resist the various physical stresses (extensions) the skin undergoes.

Collagen III: It is the collagen "of youth", produced by the young fibroblasts, but also during the early phases of wound healing. It is less resistant than collagen I, but could be partly responsible for the skin smoothness. The proportion of collagen III decreases with age to the advantage of collagen I.

Fibronectin: The fibronectins, for which there are a lot of variants, are multi adhesive soluble proteins situated in the extracellular matrix. By joining the integrins, they establish the attachment of the cells to the collagen fibrils (type I, II, III and V), and to the fibrin. Fibronectins are dimeric molecules, each chain consisting in about 2500 aminoacids. Fibronectins also exert an important function in cell migration and differentiation (see figure 1).

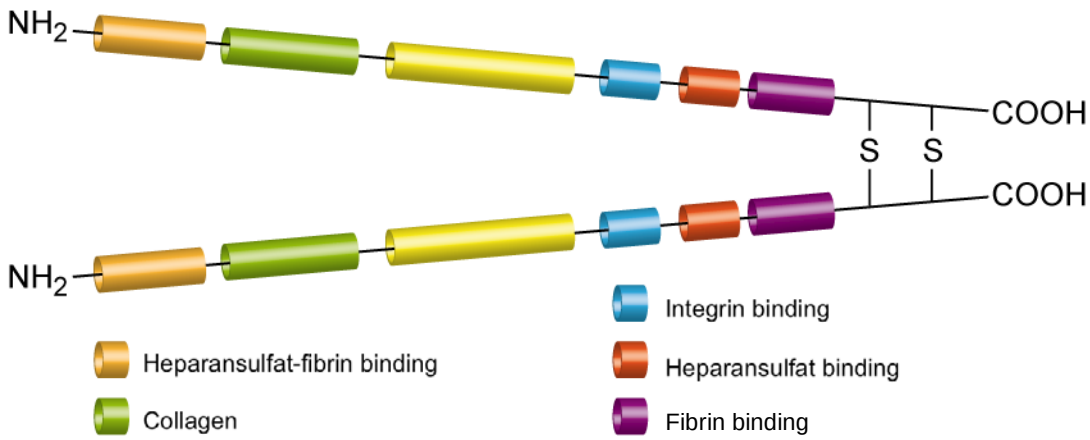


Figure 1: Structure of the chains of fibronectin

Hyaluronic acid belongs to the glycosaminoglycan group (GAG), extracellular matrix polysaccharides. It is distinctive from the other GAGs (chondroitin sulphate, dermatan sulphate, heparin...) due to the absence of sulphur, a notable anti free radical power and its very high molecular weight. Besides its role in the tissue structure, hyaluronic acid is known as an important component responsible for water retention in the tissue and for a regulatory activity in cell proliferation and migration (wound healing), in the dermis as well as at the basal layer level in the epidermis.

The **collagen IV** and **laminin-5** proteins:
The collagen type IV is a helicoidal trimeric protein being part, as laminin-5, of the anchorage proteins of the basement membrane, at the interface of the extracellular matrix (dermis) and the basal layer cells (epidermal proliferative keratinocytes).

Laminin-5 is an adhesive glycoprotein involved in the healing of injured epidermis. The laminin deposition on the dermal collagen helps migration and anchorage of keratinocytes. Its 3D structure, similar to a cross with stretch arms, could explain its structuring and adhesive properties.

HSP47

The macromolecules quoted previously are synthesised according to a precise "ritual", which often consists of a maturation process so that the molecule produced reacts appropriately to the needs of the body. Problems with these maturation processes create defective and ineffective proteins (MATSUOKA *et al*., 2004).
For example, collagen I and collagen IV, which are produced by dermal cells and deposited in extracellular spaces, undergo a maturation phase to ensure skin elasticity on the one hand and the linking of the dermis to the epidermis on the other hand.

Similarly to a builder's yard where a project manager coordinates the works, the chaperone protein HSP47 (heat shock protein 47, NATSUME *et al*., 1994) ensures that role during certain phases of the skin (re)building, in particular for collagen fibre maturation. This protein, which is specific to the collagen-producing cells, can bind to collagen I through collagen V, and in particular binds to still-immature procollagen I to prevent it from folding over on itself.
This function reinforces and protects the collagen, especially from proteases, before it exits the cell.

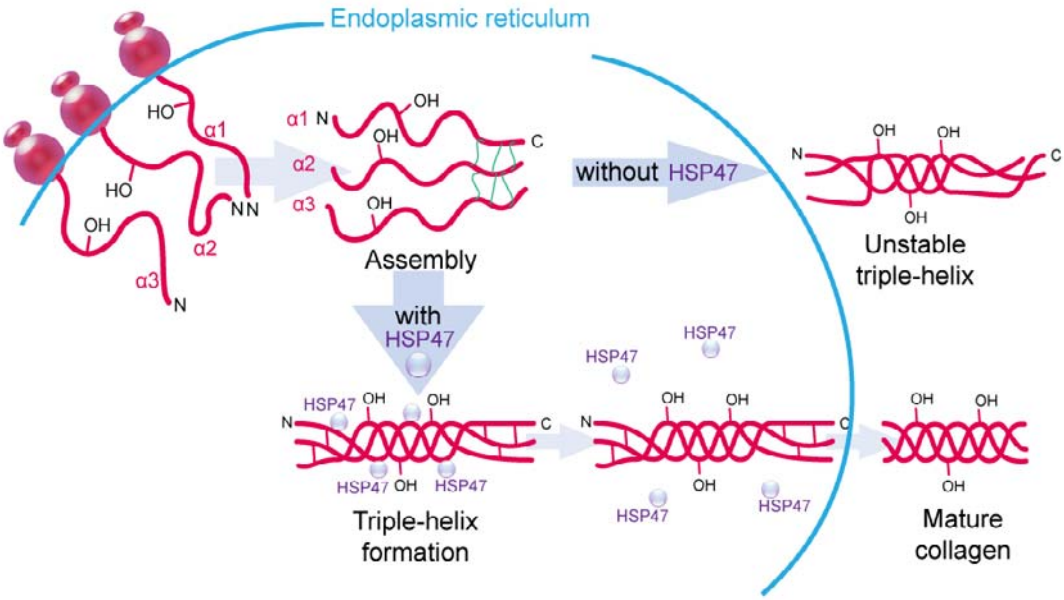


Figure 2: Role of HSP47 in the formation of collagen

Mice without HSP47 have fragile skin. Their collagen is more sensitive to proteases and thermal shock (MATSUOKA *et al*., 2004). In contrast, experimental over- expression in mice increases collagen I secretion (TOMITA *et al*., 1999; ROCNIK *et al*., 2002).

Furthermore, the absence of HSP47 reduces collagen IV synthesis, and the resultant collagen IV is fragile with respect to proteases (MATSUOKA *et al*., 2004), which is detrimental to the formation and maintenance of the dermal-epidermal junction.

In addition, HSP47 decreases with age, which contributes to lower dermal elasticity.

Therefore, it is evident that it is necessary to have coordinated and integrated synthesis of several matrix components.
In particular, it is appropriate to ensure the concurrent synthesis of collagen I and its chaperone HSP47 in order to redensify and volumise the dermis.

