TO WHOM IT MAY CONCERN

1.11.2024

DECLARATION

We, ALPAYA DOĞAL VE KOZMETİK ÜRÜNLERİ SANAYİ VE TICARET ANONİM ŞİRKETİ, situated in, Akçaburgaz Mah., 1573. Sokak TEM34 Esenyurt – 2 No:1 İç Kapı No:27 Esenyurt, İstanbul, Turkey declare that we are the owner, formulater, producer and exporter of Biobalance Eyelash Growth Serum.

Further, we declare that Biobalance Eyelash Growth Serum has been formulated with clinically proven active ingredients namely Wide Lash and we use 2% Wide Lash in our formula as suggested in the clinical studies.

In the view of above, we request you to consider the efficacy studies of the active ingredient as proof documents of claims on the labels.

Yours sincerely,

ALPAYA DOGAL VE KOZMETIK URUNLERI SAN. VE TIC. A.S.

ALPAYA DOĞAL
VE KOZMETİK ÜRÜNLERİ SAN. ve TİC. A.Ş.
Akçaburgaz Mah. 1573 Sk. No:1 İç Kapı No:27
Esenyurt/İSTANBUL
Mersis No:005 02145370015

Annex 1: Clinical Study of Wide Lash

Cosmetic Active Ingredients

WIDELASH™

sederma

ENGLISH

WIDELASH™

SEDERMA Patents: FR 2 974 297; WO 2012/143845; EP 2 699 223

FOR LONGER, STRONGER LASHES IN AS LITTLE AS TWO WEEKS

SYNOPSIS

Description: Combination of 2 complementary active ingredients Biotinyl-GHK and panthenol that act on eyelash follicles

INCI Name: Glycerin (and) Water (Aqua) (and) Panthenol (and) Biotinoyl Tripeptide-1

Demonstrated cosmetic activity

In vitro studies

➢ ***A study of the substantivity of the biotinyl-GHK peptide on hair follicles***

- The biotinyl-GHK specifically targets hair follicles.

➢ ***A study of the hair bulb keratinocyte proliferation by evaluating the mitosis rate***

- The biotinyl-GHK helps to maintain an optimal keratinocyte proliferation to ensure hair growth.

➢ ***A study of the hair follicle growth maintained in culture***

- With 2 ppm of biotinyl-GHK (0.6% **WIDELASH™**) a superior growth of +58% in comparison with T0. For 5 ppm of biotinyl-GHK (1.5% **WIDELASH™**), we obtain a +121% growth in comparison with T0..

➢ ***A study of the production of adhesion proteins collagen IV and laminin-5***

- Biotinyl-GHK reinforces the dermis/epithelium sheath junction, providing an optimal hair anchorage.

In vivo studies

Clinical study of the stimulation of eyelash growth by 30 female volunteers applying, daily, a mascara containing 2% **WIDELASH™** against a placebo mascara for 30 days. Evaluation of the length, the diameter (with calculation of the volume), strengthening and eyelash loss effect by photographic measure after 15 and 30 days, evaluation by trained experts and self-assessment after 30 days.

➢ ***Length:***

⇨ 15 days: +10,56%/T0, *(p<0,001)*
⇨ 30 days: +17,02%/T0, (*p<0,001*). The experts saw an improvement in 87% of the volunteers. 70% of the volunteers found their lashes longer.

➢ ***Diameter:***

⇨ 15 days: +11,96%/T0 *(p<0,001)*
⇨ 30 days: +18,66%/T0, (*p<0,001*). The experts saw an improvement in 74% of the volunteers. 73% of the volunteers found their eyelashes thicker.

➢ ***Volume:***

⇨ 15 days: +39,7%/T0 *(p<0,001)*
⇨ 30 days: +66,8%/T0, (*p<0,001*)

➢ ***Strengthening effect against eyelash loss:***

⇨ 15 days: **WIDELASH™** side, loss of 3.7 lashes less than the placebo side.

- ⇨ 30 days: **WIDELASH™** side, loss of 9.1 lashes less than the placebo side. 80% of the volunteers find their lashes nourished. 70%find their lashes stronger and believe that **WIDELASH™** prevents lash loss.

