Reduction of Hair-loss: Matrikines and plant molecules to the rescue

*Claire Mas-Chamberlin, Philippe Mondon, François Lamy, Olivier Peschard and Karl Lintner**

Sederma France

*author to whom correspondence should be addressed *(Tel: +331 34 57 82 80; Fax +331 34 84 11 30 ; email: karl.lintner@sederma.fr)*

Synopsis : *Excessive hair loss in men is a multifactorial problem that needs holistic treatment. The stimulation of matrix regeneration by a specifically designed peptide – biotinyl-GHK- was investigated ex vivo on human hair follicle biopsies, by immune fluorescence tagging and histology. Increased synthesis of collagen IV, laminin 5 and mitosis marker Ki67 was observed. The inhibition of 5-α-reductase by oleanolic acid was concomitantly observed in vitro. The combination of biotinyl-GHK, oleanolic acid and the well known vasoactive substance apigenin was then tested in a vehicle controlled clinical study of hair loss in a male panel of patients with high telogen ratio. Significant improvement in the A/T ratio was observed after four months in the treated group. The combined activities of activated microcirculation, inhibition of DHT production and matrix stimulation and repair clearly show measurable benefits for the anchoring and growth behaviour of the hairs on the scalp.*

Keywords: peptides, Biotinyl-GHK, hair growth, oleanolic acid, 5-α-reductase

1. INTRODUCTION

Alopecia affects 20% of men, sometimes as soon as age 20, and increases by 10% per decade. This means that more than 50% of men aged 50 suffer from baldness. Alopecia has an androgenic etiology in 95% of cases, i.e. high testosterone concentration and a high rate of conversion to DHT by 5-α-reductase. Insufficient blood irrigation of the scalp is also often cited as an important contributing factor in excessive hair loss.

The hair growth cycle is the same in both genders and consists in the 3 successive phases **anagen, catagen and telogen.** The cycle is repeated about 25 times in a lifetime.

An essential component of hair growth is the physical interaction between the dermis and epidermis, within the dermal papilla where keratinocytes and fibroblasts are condensed.

The importance of the matrix components in the survival and growth of cultured human hair follicles was also demonstrated by WARREN R. *et al.*, [1] and JAHODA *et al.* [2].

It should be noted that collagen IV and laminin 5 are mainly synthesized by keratinocytes and that laminin 5 plays a crucial and irreplaceable role in dermo-epidermal cohesion and in the migration or keratinocytes during cicatrisation [3].

Biotin or vitamin H is an essential vitamin made available to the body through the diet. Biotin deficiency gives rise to anomalies of the skin and appendages: fine, 'uncombable' hair [4], alopecia, scaling, pruritus and dermatitis [5,6].

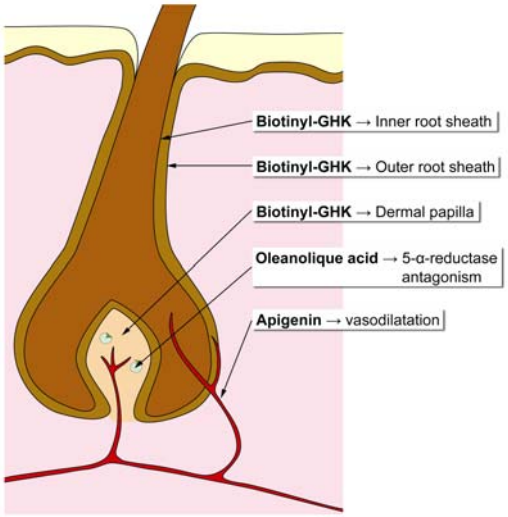

Figure 1: Strategic targets for the cosmetic treatment of hair loss.

The first target is obviously androgenic: the aim is to slow production of dihydrotestosterone (DHT) by 5α-reductase. DHT acts by atrophying the hair follicle and, according to a recently advanced hypothesis [7], through a pro-apoptotic mechanism *via* caspase 3.

The second target is the blood: good capillary perfusion is the mechanism advanced to explain the unexpected success of a peripheral vasodilator, Minoxidil®, originally used as an antihypertensive. Minoxidil® also acts by maintaining active proliferation of the already differentiated keratinocytes in the follicle [8].