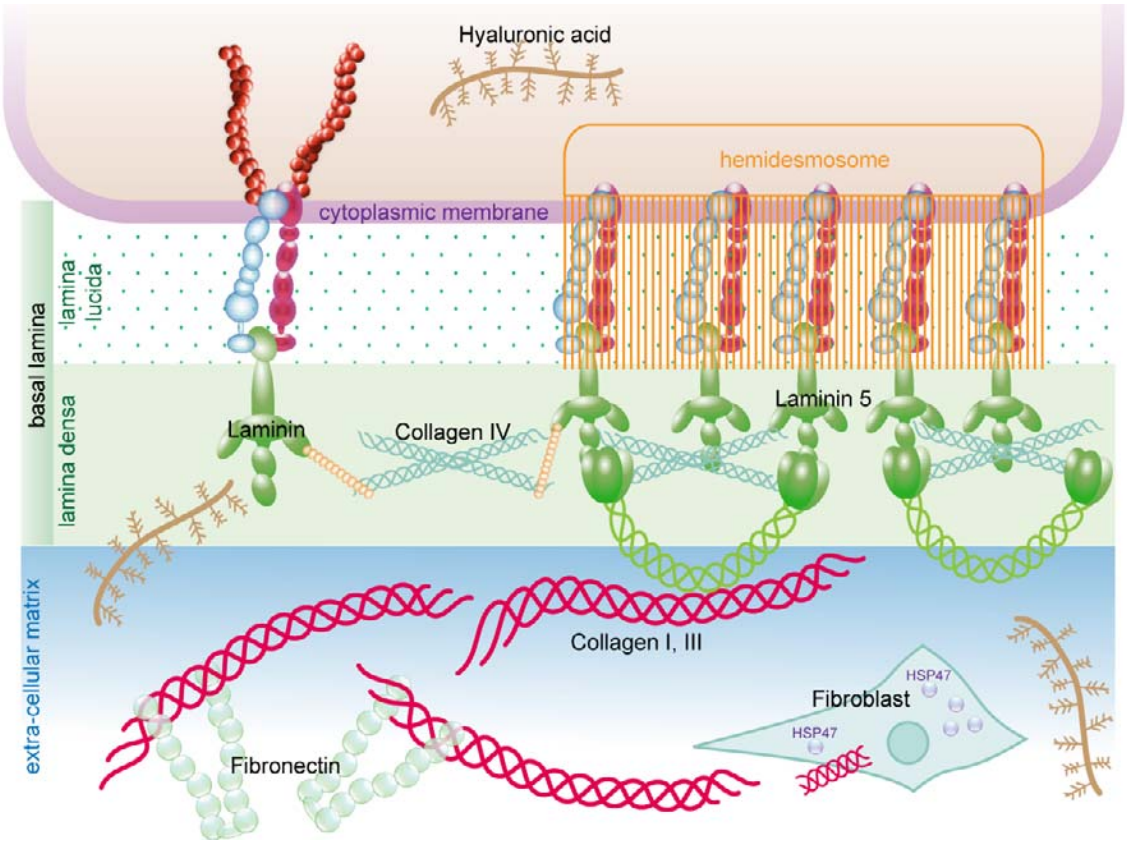


Figure 3: Structural diagram of the proteins of the dermal-epidermal junction and the dermis

THE SEDERMA CONCEPT

The decrease of forehead and crow's feet wrinkles, the major signs towards visible ageing constitute a challenge to be won: it is necessary to resendify the dermal tissue and to structure the scaffolding of the interfaces

As per the analogy of the building yard, a project manager is essential to initiate the process and specify the desired result. Thus, SEDERMA offers a matrikine-mimetic peptide:

MATRIXYL® SYNTHE'6®

Which ensures that role thanks to its capacity to:

- provide building material by stimulating the synthesis of six major molecules (hyaluronic acid, collagen I, III and IV, laminins and fibronectin),
- manage the adequate architectural organisation thanks to the chaperone protein HSP47, which completes the maturation and stabilisation of fibres.

The consumer will benefit from this product a rejuvenating lifting effect thanks to the joint reduction of the forehead and the crow's feet wrinkles, with only one intervention.

PRESENTATION OF MATRIXYL® SYNTHE'6®

MATRIXYL® synthe'6® contains the dioxygenated derivative of a natural peptide, Palmitoyl-Lysyl-Dioxymethionyl-Lysine or Palmitoyl-KMO_2K.

Our studies have demonstrated the usefulness of this matrikine-mimetic peptide in relaunching matrix macromolecular synthesis to increase the volume and firmness of the skin.

- ***Palmitoyl-KMO_2K***

The Lysine-Methionine-Lysine tripeptide (or KMK) is a sequence found naturally in matrix proteins: Collagen VI and laminins. Moreover, it is found in a DNA-protecting protein: HSP70.

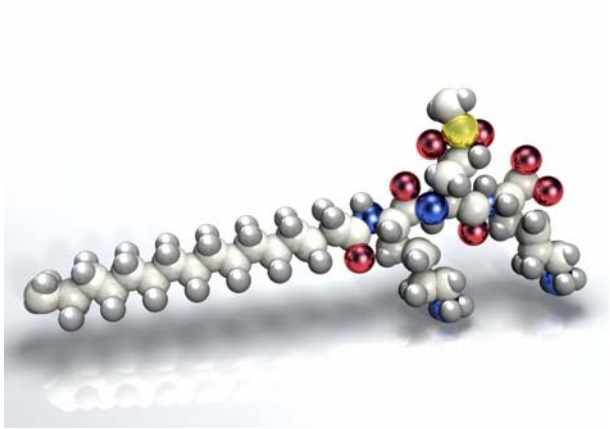


Figure 4: Palmitoyl-KMO_2K-OH

Preliminary studies demonstrated that this sequence presents strong structural and functional similarities to known matrikines. Matrikines are short amino acid chains that come from the breakdown of dermal proteins (KATAYAMA *et al*., 1993; LINTNER and PESCHARD 2000). In particular, they have a regenerating activity during healing.

The sequence used stimulates the synthesis of fibroblast collagen in culture. The studies conducted, particularly on monooxygenated or dioxygenated matrikine derivatives, have demonstrated the interest, both qualitatively and quantitatively, in such a modification in the synthesis of significant macromolecules (see table 1).

Table 1:

Neosynthesis of **collagen I**, **fibronectin** and **collagen IV** by human dermal fibroblasts in the presence of Pal-KMK and Pal-KMO_2K. Measured by Elisa (*n=5)*

		Collagen I (in % of the control)	**Fibronectin** (in % of the control)	**Collagen IV** (in % of the control)
Control	-	Reference	Reference	Reference
Pal-KMK	4 ppm	+21 ± 11 °	+8 ± 14 °	-6 ± 7 °
	6 ppm	+63 ± 22 **	-4 ± 21 °	-10 ± 6 °
Pal-KMO_2K	4 ppm	+188 ± 8 **	+42 ± 21 **	+27 ± 14 *
	6 ppm	+187 ± 86 **	+109 ± 20 **	+97 ± 32 **

NS: statistically non significant difference compared to risk control α= 0.05

*° : statistically non significant difference; *: statistically significant difference (p<0.05); ** very significant difference (p<0.01) compared to the control.*

These results clearly show the potentialisation provided by the stable dioxygenation of the methionine of the KMK tripeptide.

The effect stimulates not only collagen I synthesis, but also fibronectin synthesis and the synthesis of a critical component of the dermal-epidermal junction: collagen IV.

2. EFFICACY STUDIES

2.1. *In vitro* studies

2.1.1. Elements that reinforce matrix structures

Protocol

Human Dermal Fibroblasts (HDF) were cultivated in the presence of Pal-KMO_2K.
After contact:

- the culture supernatants were tested by Elisa (Collagen I, fibronectin),
- or the cells were broken down and a Western Blot Test was performed (HSP47),
- or the constituents of the cell layer received immunofluorescent labelling (Collagen I, III) to illustrate the deposition of these structural macromolecules.

Furthermore, tests were conducted on skin explants from a 53-year-old donor. In a first serie of trials, the explants were experimentally aged using a dermocorticoid. The explants then received the Pal-KMO_2K formulated in a cosmetic cream (see Appendix) that was applied to the surface of the explants twice a day for five consecutive days. At the same time, the control received a placebo cream.
In a second serie of trials, non-aged explants from the same donor underwent the same treatment.

After contact, the skin was frozen and cut using a microtome, and the development of the proteins was monitored using immunolabelling (Collagen I, laminin-5). Using such a study model is interesting, as a certain number of macromolecules had stronger expression due to the proximity of the two layers (MARONNET *et al*., 2006).

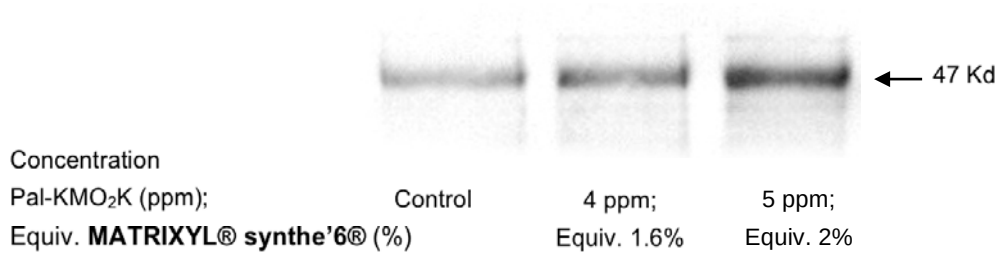
a. HSP47

For this study, the single-layer HDFs were broken down after contact with **MATRIXYL® synthe'6®.**

The quantity of HSP47 was evaluated by Western Blot after deposition and migration on an appropriate membrane, then immunolabelling was performed and results were revealed by chemiluminescence. The intensity of the bands was quantified using software intended for this purpose.