Recommendations for use:

- ***Overview:***
  - ⇨ Recommended pH: 3 - 8
  - ⇨ Add **WIDELASH** to the emulsion preferably between 25 and 65°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
HET CAM
Neutral red test HRIPT
Ames' test
Expert certification

CONTENT

10/2016/V2

1. INTRODUCTION

“The eyes are the windows to the soul” (G. Rodenbach, 1892)

In every civilisation, women, and sometimes even men, have sought to enhance their eyes (DRAELOS, 2001). Egyptians used a powder called Kohl, which protected the wearer from wind and sand, highlighted the eyes, and made the look brighter, and gave rise to the word al-kuhl, meaning "the kohl" in Arabic. Whether consciously or unconsciously, a person's eyes can be highly attractive. The appeal of the eyes depends on their shape, their colour and a person's mood or desires. Eyes can be so hypnotic that people have been known to fall in love after just one look.
“Les cosmétiques” written by Ovide in the 4th century BCE, already gave make-up advice. Today, professionals know how to work with contrast around the eyes to magnify them and create an impression of depth. To do this, they use a wide range of dedicated cosmetic products, so that regardless of eye and skin colour, there is always a way of enhancing the eyes and intensifying a personality.

In addition to eye shadow, one of the keys to making the eyes stand out is by highlighting the lashes with mascara. In fact, we know that longer, thicker lashes make the eyes more attractive, brighten the face, idealise women, give women confidence, and give a new dimension to the power of seduction.

The current market for eye makeup is the fastest growing make-up market in the USA (+6%), and in particular, the mascara market represents 1.1 billion dollars.

In the USA, people can lengthen and strengthen their lashes through off-label use of medications intended for ocular problems, but these products can have side effects. Obtaining this effect by using a mascara seems like a dream that has finally come true. SEDERMA has developed a safe ingredient that provides these results.

MASCARA

Mascara, or eyelash make-up, is a term that comes from the Italian word maschera (meaning “mask”). It has been long recognised as a cosmetic product for colouring the eyelashes. Mascara helps eyes look bigger and more expressive by colouring the eyelashes. The eyelashes, which are thinner at their tips than at their implantation site, are not easily seen against the coloured background of skin.

Thanks to the darkening provided by mascara, the ends of the eyelashes become more pronounced and the pigments deposited onto the eyelashes make them look thicker.
Mascara originally came from antimony, a black metalloid with bluish highlights that, when crushed, becomes a powder. In 1923, in effort to help his sister, T.L. Williams launched a mascara made of coal dust mixed with vaseline. Williams sold his product by mail and created the Maybelline Company.
Modern mascaras are more complex. There are two main kinds: waterproof mascaras and non-water resistant mascaras. Today, mascara provides contrast for, lengthens and thickens lashes. It helps increase their apparent volume and improve their curl. Some mascaras also contain synthetic fibres, such as nylon, which when applied with the texture of the mascara, lengthen eyelashes. Sublime eyes can also be achieved through eyelash extensions, or even eyelash transplants (STRAUB, 2008).

THE LASHES: PRESENTATION

The lashes surround the eyes and clearly have a clear protective function in mammals by helping the mobile eyelids to which they are attached. There are about 150-200 lashes on the upper eyelid and half as many on the lower eyelid. They help protect the eye from insects and dust (MOSES, 1970; RANDALL, 2007).
In addition to protecting the eyes, the lashes also play a fundamental role in feminine attraction. The literature abounds with references to love at first sight - men who fall hopelessly in love from the very first flutter of the eyelashes of a beautiful woman.

The elegant eyelash is, anatomically-speaking, a hair similar to other body hair; however, it has a few differences, such as the fact that it is larger at its base than other body hair. The eyelashes have not been the subject of intense research because there are very few eyelash-related diseases. For example, total eyelash loss has been observed following hereditary disease (Marie Unna hereditary hypotrichosis), infectious inflammatory conditions or certain medication treatments.