The third target is the matrix: By acting on cell differentiation, Minoxidil® also retards hair loss. Improving the proteinaceous tissue around the follicle complements the other two targets for action.

2. A NEW, HOLISTIC APPROACH TO REDUCE EXCESSIVE HAIR LOSS

It is clear, on the basis of current understanding of the morphogenesis of hair and the progressive discovery of the potential causes which trigger or exacerbate alopecia, that a highly complex and multifactorial mechanism is involved. Moreover, very recent genetic studies have shown a substantial number of genes (at least 5) whose mutations have consequences with respect to alopecia [9].

We therefore selected two active substances of plant origin acting on those targets: oleanolic acid (extracted from olive tree leaves) for the inhibition of 5-α1- and 5-α2-reductases and apigenin (flavonoid extracted from citrus) for vasodilatation.

Furthermore, we studied a peptide sequence endowed with tissue repair activities: the peptide Glycyl-Hystidyl-Lysine, a member of the Matrikine family [10,11], in order to ensure better 'rooting' of the hair in the skin. This peptide was prefixed with the biotinyl group to create **Biotinyl-GHK, a vitamin-bearing peptide**, with the expectation of a dual matricial and metabolic action.

The mechanisms of action, confirmed by the activation of certain genes (DNA array), matrix strengthening effects, growth of human hair follicle explants in cultures and results of a 4-month placebo-controlled clinical trial, are described in the following.

3. MATERIAL AND METHODS:

3.1. In vitro studies

3.1.1. Substantiveness of peptide Biotinyl-GHK on the hair follicle

The study was conducted on human skin explants cultured in PBS medium. Following incubation of the explants with the peptide, immunohistochemical study of sections was conducted to investigate for selective localization of the product around the pilial zone. Skin explants (with hair follicles) were incubated in the presence of 60 ppm peptide for 18 hours and compared to control explants exposed to the peptide-free excipient. The determinations were conducted in triplicate. After 18 hours, an 8-mm biopsy was removed from the centre of each well and immediately frozen in liquid nitrogen. The 15 µm thick sections were made using a freezing microtome (cryostat), then dried and fixed. Biotinyl-GHK was detected by immunolabelling coupled with streptavidin peroxidase.

3.1.2. Anti-aging study on cultured hair follicles

Excess hair follicles prepared in the context of a micrograft transplantation session were collected for culturing in a medium similar to that reported by PHILPOTT [12]. The hair follicles were individually incubated at 37°C in an air+ CO_2 (5%) atmosphere for 14 days. The explants were divided into various groups: control group in the culture medium alone, positive control group (positive reference product) and test group exposed to biotinyl-GHK peptide. The culture media were changed every 2 days. General morphology was observed on D0 and D14. Concomitantly, a fraction of the follicles was frozen with a view to conducting more advanced immunohistochemical studies. Growth was monitored using a digital camera with images taken on D0, D3, D5, D7, D11 and D14.

3.1.3. Stimulation of the adhesion proteins of the root sheath and dermal papilla

The freezing microtome sections of the D0 and D14 samples were exposed to fluorescent antibodies specific to laminin 5 (Tebu) and collagen IV (Cliniscience). The staining obtained consists in green fluorescence. Counter-staining of the nuclei was conducted using propidium iodide, yielding red staining.

The observations were conducted on the inferior zone of the follicle above and below the bulb

3.1.4. Gene activation

The DNA array study employed a panel of 600 genes selected for their interest with respect to cell function and was conducted on SkinEthic® reconstituted human epidermis samples incubated in the presence of a complex consisting of 3 active substances: peptide biotinyl-GHK, oleanolic acid and apigenin. Incubation was conducted for 18 hours. The mRNA present in the cells was reverse transcribed to yield DNA and amplified (RT-PCR method) to obtain a legible signal vs. the control cultures. The resulting image is a snapshot, at time point 18 hours, of the genes up-regulated or down-regulated by the complex.

3.1.5. 5-α-reductase inhibition

The method used was the amended ZUE-YUE method: hepatic microsomes very rich in 5α-reductases I and II were used. Microsomes were incubated at 37°C in an appropriate buffer medium, with NADPH co-factor and testosterone, for 10 minutes. Measurement of the specific absorbance of NADPH at λ = 340 nm enables calculation of the NADPH consumed by the reaction converting testosterone to dihydrotestosterone with formation of $NADP^+$. The incubations were conducted in the presence and absence (control) of various concentrations of oleanolic acid. The positive control was incubated with suramin.