Results

Table 2:
Increase in **HSP47** by **MATRIXYL® synthe'6®** (Western blot)



	Control	Equiv. 1.6% MATRIXYL® synthe'6®	Equiv. 2% MATRIXYL® synthe'6®
Mean intensity of the bands (AU)	37.5 ± 7.7	56.0 ± 10.9	83.7 ± 8.9
% Variation / Control	Reference	**+49%** *p<0.01*	**+123%** *p<0.01*

AU: Arbitrary Units

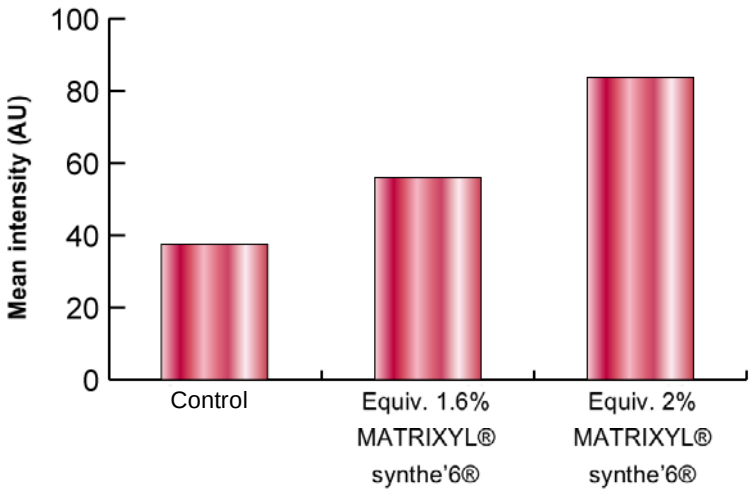


Figure 5: Increase in HSP47 synthesis by **MATRIXYL® synthe'6®** (Western Blot)

b. Collagen I

The stimulation of collagen I synthesis was evaluated by four complementary methods:

1. Elisa of the supernatants of single layer HDF culture;
2. Analysis of photo images of immunolabelled HFD cell layers;
3. Analysis by Western Blot of immunolabelled HFDs;
4. Analysis of photo images of sections of immunolabelled skin explants.

Results

Table 3:
Increase in **collagen I** in the presence of
Pal-KMO_2K / equiv. **MATRIXYL® synthe'6®**

1. Elisa of the supernatants of single layer HDF culture *(n=5)*

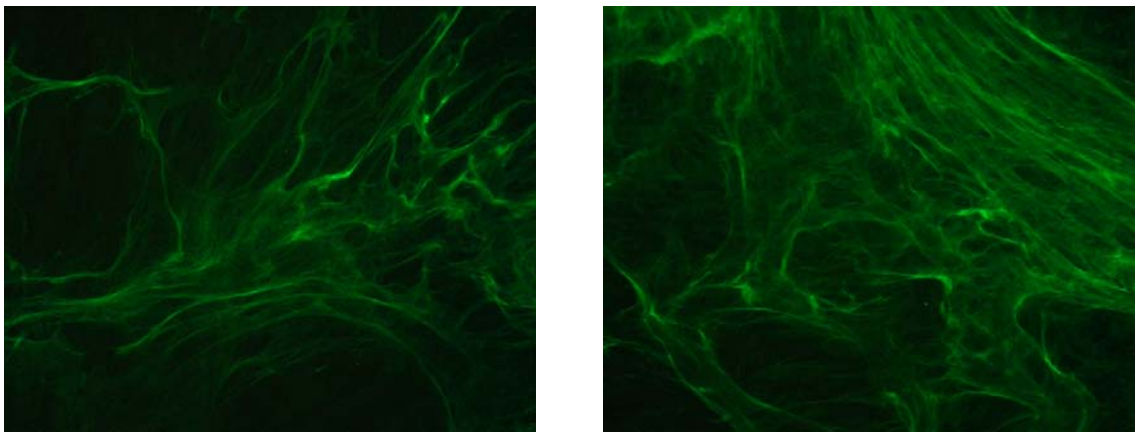
	Pal-KMO_2K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Concentration	**Collagen I in ng/10^6 cell.**	**% Variation; significance**
ELISA / HFD	Control	5128 ± 587	Reference
	2 ppm Equiv. 0.8%	6381 ± 274	**+24%** *p<0.01*
	4 ppm Equiv. 1.6%	8233 ± 794	**+61%** *p<0.01*
	5 ppm Equiv. 2%	10795 ± 659	**+111%** *p<0.01*

TGF-β1 to 10^{-6}%: +55% (*p<0.01*)

2. Analysis of photo images of immunolabelled HFD cell layers (n=3; 10 photos/case)

	Pal-KMO$_2$K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Concentration	**Collagen I In AU/10^6 cell.**	**% Variation; significance**
Matrix immunolabelling/HFD	Control	2.42 ± 1.40	Reference
	5 ppm Equiv. 2%	4.96 ± 1.86	**+105%** *p<0.01*

AU: Arbitrary Units



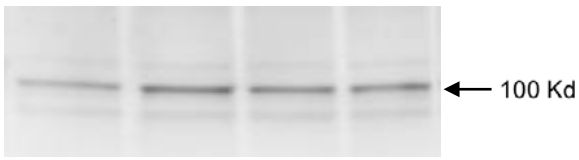
control

Pal-KMO$_2$K 5ppm
Equiv. MATRIXYL® synthe'6® (2%)

Figure 6: Variation in the synthesis of **collagen I** by the HFDs in the presence of **Pal-KMO$_2$K** equiv. **MATRIXYL® synthe'6®** (IMF Method)

3. Analysis by Western Blot of the quantity of collagen I produced by the HFDs after contact with Pal-KMO$_2$K

The quantity of collagen I was evaluated after deposit and migration on an appropriate membrane, then immunolabelling was performed and results were revealed by chemiluminescence. The intensity of the 100Kd band is quantified by special software intended for this purpose. The result was standardised through a total protein level determination.



Concentration	Control	TGF-β1	4 ppm	5 ppm
Pal-KMO$_2$K (ppm);			Equiv.	Equiv.
Equiv. **MATRIXYL® synthe'6®** (%)			1.6%	2%

Figure 7: Western Blot

	Pal-KMO_2K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Protein concentration (mg/mL)	**Intensity/mg of proteins**	**% Variation**
Control	0.441	227	Reference
TGF-β 10^{-6}%	0.454	352	+55%
Pal-KMO_2K 4 ppm; Equiv. 1.6%	0.423	311	+37%
Pal-KMO_2K 5 ppm; Equiv. 2%	0.464	336	+48%

4. Analysis of photo images of immunolabelled sections of skin explants (n=5 skins/case; 50 photos per case)

	Not experimentally aged		**Experimentally aged**	
	Fluorescence intensity	**% Variation Coll I / control**	**Fluorescence intensity**	**% Variation Coll I / control**
Control cream	15.7 ± 4	Reference	9.1 ± 1.9	Reference
Cream with 2% **MATRIXYL® synthe'6®**	18.9 ± 5.2	**+20%** *(p<0.01)*	12.5 ± 3.9	**+37.5%** *(p<0.01)*