There is a normal, less incapacitating reduction in the number and width of eyelashes starting at about 40 years of age, and this process continues as people get older. This is not due to a change in the number of eyelash follicles, which is established at birth, but rather to a shortening of the duration of follicular cycles with age (HABIF, 2003).
The normal eyelash growth cycle is about half as long as a normal hair cycle (ALONSO, 2006). By becoming even shorter, the cycle affords eyelashes less time to grow fully and diminishes the ability for the eyelashes that have fallen out to be replaced. In addition to becoming thinner, lashes lose colour, although total colour loss is very rare (eyelashes rarely turn grey). This is related to the melanocytes of the follicle, which gradually lose their ability to produce melanin and make the eyelashes appear even thinner.
Although there is a clear difference between the eyelashes of men and women, both in terms of length and thickness, in contrast to hair loss, eyelash loss does not seem to be affected by testosterone levels. 400 years BCE, Hippocrates observed that eunuchs never became bald, and hence discovered that baldness depended on a specifically masculine factor. Despite this, bald men do not lose their eyelashes (JOHNSTONE *et al.*, 2002, RANDALL, 2008).
The hair growth cycle is the same for everyone, and there are three phases (Anagen, Catagen and Telogen) (see figure 1).

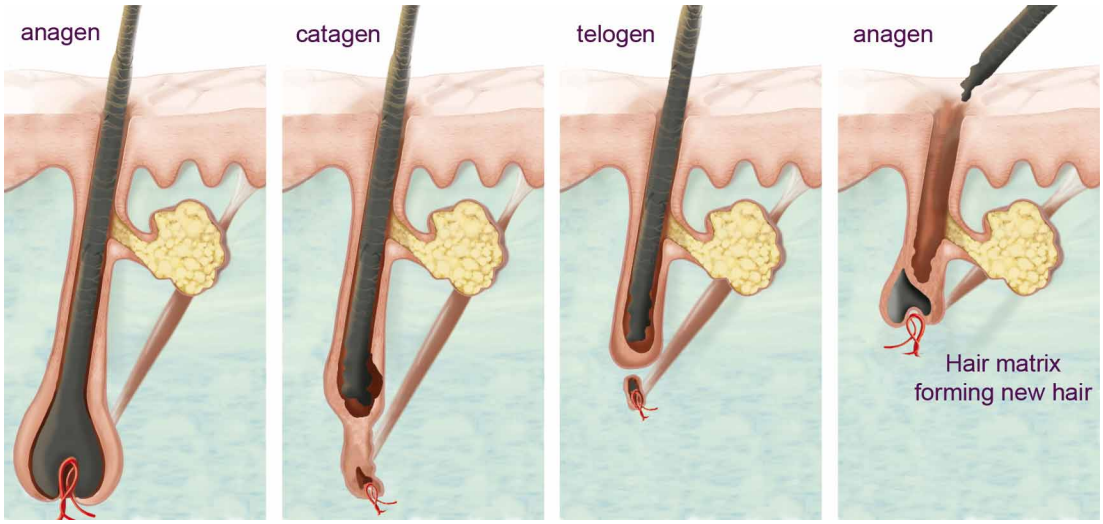


Figure 1 : Life cycle of a hair follicle

- The first phase, or growth phase, is called the **Anagen** phase. It lasts 3 to 4 years for hair and 2 to 5 months for eyelashes (ELDER, 1997; VOGT *et al.,* 2008).
- The second phase is very short - about two weeks - and corresponds to a halt in growth. It is called the **Catagen** phase.
- The third phase is called the **Telogen** phase. It corresponds to a resting phase, during which the hair falls out. This happens fairly slowly, since the hair loss occurs due to the regression of the bulb area and the detachment of the shaft (located in the hypodermis, at a depth of approximately 1.5 mm). Finally, the hair is pushed towards the surface. This phase lasts 4 to 9 months for eyelashes. More than half of the eyelashes are in this phase at a given time.

The 5- to 11-month long eyelash cycle renews itself several times during a person's life.

Each hair forms in the bulb, where the dermal papilla, composed of fibroblasts, their matrix, the blood vessels and the hair matrix, give rise first to the bulb, then to the hair itself, in which there it is intense keratinocyte cell division (ALONSO, 2006).

Each papilla, which is located at the base of the hair follicle, is governed by an "internal clock". This internal clock triggers the natural renewal cycle for hair. The cause of this trigger is still being debated. It seems that two sources of epithelial stem cells, located at the level of the bulb and lower in the outer root sheath of the anagen hair follicle, temporarily fuse to regenerate a new hair follicle (BERNARD, 2003; 2006). An interaction between the dermal cells and the epidermal cells via unidentified messengers helps establish the new structure and guarantee that it functions properly (PAUS, 1999).