3.2. *In vivo* studies: Four-month placebo-controlled clinical trial

The videotrichogram method was used to establish and monitor the time course of the ratio of the proportion of hairs in the anagen phase and the proportion in the telogen phase (A/T parameter).

Inclusion criteria Thirty-five male subjects of Caucasian origin, aged between 18 and 50 years and presenting with ***more than 20%*** *of their hair in the telogen phase were included.*

Usual exclusion criteria were used, in particular intake of corticosteroids, immunosuppressants or retinoids in the 6 months or anti-inflammatories in the week preceding the study; local application of Minoxidil® or any local 'anti-hair loss' treatment, applied topically or taken orally; or trophic treatment of the hair in the last 3 months.

Product application The product or placebo was applied twice daily to the scalp using gentle massage.

An alcoholic lotion (8%) with the appearance of a colourless liquid was formulated. It contained 15 ppm of apigenin, 9 ppm of oleanolic acid and 6 ppm of Biotinyl-GHK as well as 1% cetrimonium chloride, 0.4% polysorbate 20, citrate buffer and preservatives. The placebo (without the three actives) was indistinguishable. At time points T0 and T4 months, a physical examination of the scalp was conducted by a dermatologist and safety was assessed by subject interview.

Videotrichogram: The system used consisted in a MORITEX SCOPEMAN® MS-500 videomicroscope fitted with a mobile 25X objective with optical fibre, connected to a digital image acquisition system.

The images were analyzed by the COUNT-HAIR® program developed by Laboratoires DERMSCAN.

Image acquisition at T0 and after 4 months was conducted on the same shaved hair zone (about 1 cm^2/ 200 hairs, on average), after marking. The parameters monitored were the length and growth rate of the hair and the proportion of hairs in the anagen phase and proportion in the telogen phase.

Hair samples: morphological analysis and immunolabelling of collagen IV and laminin 5. At T0 and at the end of the study, 24 hairs were sampled from the border of the alopecic zone using tweezers. Six subjects in the treatment group and 6 in the placebo group underwent sampling. The hairs were fixed in Bouin's fluid (12 hairs) or frozen immediately (12 hairs) prior to analysis.

4. RESULTS AND DISCUSSION

4.1. *in vitro* and *ex vivo* studies

4.1.1. 5-α-reductase inhibition

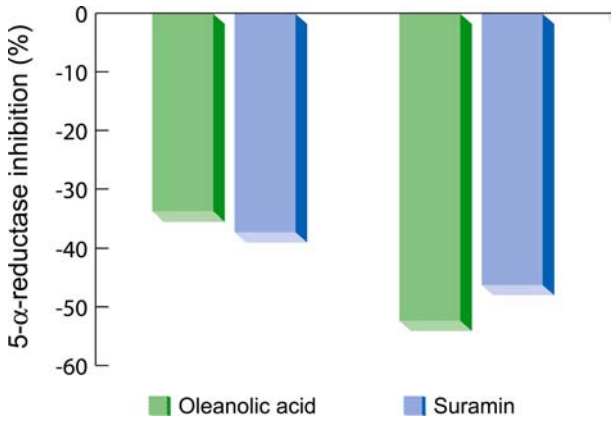

Figure 2: Concentration dependent inhibition of testosterone conversion (Oleanolic acid: conc I = 3 ppm; conc II = 9 ppm, Suramin: conc I = 70 ppm; conc II = 140 ppm).

The percentages shown in Figure 2 were determined from the values obtained for the various incubations by comparison with a control incubation with testosterone. The values reported are the means of triplicate assays. The suramin positive control yielded the expected results, namely, dose-dependent inhibition. Thus oleanolic acid is clearly shown to inhibit 5-α-reductase with a dose effect enabling inactivation of 54% of testosterone conversion to dihydrotestosterone at a concentration as low as 9 ppm.