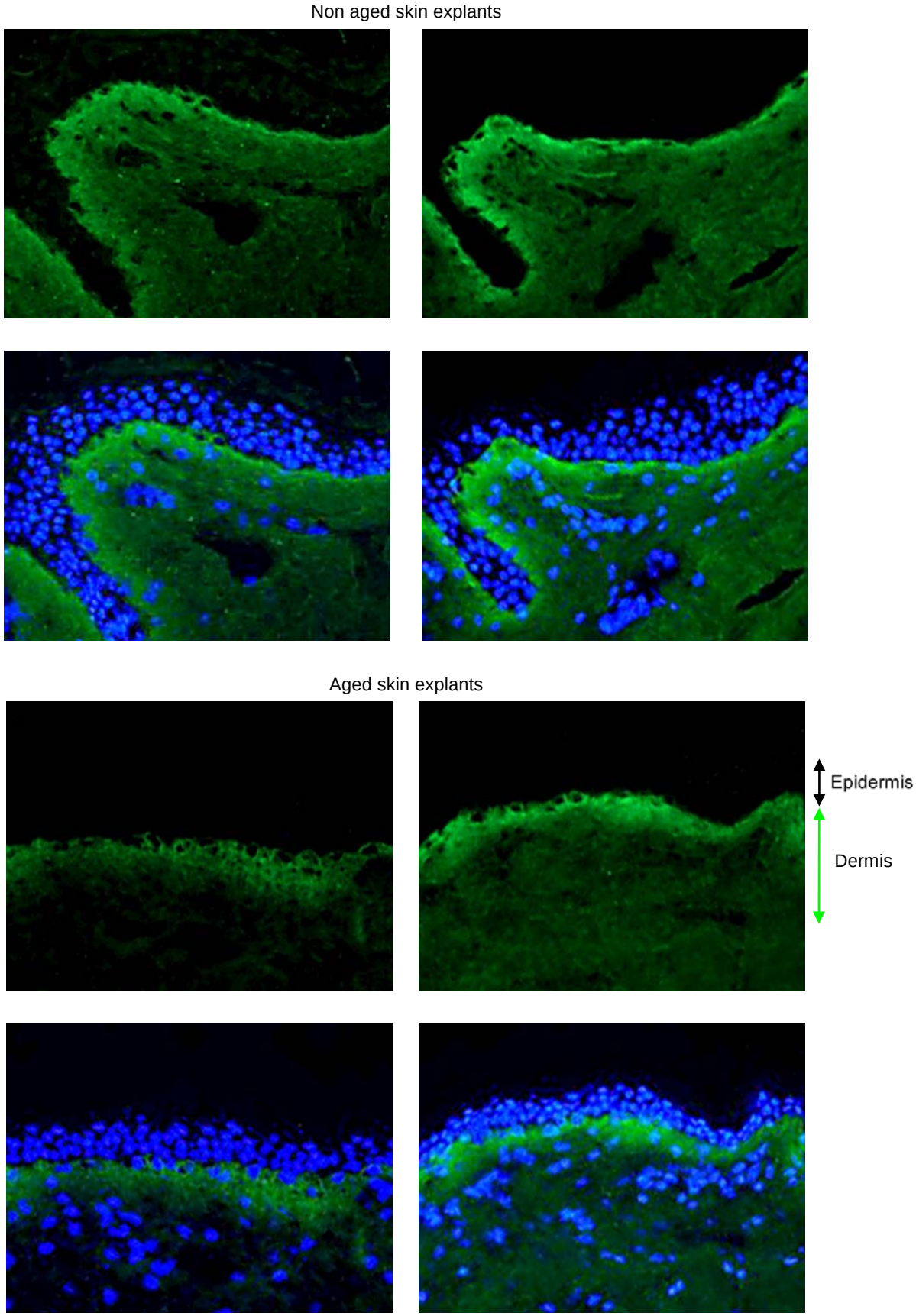


Figure 8: Variation of the synthesis of **collagen I** in the superficial dermis after application of a cream with 2% **MATRIXYL® synthe'6®**

The results obtained with these different techniques corroborate and demonstrate a stimulation of collagen I synthesis by the Pal-KMO_2K and by the cream containing 2% **MATRIXYL® synthe'6®**.
The experimental ageing with corticoids helped to show the benefit of using this kind of product to support collagen I synthesis.
This Pal-KMO_2K stimulation of collagen I is accompanied by a concurrent increase in its chaperone HSP47 protein.

c. The boosting effect of luminotherapy on collagen (Subcontracted study, Gredeco)

Skin explants from six different donors were kept alive artificially in an appropriate environment.
These explants were experimentally aged using a single dose of UVA + UVB radiation. The explants were then exposed to the light of a TriWings lamp (600 - 650 nm) for 10 minutes and the creams, either the control or a cream containing 2% **MATRIXYL® synthe'6®**, were applied to the skin explants.

This combined light and cream treatment was repeated eight times. The skin explants were fixed and stained using red Sirius in order to quantify the collagen of the superficial and middle dermis by image analysis.

Table 4:
Increase in collagen under the effect of luminotherapy in the presence of **MATRIXYL® synthe'6®**

Aged Skin + Lamp and:	**Collagen in % of occupied surface**	**% Variation; significance**
-	55.0 ± 8.6	**Reference**
Control cream	57.3 ± 7.3	# +4%; *ns*
2% **MATRIXYL® synthe'6®** cream	**61.5 ± 6.6**	***# +12%; p<0.03***

The skin explants that were aged and exposed to the lamp show a 7.4% increase in staining compared to the aged skin that was not subsequently exposed to light. Therefore, the lamp used did indeed stimulate collagen synthesis in the experimentally aged skins.

The application of 2% **MATRIXYL® synthe'6®** further stimulated this synthesis. Hence, it is clear that **MATRIXYL® synthe'6®** reinforced the effect of luminotherapy as an anti-ageing treatment. Together, the stimulation was approximately +20%.

d. Collagen III

The stimulation of collagen III synthesis was evaluated using two additional methods:

1. Analysis of photo images of immunolabelled HFD cell layers *(n=3)*;
2. Study of the DNA microarray of cellular extracts (BioAlternatives).

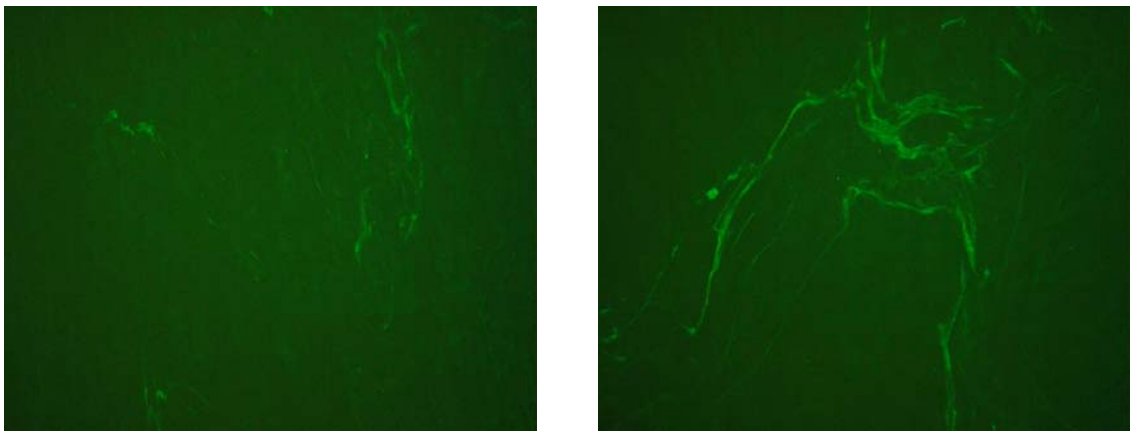
Results

Table 5:

Increase in **collagen III** in the presence of Pal-KMO_2K / Equiv. **MATRIXYL® synthe'6®**

	Collagen III in AU/10^6 cell.	**% Variation of the synthesis of Collagen III (IMF)**	**% Variation in the expression of the mRNA of COL3 α1**	**BioAlternative's Conclusion**
Control	3.36 ± 1.83	Reference	100%	Reference
Pal-KMO_2K 5 ppm / **MATRIXYL® synthe'6®** equiv. 2%	6.84 ± 3.97	**+104%** *p<0.01*	+268%	Clear stimulation

IMF: Immunofluorescence; AU: Arbitrary Units



control — Pal-KMO_2K 5 ppm / Equiv. **MATRIXYL® synthe'6®** (2%)

Figure 9: Variation in the synthesis of **collagen III** by the HFDs

The results obtained above with the two different techniques demonstrate that the matrikine-mimetic peptide Pal-KMO_2K exerted a stimulating effect on the synthesis of collagen III.

e. Fibronectin

In addition to the preceding studies, the synthesis of fibronectin was evaluated by ELISA.
Fibronectin is another of the major constituents of the dermis. It serves, in particular, as an anchoring point for numerous components, such as collagens, fibrin, heparin and cells. It participates in many physiological processes (healing, growth, contraction…).