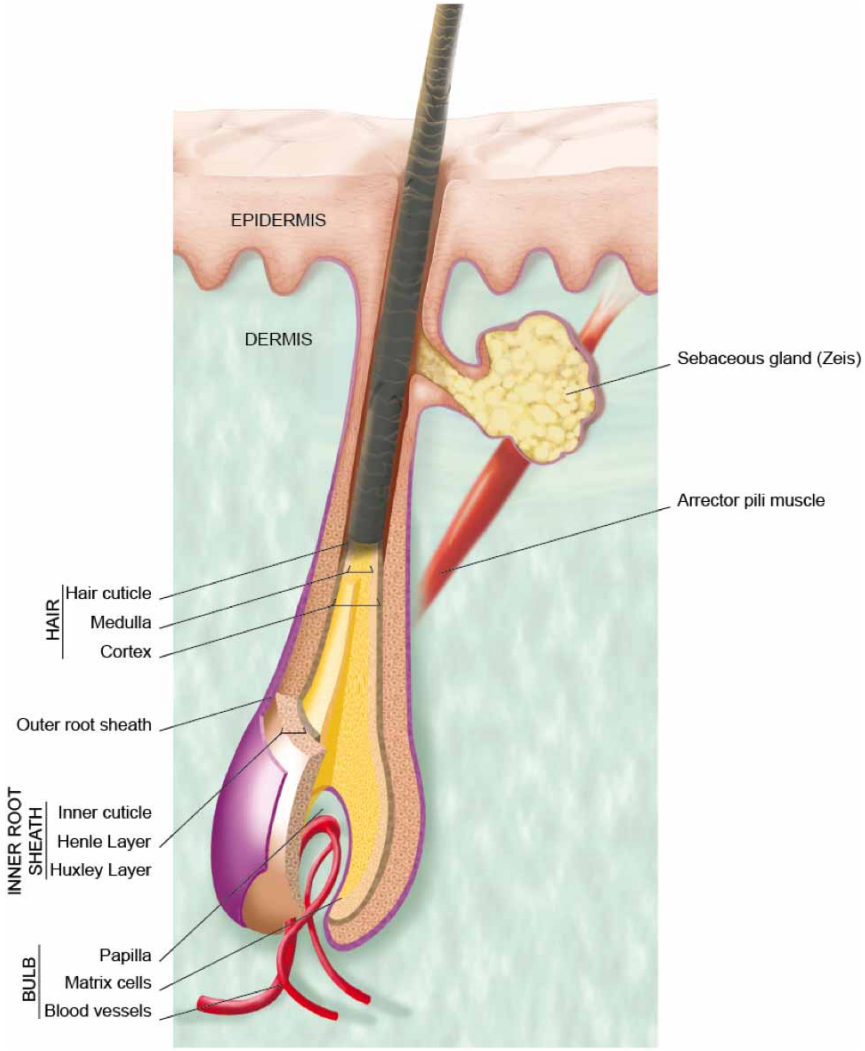


Figure 2: Structure of a hair follicle

Recently, bimatoprost, a synthetic prostaglandin F2 analogue always prescribed to reduce intraocular eye pressure in glaucoma patients, was approved by the US FDA in ophthalmic solution form as a prescription treatment to lengthen eyelashes, thicken them, and increase their pigmentation in patients suffering from eyelash hypotrichosis (loss or reduction).

Bimatoprost acts on prostaglandin receptors. A randomised, placebo-controlled study demonstrated that it enhanced natural eyelash growth as well as eyelash thickness and pigmentation (SMITH *et al.*, 2009). It is possible that it works by increasing the number of follicles in the anagen phase, as well as the duration of this phase, by stimulating keratinocyte production.

Although the list of potential side effects is long (blurry vision, eyelid redness, permanent darkening of the iris, burning sensation, darkening of the eyelid or the area beneath the eye; SAINT-LOUIS, 2010), bimatoprost is generally well-tolerated.

This US FDA authorisation for a specific product does not apply, however, to other prostaglandin analogues (known for being frequently pro-inflammatory) or to other countries.

So, what are the alternatives for safely meeting the market needs with proven efficacy? Studying hair structure provides possibilities, since different hair types have certain points in common.

HAIR AND THE EXTRACELLULAR MATRIX

An essential part of hair growth is the interaction between the dermis and the epidermis, at the level of the dermal papilla. This small structure is made of fibroblasts embedded in their abundant matrix and surrounded by a layer of highly proliferative keratinocytes.