4.1.1. Protein affinity:

Biotin (vitamin H) is known to possess a very high affinity to certain proteins (avidin, streptavidin). It was of interest to investigate if the biotinylation of a matrikine (Gly-His-Lys) would lead to increased affinity of the peptide to hair keratins. By the technique described in the Materials & Methods section we could observe, in histological examination, the high affinity of the peptide to the proteins surrounding the hair shaft. The sections of the biopsies showed clear peri-pilial localization of peptide biotinyl-GHK (data not shown here for reasons of space). Biotinyl-GHK is thus a substantive peptide that exhibits specific localization around its target: the hair follicle.

4.1.2. Hair shaft growth

The isolated hairs survived in the growth medium for about 15 days. Visual inspection and growth determinations with a camera – the latter conducted on the free part of the hair shaft – showed differential hair growth, depending on the culture medium.

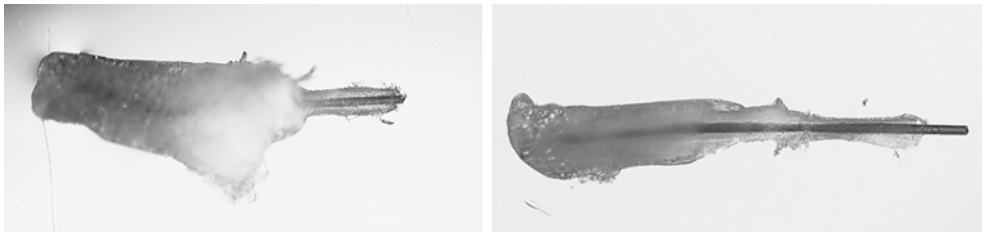

Figure 3: Control hair on Day 14 (left), biotinyl-GHK treated hair on D14 (right).

The results obtained are shown in the photographs (Fig. 3); quantitatively they represent a growth rate increase of 58% over baseline at 2 ppm of peptide and of 121% at 5ppm. Minoxidil® effects a similar increase. In a further experiment (data not shown), the mitosis (cell proliferation) rate was monitored by the Ki67 marker. Freezing microtome sections were made on D0 and D14 and exposed to peroxidase-bound anti-Ki67 antibody. On the sections, the dividing cells were stained dark brown. For the control bulb, the results showed a decrease in mitotic keratinocytes on day 14 of culture, reflecting cell aging. 10µM Minoxidil® maintained proliferative activity (as reported by BOYERA [8]); the much lower concentrations of 0.3 and 1.0 µM biotinyl-GHK led to similar staining intensity, expressing Ki67. Clearly, the matrikine peptide exerts stimulatory activity on the cellular metabolism. The tripeptide Gly-His-Lys had already been described [13,14] as stimulatory in various forms (bound to copper, palmitoylated…). The biotinylation, while contributing to improved affinity, was not expected to, and does not, interfere with the stimulatory message of this potent peptide.

4.1.3. Adhesion proteins of the root sheath and dermal papilla

The quality of the dermo-epidermal junction depends on the formation of a very dense basal lamina rich in laminin 5 and collagen IV, on which the keratinocytes of the first basement layer rest and to which they adhere.

Laminin 5 and collagen IV are two proteoglycans of capital importance in the constitution of the basement membrane, the attachment zone for the epidermis and dermis, and, in the case of hairs, between the root sheath and dermis. Laminin 5 and collagen IV are also strongly present in the dermal papilla [15] as shown by the control sections made on D0 using cultured hair follicles.

In the control follicle, the **laminin 5** band, outer root sheath side (periphery of the follicle), lost thickness after 14 days. Following exposure to 2 ppm biotinyl-GHK, laminin 5 remained strongly present at papilla level and in the outer root sheath after 14 days (Fig. 4a and 4b).