Results

Table 6:

Increase in **fibronectin** in the presence of Pal-KMO_2K / Equiv. **MATRIXYL® synthe'6®**

	Pal-KMO_2K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Concentration	**Fibronectin in ng/10^6 cell.**	**% Variation; significance**
ELISA / HFD	Control	16039 ± 2604	Reference
	2 ppm Equiv. 0.8%	20576 ± 772	**+28%** *p<0.01*
	4 ppm Equiv. 1.6%	22258 ± 1697	**+39%** *p<0.01*
	5 ppm Equiv. 2%	25468 ± 1300	**+59%** *p<0.01*

TGF-β1 to 10^{-6}%: +194%

Fibronectin synthesis is therefore promoted through the use of **Pal-KMO_2K**. This stimulation, in addition to what was observed for the collagens, helps envisage a strengthening and volumising effect for the dermis

f. Hyaluronic acid

Hyaluronic acid is known as one of the major constituents of the skin. It is a good moisturiser due to its capacity to capture and retain water molecules, and it is also an important volumising agent. Its presence is observed both in the dermis and in the epidermis in the periphery of the keratinocytes (TAMMI *et al.*, 1989). The stimulation of hylauronic acid synthesis provides the skin with both a volumiser and a natural moisturiser.
Human keratinocytes (HK) were put in contact with **MATRIXYL® synthe'6®** and an ELISA type test was employed to evaluate the induction of synthesis shown by these cells. *(n=5).*

Results

Table 7:

Increase in **hyaluronic acid** in the presence of Pal-KMO_2K / equiv. **MATRIXYL® synthe'6®**

	Pal-KMO_2K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Concentration	**Hyaluronic Acid in ng/10^6 cell.**	**% Variation; significance**
ELISA / HK	Control	1373 ± 56	Reference
	4 ppm Equiv. 1.6%	2633 ± 177	**+92%** *p<0.05*
	5 ppm Equiv. 2%	3767 ± 169	**+174%** *p<0.01*
	6 ppm Equiv. 2.4%	5240 ± 292	**+282%** *p<0.01*

Retinoic acid at 1 µM: +162%

2.1.2. Study of the elements that reinforce the DEJ

The dermal-epidermal junction is fundamental for the skin. It nourishes and hydrates the epidermis, which is not vascularised. Moreover, it protects the epidermis from tearing.
Among the many components of this junction, we targeted certain essential ones: Collagen IV and Laminins.

a. Collagen IV

The stimulation of collagen IV synthesis was evaluated by ELISA on the supernatants of the monolayer HDF culture *(n=5).*

Results

Table 8:

Increase in **collagen IV** in the presence of Pal-KMO_2K / Equiv. **MATRIXYL® synthe'6®**

	Pal-KMO_2K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Concentration	**Collagen IV in ng/10^6 cell.**	**% Variation; significance**
ELISA / HFD	Control	24 ± 4	Reference
	2 ppm Equiv. 0.8%	29 ± 3	**+19%** ns
	4 ppm Equiv. 1.6%	31 ± 2	**+28%** *p<0.05*
	5 ppm Equiv. 2%	34 ± 3	**+42%** *p<0.01*

b. Laminin-5

The stimulation of laminin synthesis, and more specifically, of laminin-5, was studied using two methods:

- First, the laminin level was determined quantitatively on human keratinocytes (HK) using ELISA on the culture supernatants *(n=5),* in order to assess the possibility of synthesis induction with **MATRIXYL® synthe'6®**.
- Next, 2% **MATRIXYL® synthe'6®** cream or the control cream was applied to the skin explants (see Collagen I §). This accurately helped target the induction of laminin-5 synthesis at the level of the dermal-epidermal junction using immunofluorescent labelling of the frozen sections *(n=5).*

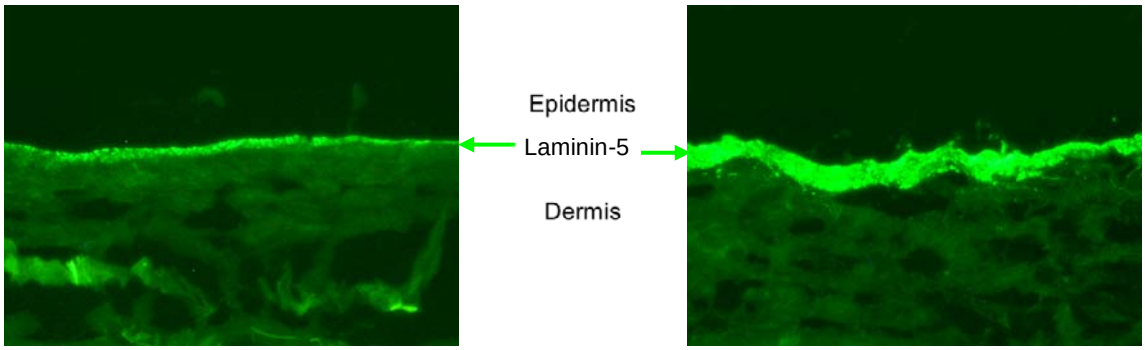
Results

Table 9:

Increase in **laminins** in the presence of Pal-KMO_2K / equiv. **MATRIXYL® synthe'6®** (ELISA levels)

	Pal-KMO_2K (ppm) / MATRIXYL® synthe'6® (equiv. %)		
	Concentration	**Laminins in ng/10^6 cell.**	**% Variation; significance**
ELISA / HK	Control	61 ± 9	Reference
	4 ppm Equiv. 1.6%	85 ± 19	**+39%** *p<0.05*
	5 ppm Equiv. 2%	107 ± 20	**+75%** *p<0.01*
	6 ppm Equiv. 2.4%	196 ± 20	**+221%** *p<0.01*

TGF-β1 to 10^{-6}%: +275%



Control cream **+15%; p<0.05** **2% MATRIXYL® synthe'6® cream**

Figure 10: Variation in the synthesis of Laminin-5 at the level of the dermal-epidermal interface in the presence of 2% **MATRIXYL® synthe'6®** applied to the surface (2/d x 5d) of skin explants

These two results demonstrated a clear reinforcement of the DEJ.

2.2. *In vivo* studies

SEDERMA (JANUARY - MARCH 2010)

Principle

The anti-wrinkle efficacy study for **MATRIXYL® synthe'6®** was conducted on a panel of 25 volunteers. During this study, the wrinkles and lines were monitored on two particularly important sites: the forehead and the crow's feets.

Several additional methods were combined during this study:

- **Fringe Projection** (FOITS) for crow's feet and forehead lines
- **Negative imprints and standardised photographs,** for crow's feet
- **Clinical evaluation with photographic scale**, for the forehead

Protocol

SPECIAL INCLUSION CRITERIA FOR THE STUDY

Women with enough crow's feet and forehead lines were included. In order to obtain a sufficient, well-distributed panel, the severity of wrinkling was verified on a photographic scale of 0 to 9 including the number of wrinkles and their severity. (9 was the rating that corresponded to the highest number of wrinkles).

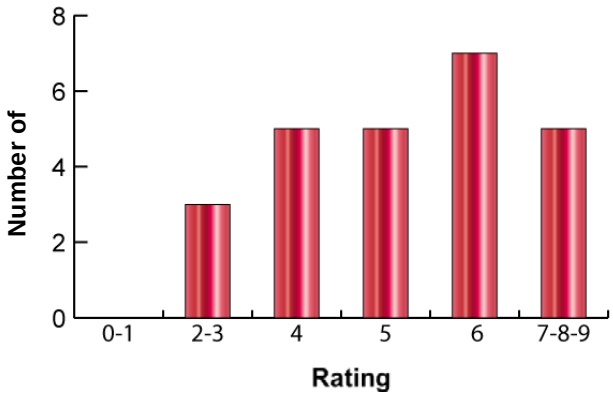


Figure 11: Description of the panel by rating

The volunteers had to respect a 15-day wash-out period that consisted of using the placebo on the entire face. Hormonal consistency was requested for the three months preceding the test and during the test (i.e., no change in contraception, substitution treatment or curative treatment).
Only the cosmetic products provided were allowed to be used for the duration of the study.

