INTRODUCTION

Wrinkles are generally the first visible signs of ageing and thus a major challenge for the cosmetic formulator and marketer. Between the promise of reducing fine lines and smoothing the skin and the achievement of real consumer perceived benefits in wrinkle reduction there often lurks a wide gap, difficult to bridge.

Research over the last decades has revealed much about the skin, its structure and physiology, the ageing process and possible remedies to repair some of the damage of time. Even if the genetic causes of ageing cannot be reprogrammed, some of the external causes of skin ageing (UV induced changes, oxidative damages) can at least be partially prevented by modern cosmetic products (sun screens, antioxidants), thus leading to slower rates of visible degradation of the skin. Reversal of existing damage, however, is even more difficult to achieve as it requires biological activity within the skin, not unlike skin repair after a wound, such as stimulation of extra cellular matrix synthesis.

Retinoids (retinoic acid, retinol and its esters, all derivatives of vitamin A) have been recognised by cosmetologists and dermatologists alike as truly active molecules in skin tissue repair. Two drawbacks are, however, to be noted with these retinoids: all these substances have a high irritation potential, sometimes carry warnings of teratogenic activity and are difficult to stabilise in finished consumer products, partly because these molecules possess biological activity interfering in numerous biochemical and cellular processes far beyond extra cellular matrix stimulation. Cosmetic scientists are constantly seeking viable alternatives.

Wound healing is a very complex process, the mechanics of which have not yet been completely unravelled. Although some general features are common to the wound repair mechanisms in different organs and tissues, focused on here are the activities observed in skin tissue, which in any case, when a “wound” is thought of, comes to mind first.

What happens when we cut our skin?
The first step in damage control is to stop the potentially deadly loss of blood: coagulation occurs in the micro-vessels and fibrin is deposited to obliterate any holes or openings.

The peptides cleaved from the circulating proteins in their activation process are not just by-products, but have their own purpose and activity. They are often chemotactic – that is they attract cells (platelets, leucocytes, macrophages and fibroblasts) to the site of the wound. Some peptides then act on the damaged cells and provoke their death (apoptosis, a safety feature), others act on the remaining cells and on those chemically attracted cells to stimulate them into the synthesis of new tissue.

Indeed, once the blood flow has been taken care of, and edema has been circumscribed, the process of reconstruction of the conjunctive tissue begins by deposition of collagen fibres and glycosaminoglycans. The covering of the wound site by epithelial cells also initiates the synthesis of matrix macro-molecules [Siméon et al 1999].

Fibroblasts first secrete proteolytic enzymes to help clean out the wound, then start to rebuild the tissue.

What makes the fibroblasts change their activity? It appears that an external signal,
Figure 2: Stimulation of the synthesis of soluble collagen IV. 4% PalKTTPS (4 ppm of pure peptide) are as much efficient as 20 ppm vitamin C.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vitamin C</th>
<th>TGFβ</th>
<th>PalKTTPS 2%</th>
<th>PalKTTPS 4%</th>
<th>PalKTTPS 8%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0/µm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stimulation of Collagen IV synthesis

like a local hormone, a messenger molecule that interacts with the fibroblast cell membrane receptors, induces this change. And what kind of signal might that be? Obvious candidates are small signal peptides, those that are released from the degradation process of the macromolecules. This feedback mechanism is a logical consequence of the wound-healing cascade and confirms that mother nature is economical: use every waste product possible for some beneficial purpose!

Some of these peptides, fragments of the degraded macromolecules, have now been isolated, identified and synthesised in larger quantities for further investigation. Pr Maquart has coined the very useful and descriptive name "Matrines" for these peptide fragments.

It is considered that the ageing process (induced by sun exposure and the ensuing inflammatory reactions, the free radicals, the upregulation of elastolysis and collagenolysis) is a slowly occurring wound – the "injuries" of time as Horace put it so aptly [Horace, 50 B.C.] – then the use of these signalling “wound healing peptides” (Matrines) in cosmetics seems worth pursuing.