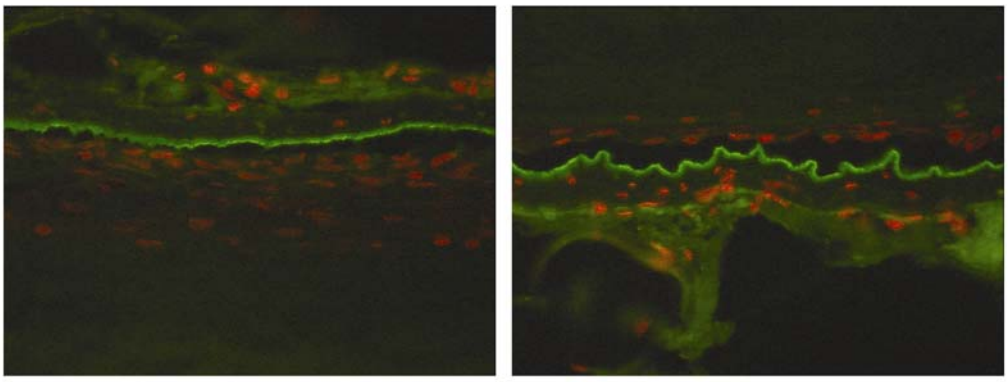

Figure 4: Laminin 5 staining: root sheath after 14 days without treatment (left), after treatment with Biot-GHK (right).

In the control, the **collagen IV** band, very thick at T0, has lost density and coherence at T=14 days. The papilla lost its labelling (photos not shown). In the presence of biotinyl-GHK, after 14 days, collagen IV remained strongly visible in the dermal papilla (e) and was very thick and structured at root sheath level. The structure observed is almost the same as the one at D0.

Morphological observation after 14 days of incubation showed, in the control, a flattened dermo-epidermal junction, on the outer sheath side, that had lost its basal lamina. In contrast, when the hair follicle was incubated with biotinyl-GHK for 14 days, the basal lamina persisted and was clearly visible, showing its sinusoidal character (photo not shown). These two findings reflect a strongly adherent and living dermo-epidermal junction.

4.1.4. DNA array: gene activation

The DNA array study was conducted on SkinEthic® reconstituted human epidermis samples incubated in the presence of a complex consisting of 3 active substances, as justified in a previous paragraph: the peptide biotinyl-GHK, oleanolic acid and apigenin[1]. The study showed the marker genes up-regulated and down-regulated, thus enabling definition of a profile of the mechanisms responsible for the action of the cosmetic active substances on keratinocyte and fibroblast populations.

For reasons of space it is not possible to list here and comment on all 23 genes that were up- and 9 genes that were down-regulated after addition of the synergistic blend of actives. In summary, the up-regulated genes reflect a cell profile oriented towards high growth activity with very strongly expressed cell metabolism enzymes. Antioxidant protective enzymes were also associated since it is necessary to protect the cell against the oxygen free radicals systematically generated by the high level metabolic activity.

Markers of cell proliferation such as proliferating cell nuclear antigen (PCNA) and steroid receptor co-activator were markedly up-regulated, but also associated with protein HSP27 (164%), indicating pro-differentiation activity [16]. The differentiation was accompanied by an increase in several adhesion proteins: Desmogleins, Vimentin and Cytokeratins 10 (differentiation), 14 and 16 (morphogenesis of the hair and keratinocyte proliferation) are among those.

Desmogleins are adhesion proteins that are indispensable for between-keratinocyte adhesion and which contribute to the formation of the outer root sheath of the hair. They are also involved in anchoring the root sheath to dermal structures: mice in which the desmoglein genes have been knocked out loose their telogen hair prematurely. Vimentin is a constituent of the matrix synthesized by keratinocytes at the junction between the epithelial tissue and mesenchyma (dermis) which plays a role in the morphogenesis of hair. Cytokeratins 10 (differentiation), 14 and 16 (morphogenesis of the hair and keratinocyte proliferation) and the metabolic enzymes and markers of cell mitosis (proliferating cell nuclear antigen) characterize keratinocyte hyperactivity oriented towards the morphogenesis of new tissues. Gene down-regulation was reflected in decreased expression of the interferon receptor (-57%), associated with an increase in the interferon antagonist (+135%), both making a strong anti-inflammatory contribution.

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[1] *Tradename : PROCAPIL™*

4.2. Clinical study

Since men are mainly affected by a receding hair line and incipient baldness, a study in male subjects presenting with that problem was set up. Study duration of 4 months was selected in order to totally span the telogen phase.

4.2.1. A/T ratio

The videotrichogram method was used to measure the anti-hairloss activity of the above described synergistic blend of actives (oleanolic acid, apigenin and biotinyl-GHK). We retained the classical parameter of A/T ratio as the endpoint of the study.

Out of the 35 subjects included in the study, 18 were randomly allocated to the ***verum*** group (37 ± 2 years) and 17 to the ***placebo*** group (38 ± 1 year).