TYPE OF STUDIES AND DURATION

Single-blind clinical studies using non-invasive measures were conducted on 25 volunteers (mean age 57 years [42 to 70 years]). During these studies, 2% **MATRIXYL® synthe'6®** cream was applied randomly (see Appendix) on the entire forehead and on the crow's feet on one side of the face. The placebo cream was applied on the other side of the face. For study compliance reasons, only 2% **MATRIXYL® synthe'6®** cream was applied to the forehead, because this area was difficult to divide into two.

The 2% **MATRIXYL® synthe'6®** cream or the placebo cream was applied by massaging it in twice a day for two months.

The study synopsis can be seen in the diagram below:

T0	T2 months
FOITS	FOITS
Imprints	Imprints
Photos	Photos
Clinical evaluation	Clinical evaluation

TOLERANCE

The product was well tolerated by the volunteers.

2.2.1. Study of the relief of the forehead

The 3D topography of crow's feet was obtained using different methods. The FOITS technique (*Fast Optical In vivo Topometry System*) was used to map the surface of the skin without coming into contact with it. The machine used, a Dermatop (Breukman - Eotech), consisted of an interdependent fringe projector and camera, which formed a specific angle, thereby enabling triangulation. The study of the fringe deformation by the relief of the area provided a 3D reconstruction of the relief.

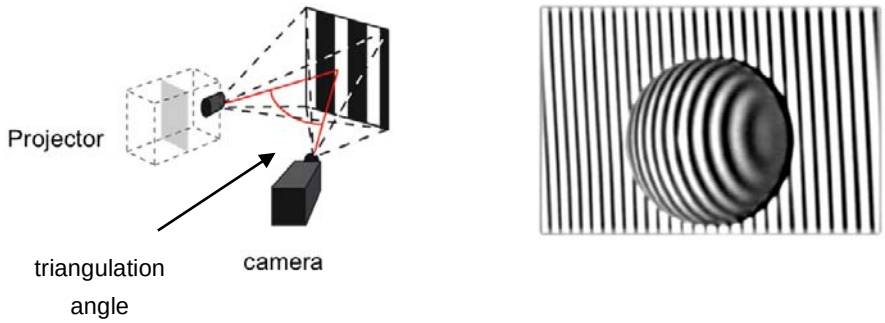


Figure 12: FOITS technique

An analysis was then made using Optocat software (Breukman - Eotech) in order to extract the volume occupied by the wrinkles on the one hand and the complexity of the surface on the other hand. The complexity is the percentage of the total surface developed with all of its reliefs on a perfectly flat surface. The decrease in this percentage demonstrates a smoothing effect of the product (lifting effect).

Regarding the forehead, its relief is accentuated with age due to the continuous stress that facial expressions exert on the skin, and lines can be observed. Choosing this area, with computer assistance, helped us to extract from the same site each time. Using one scan, the volume, the complexity and the maximal depth were calculated.

Table 10:

Improvement in the topographic parameters after application of 2%

MATRIXYL® synthe'6® (25 volunteers)

	Volume (mm^3)		**Complexity (%)**		**Maximum Depth (µm)**	
	T0	T 2 months	T0	T 2 months	T0	T 2 months
Mean ± Standard Deviation	3.12 ± 1.68	2.16 ± 1.54	1.25 ±0,92	0.90 ± 0.44	124 ± 57	103 ± 30
Variation (%) Significance Maximum observed	**-31%** *p=0.055* →-100%		**-28%** *p<0.05* →-77%		**-16.3%** *p<0.05* →-62%	

These data indicate that the volume of wrinkles significantly diminishes by 31% from T0 to T2 months.
Smoothing of the forehead was also observed with a reduction in the complexity of the relief, -28% (*p<0.05*) compared to T0. This reduction was seen as a decrease in the entire cutaneous relief.
Confirming this, the maximum depth of the evaluated wrinkle significantly diminished by 16%, which is important because this parameter has a direct effect on the perception of wrinkling.
The 3D acquisitions below perfectly illustrate the decrease in forehead furrowing.

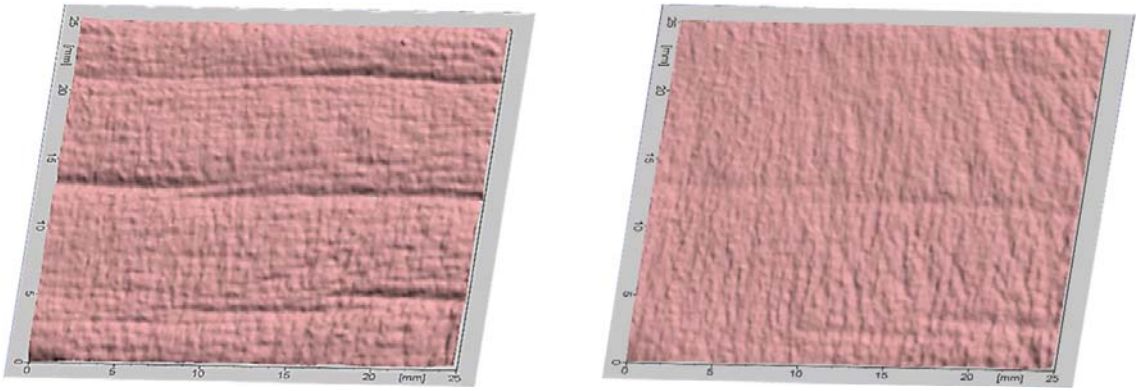


Figure 13: Visualising the smoothing effect on the forehead using the FOITS technique
(left: T0 and right T2 months)

2.2.2. Clinical evaluation with photographic scale, for the forehead

The clinical evaluation was performed by two trained technicians. An evaluation of the number and depth of wrinkles was performed using a photographic scale with nine ratings. The environmental lighting was carefully controlled using two daylight lamps.
The mean of the scores obtained by the two evaluators is presented in the table below:

Table 11:
Variation of the rating characterising the severity of wrinkling
after application of 2% **MATRIXYL® synthe'6®** (25 volunteers)

	MATRIXYL® synthe'6®	
	T0	T 2 months
Mean of the evaluation	**4.26**	**3.95**
% variation *vs*. T0 **significance**	**-7.1** ***p<0.05***	

The results of this evaluation by clinical score demonstrate a significant improvement at T 2 months.

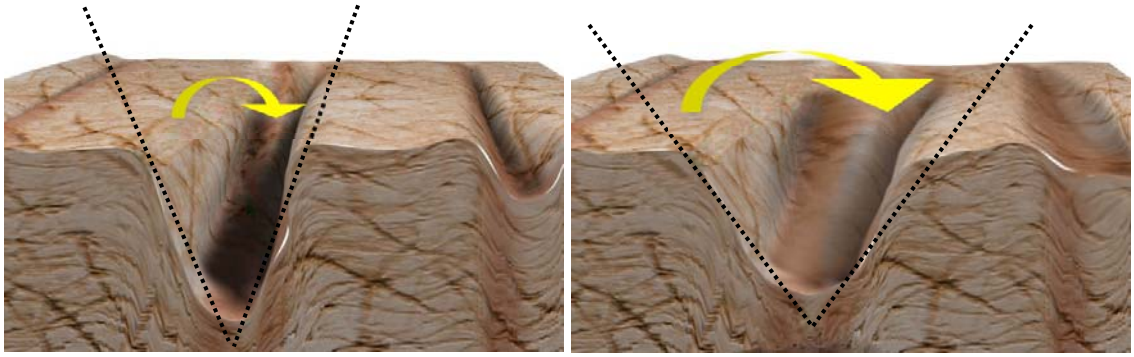
2.2.3. Study of the crow's feet

As for the forehead, the cutaneous relief of the crow's feet was estimated using the FOITS technique directly on the skin and by using negative imprints. These standardised negative imprints of the crow's feet performed using Silflo® were analysed using the projected shadow technique. The method consisted of projecting a light ray on the imprint, thereby generating different sized shadows according to the relief. The shadows were analysed by a special analytical computer program that creates a representation of the surfaces. This method provided access to certain parameters.