The dermal papilla is an area that is especially rich in collagens and glycosaminoglycans, which maintain close contact between the two cellular populations and promote the biochemical communication needed for hair shaft growth. The importance of collagen IV and laminins must be underscored, since these elements, along with fibronectin, constitute the basement membrane of the dermal-epidermal junction as well as the majority of the dermal papillae to create a sort of matrix for promoting hair growth (JAHODA *et al.*, 1992; ALMOND-ROESLER B*. et al.*, 1997).

The central role played by these matricial macromolecules in the growth and differentiation of the skin and skin appendages is well-illustrated by the work of TAMIOLAKIS *et al.*, (2001) on the embryogenesis of human skin. In fact, these authors demonstrated high concentrations of laminin, collagen IV and fibronectin in the epithelial root sheath of the developing hair shaft.

The importance of these components in improving the survival and growth of cultured human hair follicles was also demonstrated by WARREN *et al*., (1992). Collagen IV and laminin-5 are synthesised by both the keratinocytes and the fibroblasts. Laminin-5 plays a crucial, irreplaceable role in dermal-epidermal cohesion and in the migration of keratinocytes during healing.

The role of interfacial matricial proteins is particularly well demonstrated in the sequence of events that lead to the regeneration of a new hair

When the bulb is artificially severed and removed, the keratinocytes of the external epithelial root sheath migrate beneath the damaged area and organise themselves opposite the fibroblasts. It is within this new interface that a matrix comprising collagen IV, laminin-5 and fibronectin regenerates: a new dermal papilla forms and once again becomes functional (JAHODA *et al.*, 1992).

HAIR AND DEFICIENCIES

Like for the rest of the body, vitamins play an important role in the proper growth and maintenance of healthy skin appendages, hair and lashes.

- ***Biotin***

Biotin, or vitamin H, is an essential vitamin that is ingested into the body through food. A biotin deficiency is manifested through problems of the skin and skin appendages: thin hair that is impossible to style (SHELLEY *et al*, 1985), alopecia, scaling, pruritus and dermatitis (FRIGG *et al.*, 1989, FRITSCHE *et al.*, 1991).

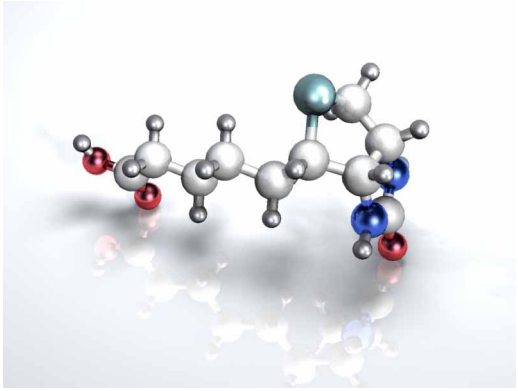


Figure 3 : Biotin

The neurons and keratinocytes are among the structures that are most sensitive to biotin deficiencies (SUORMALA *et al.*, 2002), and the resultant human physiological deficits include mental retardation and skin disorders. This is not unexpected, given the shared embryological origins of the skin and the brain.

In particular, biotin regulates the formation of late-differentiating cytokeratins in the epidermis (FRITSCHE *et al.*, 1991).

From a biochemical standpoint, biotin is an enzymatic cofactor that is essential for the proper functioning of mitochondrial carboxylases, of which it constitutes a prosthetic group. Biotin is covalently bound to the lysine residues of mitochondrial enzymes (pyruvate, priopionyl-CoA, 3-methyl crotonyl-CoA and acetyl-CoA carboxylases), and by passing through an activated form of carboxy-biotin, it assists in the transfer of the CO2 group to accepting molecules, such as pyruvate (Krebs cycle) and oxaloacetate (lipogenesis).

- ***Biotinyl-GHK***

The Glycyl-Histidyl-Lysine peptide sequence belongs to the Matrikine family (MAQUART *et al.*, 1999). The various forms of GHK: Palmitoyl-GHK (LINTNER and PESCHARD, 2000) and biotinyl-GHK (LINTNER 2010; MAS CHAMBERLIN *et al.*, 2005; ARUL *et al.*, 2007) both of which actively participate in the reconstruction of the extracellular matrix of connective tissue, stimulate macromolecule synthesis, and even promote healing