KTTPS is the abbreviation for a pentapeptide composed of the amino acid sequence Lys-Thr-Thr-Lys-Ser, which is described as the shortest fragment of pro-collagen I, released during collagenolysis and able to stimulate the synthesis of collagen I, III and fibronectin in human fibroblasts [Katayama et al 1993]. Starting with this observation, we developed a derivative, Palmitoyl-Lys-Thr-Thr-Lys-Ser (Pal-KTTPS), dissolved at the concentration of 100 ppm in a cosmetic hydroalcoholic excipient (trade name MATRIXYL®) in order to investigate its potential use in topical cosmetic products. The following text summarises the results obtained from numerous in vitro and in vivo (clinical) studies carried out with this peptide.

Material and methods
Pal-KTTPS is obtained by classical peptide synthesis. All in vitro studies are carried out on normal human fibroblasts from neocultured cells. Precise protocols and detection methods of the endpoints are described elsewhere and can be obtained from the authors. Four clinical studies on human volunteers were carried out over the last two years, with a time span of a minimum four months' application per study, and six months in two protocols. Methods to measure anti-wrinkle effects are based on visual dermatological evaluation, image analysis of skin replicas, self-assessment, macro-photographs and ultrasound echography. One study included the taking of skin biopsies and staining for various macromolecules after two and four months treatment. All studies were vehicle- or benchmark-controlled, either half face or in two separate groups. Statistical analysis (ANOVA) is based on student’s t-test.

Results
The original KTTPS peptide was palmitoylated in order to improve bioavailability and skin penetration through the stratum corneum. Radioactive labelling studies have shown this strategy to facilitate the diffusion of otherwise highly hydrophilic peptides into the skin, all the while helping to maintain the peptide in the skin and to prevent its diffusion into systemic bloodstream (Lintner and Peschard, 2000). A wide range of toxicological studies in a broad spectrum of concentrations (oral toxicity, skin and eye irritation, mutagenicity, sensitisation, human trials) have shown the Pal-KTTPS peptide and the commercial form MATRIXYL to be thoroughly without risk, as safe as can be expected from a naturally occurring molecule.

In vitro studies
The stimulation of the synthesis of macromolecules by the pentapeptide is shown in the figures 1 and 2.

Incubating human fibroblasts with amounts of a few ppm of the peptide leads to a very strong increase in the rate of secretion of collagen I, the major collagen type of skin tissue and of collagen III, an important structural protein in the 3D network of connective tissue [Katayama 1993]. Collagen IV (Fig. 2), the role of which is to participate in and maintain the epidermal-dermal junction (EDJ) and fibronectin, a connective tissue adhesion protein, are also boosted. As the figures show, the peptide possesses these powerful stimulatory activities at concentrations at the ppm (µg/kg) level, that is micromolar (10⁻⁶M) concentration. Vitamin C and TGF-β serve as positive controls in these experiments.

Investigated parameters include: wrinkle surface (left), wrinkle volume (middle) and overall surface roughness (right).
Proposed mechanism of action:
DNA array investigations indicate that the peptide is specifically active on genes that participate in wound healing processes. In comparison to Retinol, Pal-KTTKS activates about 16 genes as opposed to more than 50 for the vitamin A molecule. Again, the analysis of these data shows that Pal-KTTKS is highly specific, without side effects, whereas Retinol turns on many more genes, some of which are of inflammatory nature and might explain the irritancies observed in connection with the use of this active. These in vitro studies were designed to investigate and understand the mechanism of action of the peptide in human skin cells. All results concur to indicate that the palmitoyl-peptide traverses the stratum corneum, interacts with the fibroblasts and keratinocytes of the skin and triggers a genetic response closely related to wound healing activities. This leads to neo-synthesis of skin matrix molecules, thickening of the skin, regeneration of tissue, and thus a lasting decrease of surface wrinkles, as the following paragraphs will show.

Clinical studies
The all-important test for the activity of a cosmetic ingredient is, however, based on human trials. As of today, more than five independent clinical trials have been carried out, over a total of close to 300 panelists. The results of three placebo controlled and two benchmark controlled studies will be summarised briefly. Image analysis is one of the quantitative and proved methods to measure the wrinkly appearance of skin and to determine changes in this appearance. Wrinkled surface at various depths, wrinkle volume and overall roughness are among the parameters measured. Dermatological evaluation and self-assessment (both subjective methods) are often done in parallel to the analysis of skin replicas. All five clinical trials of our knowledge employed this method and found consistent results of highly significant improvement of the overall appearance of the skin in the eye zone ("crow’s feet") and other sites in the face. Figures 3-5 are illustrative of one of these studies. The percent changes in each parameter depend of course on the length of the treatment period, which varied from one clinical trial to another, going from one to six months. Best results are obtained after four to six months, as the biological recovery of the skin is a slower process than simple miniaturisation and superficial skin smoothing. The side of the face having received the placebo cream shows no significant improvement.