After 4 months of the described treatment, the volunteers showed a marked improvement in the proportion of anagen phase hairs, significantly superior compared to T0 (+10%, $p<0.05$). The placebo is inactive. The comparison with the data published for orally taken Finasteride® [17] shows that this topically applied blend has remarkable activity. In fact, an only moderate 8% variation of A/T ratio (compared to T0) is reported for Finasteride® after 5 months. In our clinical study, 67% of the subjects using the *verum* lotion presented an improvement in A/T ratio which reached 31, 33 and 46% in the most responsive panellists, respectively (Figure 5). In contrast, in the placebo group, there was a trend toward a decrease in anagen hairs.

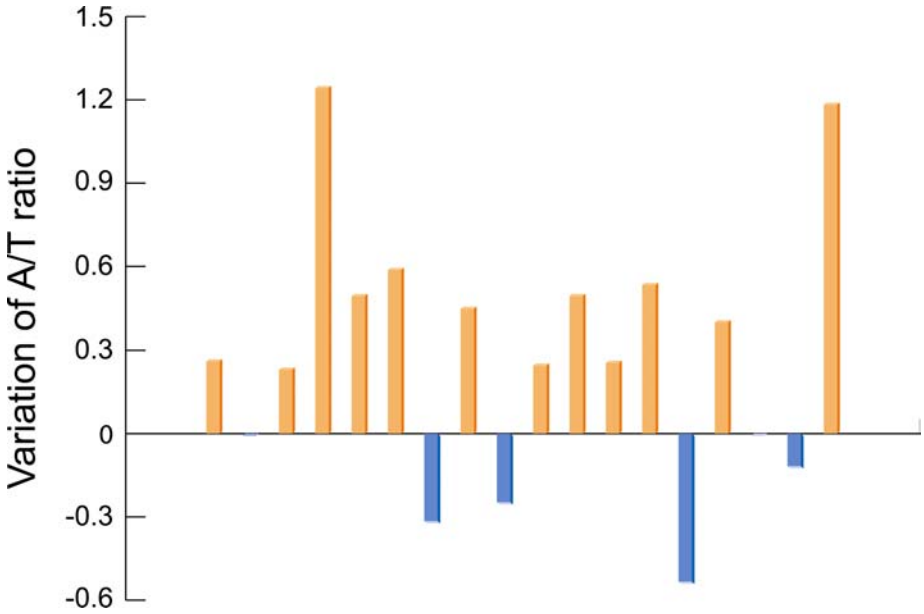

Figure 5: Individual results of A/T changes after 4 months of treatment with verum lotion.

4.2.2. Morphological changes in the hair after 4 months

The between-group differences were observed using telogen hair (sampled by pulling out) after 4 months. The immunofluorescence findings with respect to the markers collagen IV and laminin 5 further reinforced the previous findings: Greater laminin- 5 fluorescence and collagen IV labelling of the root sheath was observed for the telogen bulbs in the ***verum*** group (Fig 6).