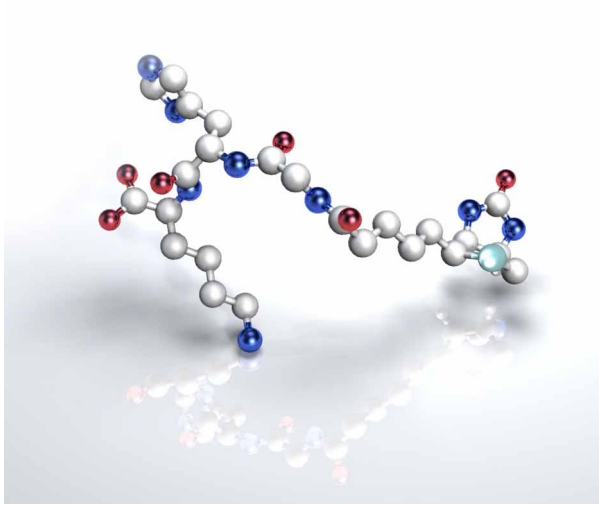


Figure 4 : Biotinyl-GHK

Furthermore, recent studies by HONG et al, (2010) indicate that GHK may have anti-cancer properties.

- ***Panthenol***

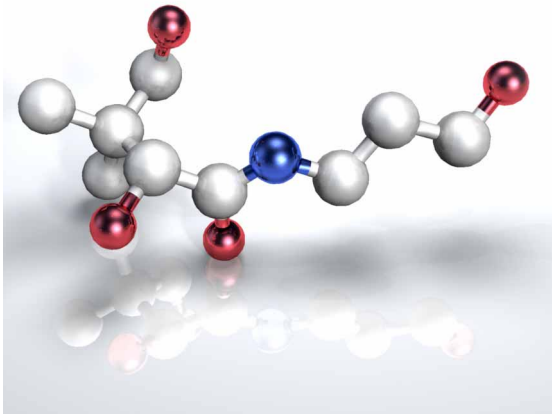


Figure 5 : Panthenol

Pantothenic acid, which is formed from ß-alanine and pantoic acid, is a component of coenzyme A (CoA) and acyl carrier protein (ACP).

Pantothenic acid exerts its effects through coenzyme A and ACP, which are acyl group transporters. Coenzyme A produces high energy bonds with acetic acid (acetyl group) to form acetyl-coenzyme A and with other carboxylic acids to form acyl-CoA molecules. Pantothenic acid deficiencies in animals can lead to fur loss.

Publications on the use and benefits of vitamin B5 supplementation (and panthenol in particular) are rare. However, a study on the growth and differentiation of keratinocytes in the presence of pantothenate and two articles on the positive effects of panthenol on trichopathies (BUDDE *et al.*, 1993; OBRIGKEIT *et al.*, 2006; STANGL, 1950) point out the benefits of this vitamin for the connective tissue.

Pantothenic acid administered in its alcohol form, which rapidly oxidises into an acid in the body, was recommended to prevent hair whitening and hair loss as well as to promote epithelial regeneration in the respiratory tract and wound healing. In cosmetics, panthenol's uses, among others, are as a moisturiser and an emollient. In particular, it easily binds to and lubricates the hair shaft.

Panthenol has not been extensively studied in cosmetics and/or hair care; at least, very few studies have been published. Nevertheless, WEISER and ERLEMANN (1987) demonstrated that even low concentrations of panthenol can improve epithelialisation and healing by up to 30%.

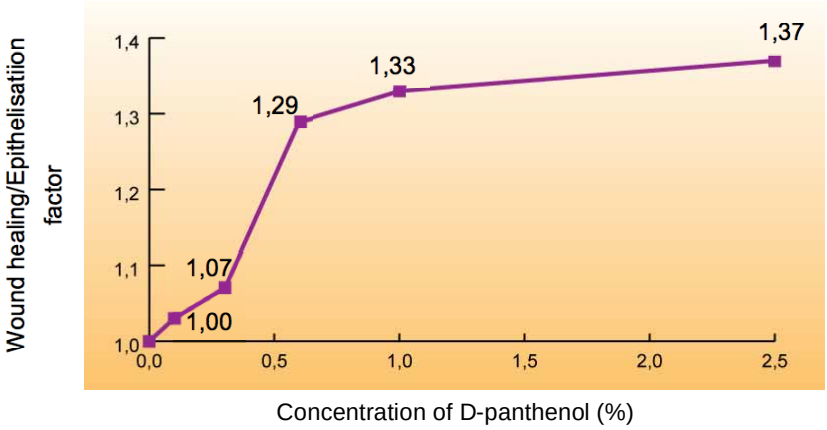


Figure 6: Wound treatment of D-panthenol formulated in a W/O cream

The ease with which panthenol penetrates the hair and root explains its hydrating power, which contributes to nourishing and reinforcing the sheath and shaft as well as anchoring the shaft in the infundibulum