Dermatological evaluation of the skin appearance, of fine and deep lines around the eyes, the nose and on the front also confirms the effect of treatment with the pentapeptide.

A smaller study on 10 panelists was conducted against a vitamin C containing cream, also over six months, where the above results are confirmed for the peptide, whereas the vitamin C cream (off the shelf brand) was ineffective (data not shown).
Comparison to Retinol

A step further was taken with a comparative study in which 20 panellists used either a 3% MATRIXYL (3 ppm of Pal-KTTKS) cream or a 700 ppm Retinol cream of otherwise equal composition, for two and four months. An additional parameter measured was skin thickness (determined by ultrasound echography). Figure 6 indicates that both actives give the expected performance: skin thickening of about 9% after four months, but the peptide appears to have an advantage at two months.

Classical image analysis of the replicates and dermatological visual assessment confirmed the observations after four months. Three ppm of Pal-KTTKS thus perform as well as 700 ppm (about the maximum concentration tolerated without skin irritation) of Retinol.

Biopsies and consumer perception

The two most recent clinical studies, however, reveal the most spectacular results on the Pal-KTTKS peptide.

One, conducted and carried out by L. Robinson et al. [2002] included 92 panellists who used a basic moisturiser with (on one side of the face) and without (the other side) the 3 ppm of Pal-KTTKS for three months. Again image analysis confirmed the wrinkle-reducing efficacy of the peptide in comparison to the vehicle: results were measurable after only four weeks of use. Visual scoring of high precision photography was reported in this paper to have resulted in significant skin improvement after only two months. The results of this severe methodology of product evaluation testify to the truly consumer perceivable benefits of Pal-KTTKS.

Our own most recent trial [Mas-Chamberlin et al. 2002] involved 80 volunteers, divided into two groups, one of which received a placebo cream, the other one the cream containing 5 ppm of peptide. Two and four months time points were determined, again by image analysis, photography and assessment. More important, however, was the possibility of taking skin biopsies from selected volunteers, both from the placebo group and the treated group. The biopsies were frozen and then used for histological evaluation, looking for specific markers such as fibronectin, collagen I, collagen IV, collagen VII and elastin. The collagen IV data show an improved epidermal-dermal junction in the treated samples (Fig. 7), another observation concerned elastin.

It appears that the Pal-KTTKS peptide enhances elastin synthesis in the skin in significant amounts, whereas the placebo (vehicle) had no effect on this parameter (Fig. 8).
Visual effects
Biopsies and histology, image analysis and percent variation of wrinkle density are, however, not items that “talk” directly to the consumer. Nothing is potentially as convincing as pictures and visual effects. The photographs shown here are representative of the results that can be obtained with the pentapeptide in two to six months (they are each of course from different subjects).

Conclusion
KTTHS is a natural fragment of procollagen I molecule, thus a Matrilin according to the definition of Pr Maquart, that appears to participate in the wound healing process, possibly in various tissues and organs. Nature is economical and uses naturally occurring fragments such as this to act as signal molecules for repairing tissue damages. In order to make use of this natural principle, we have modified the pentapeptide by attaching the palmitoyl chain and affording a more lipophilic character to it, thus making sure that penetration into the skin but not through the skin occurs. Topical application of a peptide thus became possible.

In this article we have attempted to summarise the pertinent data on an exciting new cosmetic active ingredient:

Pal-KTTHS (INCI name: Palmitoyl-pentapeptide 3). The range of experiments done on this material spans from molecular biology (to understand the underlying principles of action), to cell culture studies and then to profilometry with image analysis for objectively documenting the wrinkle improvements, and finally to visual assessment by dermatologists and consumers alike. All these data, coupled with extensive toxilogical evaluation and in-use tests, concur to demonstrate that the Matrilin pentapeptide Pal-KTTHS is a stable, safe and very potent ingredient that allows the cosmetic formulator to go a step beyond the classical vitamin A derivatives.

References
2. Horace, 50 B.C., Odes III.