An imprint analysis helped measure the percentage of the surface occupied by the deepest wrinkles (depth > 100µm), for which the sizes indicate the general "severity" of the wrinkled appearance; the procedure then helped perform a more in-depth analysis of one of the main wrinkles. This provided the mean depth and the magnitude of this wrinkle.

In the visual perception of a wrinkle, there are several parameters to examine: the extent of the wrinkle, as shown by its volume, depth and opening. In fact, for a given depth, a wrinkle with a narrow furrow will let less light in than a wrinkle with a wider furrow (i.e., more open), as the diagram on the next page illustrates.

Therefore, to diminish the perception of a wrinkle, we must try to decrease its depth and increase the size of its opening, for when it is more open, more light will enter and diminish the perceived size of the wrinkle.



Closed wrinkle Open wrinkle

Figure 14: Parameter of the wrinkle spread (angle)

Table 12:

Variation in the topographic parameters of crow's feet

after application of 2% **MATRIXYL® synthe'6®** (on 25 or 24* volunteers)

Surface occupied by deep wrinkles (%)	**MATRIXYL® synthe'6® (2%)**		Placebo	
*	**T0**	**T 2 months**	T0	T 2 months
Mean	**5.28 ± 2.68**	**4.23 ± 2.00**	4.48 ± 2.26	4.86 ± 2.91
% variation *vs.* T0 **significance** **Maximum**	**-20.0%;** ***p<0.01*** **→ -71%**		8.5%; *ns*	
Difference in variations; **Significance**	**-28.5%** ***p<0.05***			

Volume of the wrinkles (mm^3)	MATRIXYL® synthe'6® (2%)		Placebo	
	T0	**T 2 months**	T0	T 2 months
Mean	**4.57 ± 1.54**	**4.14 ± 1.70**	5.21 ± 2.18	5.82 ± 2.99
% variation *vs.* T0 **significance** **Maximum**	**-9.4%;** ***p<0.05*** **→ -52%**		11.7%; *ns*	
Difference in variations; **Significance**	**-21.1%** ***p<0.05***			

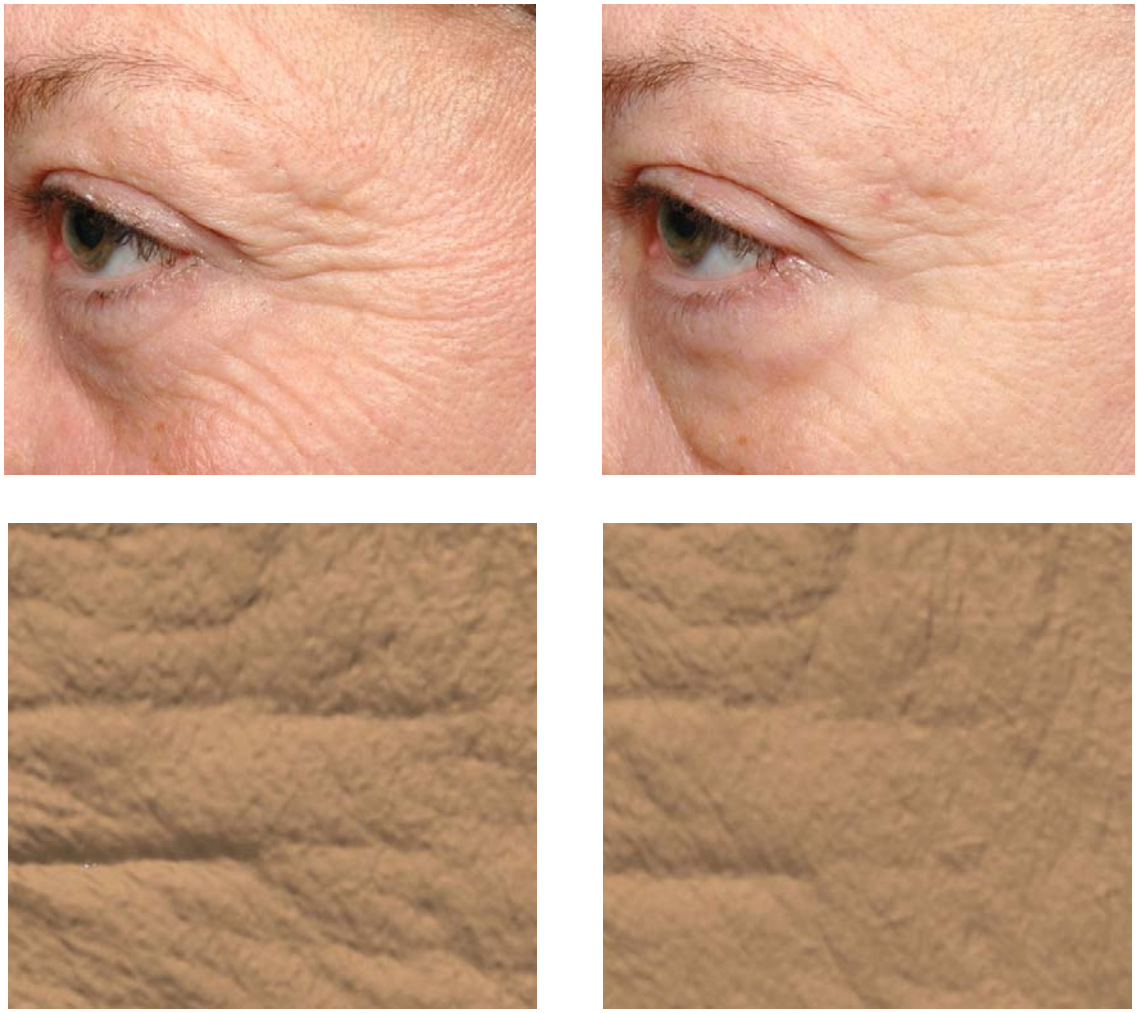
Mean depth of a main wrinkle (µm)	MATRIXYL® synthe'6® (2%)		Placebo	
*	**T0**	**T 2 months**	T0	T 2 months
Mean	**107.87 ± 26.10**	**95.81 ± 24.54**	97.98 ± 24.54	101.70 ± 30.46
% variation *vs.* T0 **significance** **Maximum**	**-11.2%;** ***p<0.01*** **→ -46%**		3.8%; *ns*	
Difference in variations; **Significance**	**-15.0%** ***p<0.05***			

Complexity of the crow's feet; lifting effect	MATRIXYL® synthe'6® (2%)		Placebo	
	T0	**T 2 months**	T0	T 2 months
Mean	**3.85 ± 1,04**	**3.54 ± 0.84**	3.80 ±1.00	3.97 ± 1.55
% variation *vs.* T0 **significance** **Maximum**	**-8.1%;** ***p=0.05*** **→ -42%**		4.5%; *ns*	
Difference in variations; **Significance**	**-12.6%** ***p<0.05***			

Openness of the wrinkle (angle)	MATRIXYL® synthe'6® (2%)		Placebo	
*	**T0**	**T 2 months**	T0	T 2 months
Mean	**98.40 ± 10.49**	**105.39 ± 14.01**	98.20 ± 15.11	96.80 ± 14.16
% variation *vs.* T0 **significance** **Maximum**	**+7%;** ***p<0.01*** **→ +28%**		-1.4%; *ns*	
Difference in variations; **Significance**	**+8.4%** ***p<0.05***			

(*) One set of impressions could not be used because it had too many defects; therefore, the results were obtained on 24 volunteers.

The example below illustrates the effect of **MATRIXYL® synthe'6®**:



T0 T2 months

The application on the crow's feet of **MATRIXYL® synthe'6®** and, contralaterally, of the placebo, demonstrated a very favourable change in the crow's feet wrinkles with the use of **MATRIXYL® synthe'6®.**

We observed a significant decrease in the severity of the wrinkled appearance (-28.5% *vs.* placebo), wrinkle volume (-21% *vs.* placebo), mean depth (-19% *vs.* placebo) and in the complexity (-13% *vs.* placebo). The wrinkles were not as deep, the skin was smoother and the wrinkles spread (angle) (+8.5% *vs.* placebo).

The lower percentages observed for the crow's feet with respect to volume and complexity can be explained by a better application of the cream on the forehead. In fact, the volunteers applied the cream more generously to their forehead and massaged it longer than they did to their crow's feet. Since the surface area of the crow's feet is smaller, it was not possible to apply the same quantities (74% less was applied, $p<0.01$; N=15 volunteers). Furthermore, its proximity to the eye prevents long massage time (41% shorter massage time; $p<0.01$).