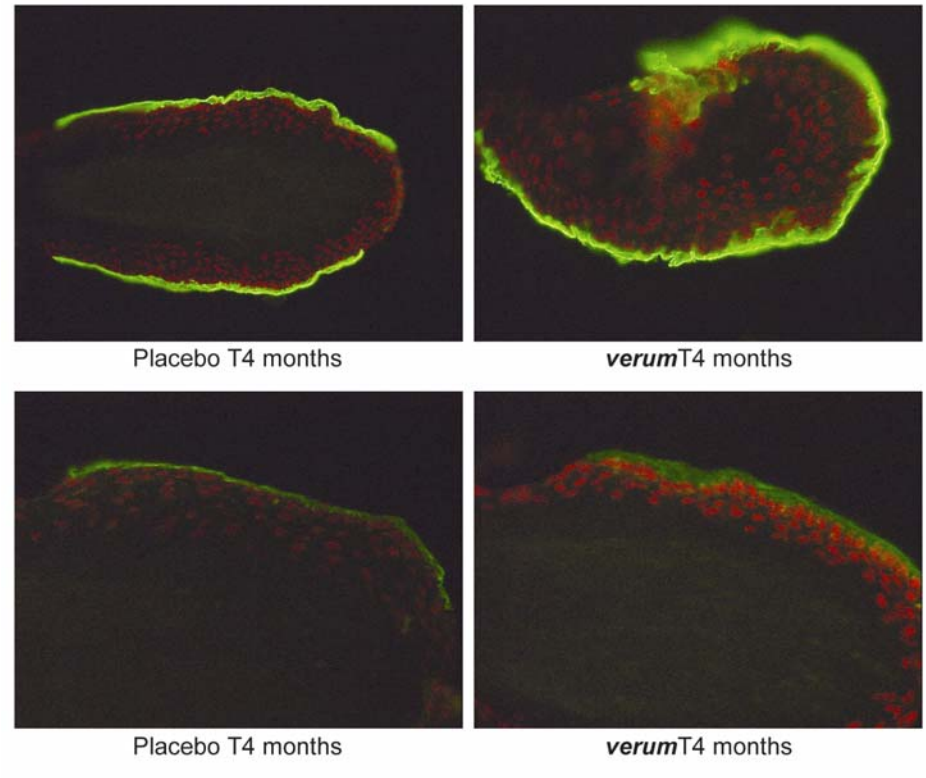

Figure 6: Telogen hair treated with the active complex:
above*: collagen IV staining /* ***below****: laminin 5 staining*

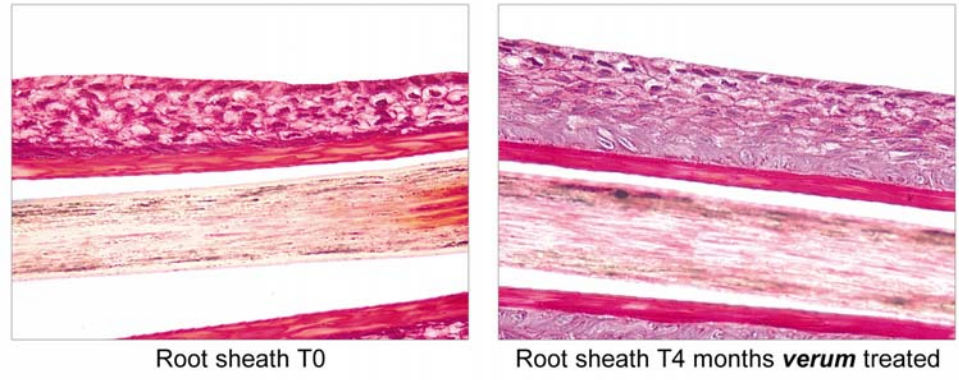

Figure 7: Anagen hair treated with the active complex

The root sheath of anagen hairs also improved, with thickening and clearly defined cell bases: in the *verum* treated hairs (Fig. 7), the root sheath was observed to be of higher quality with a perfectly structured basal lamina ensuring optimum dermal-epidermal adhesion on the outer side of the hair.

5. CONCLUSION

We have identified, from the study of the literature, three phenomena responsible for excessive hair loss. By combining the peptide biotinyl-GHK, a matrikine [10], with oleanolic acid, a 5-α-reductase inhibitor, and a known vasodilatating substance such as apigenin in a specific blend, we were able to show in a coherent battery of *in vitro* and *ex vivo* tests followed by a 4 month clinical double blind vehicle controlled *in vivo* study, that

- the biotinylated peptide is highly substantive to the hair shaft and its surroundings in the epidermis
- adhesion proteins, such as vimentin, desmogleins, desmocollins, laminin 5 and collagen IV are stimulated by this peptide/flavonoid/phytosterol combination
- Potent activity on keratinocyte multiplication and hair morphogenesis can be achieved in various models.

The 4-month clinical trial (covering a complete telogen cycle) compared this active blend to placebo and confirmed the marked anti-hair loss activity of the complex:

- Out of 18 volunteers in the active group, 67% showed significant improvement in the mean anagen/telogen ratio ($p<0.05$), with certain subjects showing an improvement greater than 30%.
- The morphological and immunohistological analyses of the hair samples taken at the start and the end of the study showed that the bulb of telogen hair, root sheath and laminin 5 and collagen IV densities were markedly improved in the treated group, in contrast to what was observed in the placebo group.

The above set of results suggests that this active blend acts by promoting enhanced anchorage of telogen hair in the dermis via regeneration of the root sheath. It thus slows hair loss and improves the health of hair follicles.

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