3. CONCLUSION

For all women, wrinkles represent a clear sign of ageing that they see in their mirror every morning. Face lifts, which are major surgical procedures, are indeed effective solutions. Repeated injections of fillers, such as collagen and now hyaluronic acid, also work. However, in order to avoid the invasive nature and sometimes devastating and irreversible results of these techniques, it is possible to obtain a plumping action using sophisticated ingredients that work on the very structure of skin tissue.

MATRIXYL® synthe'6® was designed to provide an alternative to hyaluronic acid and collagen injections while supporting an anti-ageing claim. Its active ingredient, Pal-KMO_2K peptide, provides visible, proven filling and anti-wrinkle effects on the epidermis, the dermal-epidermal junction and the dermis.

We observed, during *in vitro* studies, that 2% **MATRIXYL® synthe'6®** strongly stimulated:

- the synthesis of matrix molecules (Collagen I: +111%, collagen III: +104%, fibronectin: +59% and HSP 47: +123%),
- the synthesis of molecules of the dermal-epidermal junction (Collagen IV: +42% and laminins: +75%),
- the synthesis of hyaluronic acid (+174%), which through its highly branched structure and moisture-retaining capabilities provides volume, and is found at all levels of the epidermis, dermal-epidermal junction and dermis.

The results of *in vitro* tests demonstrated a mechanism of action that leads to facial wrinkle smoothing by promoting a restructuring of the cutaneous tissues.

Furthermore, the peptide demonstrates that it boosts luminotherapy since, on artificially aged skin, the application of the equivalent of 2% **MATRIXYL® synthe'6®** significantly reinforced (+12%) the positive effect of this emerging technology.

A clinical study was conducted on 25 female volunteers, who applied a 2% **MATRIXYL® synthe'6®** cream twice a day. The assessments specifically monitored the smoothing and filling effect on forehead lines and crow's feet.

The anti-ageing effect of **MATRIXYL® synthe'6®** was proven using predominant techniques.

On the forehead, measurement by fringe projection, also known as FOITS, demonstrated a visible, mean reduction in main wrinkle volume of 31%. This was complemented by a 28% reduction in cutaneous roughness, which can be associated with a lifting effect. Finally, the maximum depth of this main wrinkle decreased by a mean of 16.3%.

For the crow's feet, the anti-wrinkle effect was measured through a decrease in the surface occupied by deep wrinkles (-28.5%), a decrease in the volume of a major wrinkle (-21%), and a reduction in the mean depth (-15%). There was also a lifting effect, since there was an approximate -13% reduction in cutaneous roughness. The wrinkle further opened by 8.4%, which reduced the shadow effect.

These results are directly related to the *in vitro* results obtained, which demonstrated an increase in the quantity of six major components of cutaneous tissue.

MATRIXYL® synthe'6®, by stimulating the synthesis of 6 component molecules, demonstrates visibly effective smoothing of facial wrinkles. Its effect on forehead lines and crow's feet is similar to the effect of

hyaluronic acid and collagen injections. **MATRIXYL® synthe'6®** provides a rejuvenating effect by significantly reducing wrinkle severity.

It is recommended to add 2%
MATRIXYL® synthe'6® to cosmetic formulations.

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5. APPENDIX

Formula for the products used in the *in vivo* evaluations.

Starting materials	INCI name	Supplier	Placebo product (%)	Active product (%)
Phase A				
H_2O	Water		qsp 100	qsp 100
Ultrez 10	Carbomer		0.25	0.25
Phase B				
Butylene glycol	Butylene Glycol		2.00	2.00
Phenoxyethanol	Phenoxyethanol		qs	qs
Phase C				
Brij S2 SS	Steareth-2	CRODA	0.40	0.40
Brij S10 SO	Steareth-10	CRODA	1.20	1.20
Crodafos CES	Cetearyl Alcohol (and) Dicetyl Phosphate (and) Ceteth-10 Phosphate	CRODA	4.00	4.00
Crodacol CS90	Cetearyl Alcohol	CRODA	1.00	1.00
Laurocapram	Laurocapram		2.50	2.50
DC 345	Cyclopentasiloxane (and) Cyclohexasiloxane		2.00	2.00
Crodamol OSU	Diethylhexyl Succinate	CRODA	7.00	7.00
Phase D				
Potassium sorbate	Potassium Sorbate		0.10	0.10
Phase E				
H_2O	Water		3.00	3,00
NaOH 30%	Sodium Hydroxide		0.40	0.40
Phase F				
Excipient			2.00	-
MATRIXYL® synthe'6®	(see synopsis)	SEDERMA	-	2.00

OPERATING PROCEDURE:

(laboratory preparation)

Step 1. Weigh phase A and let it inflate without mixing for 30 minutes.

Step 2. Heat phase A to 75°C in a water-bath.

Step 3. Weigh phase B and mix well.

Step 4. Weigh Phase C and heat to 75°C in a water-bath. Mix well.

Step 5. Add phase B to phase A.

Step 6. Pour phase C into phase A+B while performing staro agitation at a speed of 1000 rpm. Homogenise well.

Step 7. Add phase D, extemporaneously,

Step 8. Add phase E, homogenise well,

Step 9. Then add phase F, homogenise well.

Cosmetic
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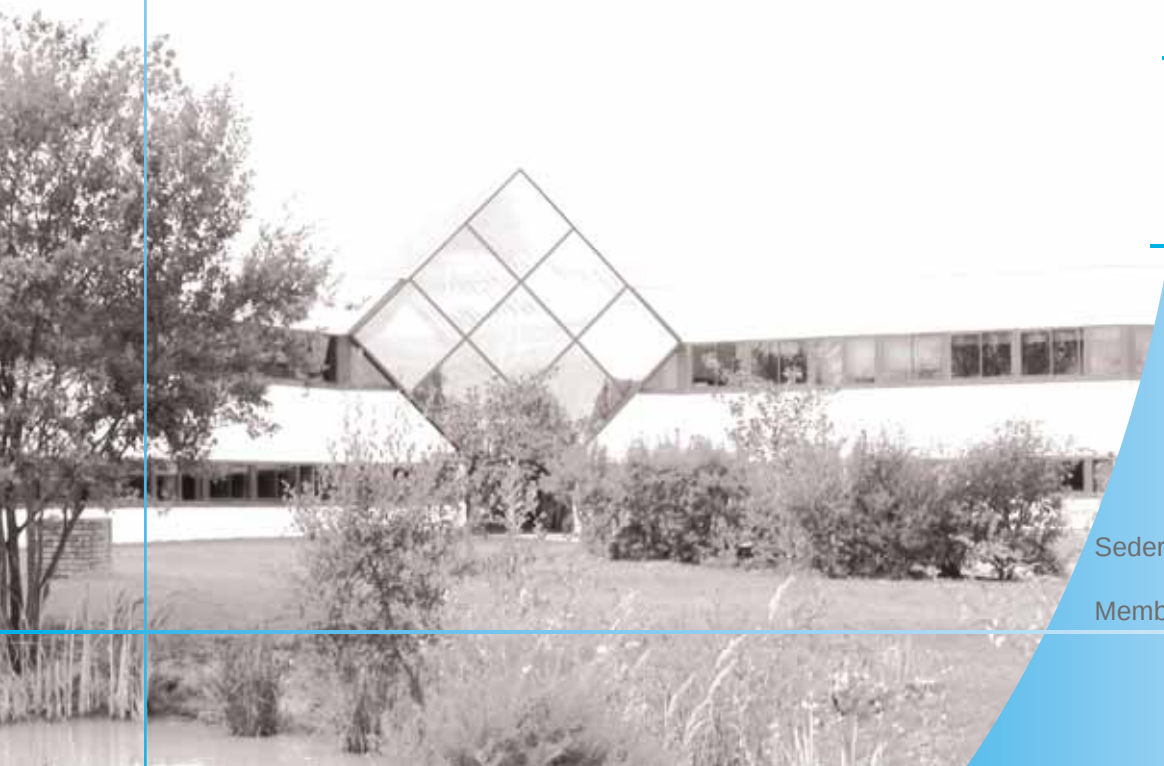

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