THE SEDERMA CONCEPT

It clearly seems, according to current knowledge on the morphogenesis of hairs, that the mechanism of their formation is complex and multifactorial. Promoting the genesis of the follicle and the progression in the hair cycle is thus a challenge. We chose two compound actives to try to act on these targets: the biotinyl-GHK peptide associated with the panthenol. The preparation containing the vitamin biotinyl-GHK peptide (330 ppm) and the natural precursor of the provitamin B5 (panthenol), for cosmetic use in treating lashes constitutes our new active ingredient:

WIDELASH™

The properties described by the three constituents biotin, peptide GHK and panthenol seem ideal, in synergy, to improve, growth, volume and the preservation of lashes, without prostaglandins and medical approval.

The following chapters describe the diverse in vitro and clinical studies carried out on the peptide, the panthenol and the formulated preparation.

2. EFFICACY TESTS

2.1. *In vitro* studies

2.1.1. Study of hair follicle explants maintained in culture

(SUBSTANTIVITY OF PEPTIDE BIOTINYL-GHK ON THE HAIR FOLLICLE - BIOALTERNATIVES STUDY)

Contact between active substances, such as **biotinyl-GHK** and the skin, hair follicle or connective tissue, is important to obtain biological activity. Compounds that are too water soluble will not provide sufficiently substantive to demonstrate efficacy. The biotinylation of the active GHK peptide provides not only the peptide and a vitamin, but also the simultaneous assurance that the compounds show higher affinity for the target, as demonstrated in the following study.

PRINCIPLE

The selective immune-histochemical search for the **biotinyl-GHK** peptide around the hair area was performed by exploiting biotin's strong affinity for streptavidin. Streptavidin is bound to a peroxidase revealing system, which adds local colour where it binds.

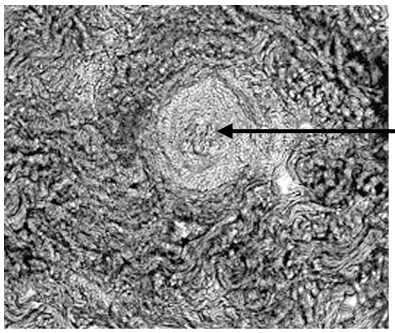
PROTOCOL

Obtaining lash explants is impossible for understandable reasons. Therefore, the study was performed on human skin explants retrieved during an abdominal plastic surgery procedure. The explants were kept alive in a humid chamber.

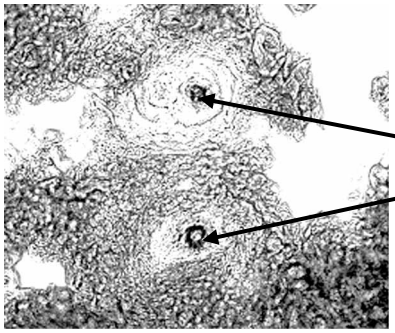
The hair follicle-containing skin explants received **biotinyl-GHK** at a concentration of 60 ppm in order to potentiate a single application and visualise the peptide. For comparative reasons, control explants were treated with the non-peptide containing medium. The tests were performed in triplicate.
After an 18-hour incubation period, an 8 mm biopsy was taken from the centre of each follicle. Next, 15 µm cryostat sections were performed on the biopsies, which were frozen in nitrogen. Revealing the presence of **biotinyl-GHK** was performed as previously mentioned, using an antibody with streptavidin-peroxidase.

RESULTS

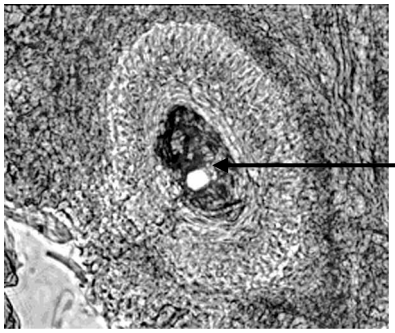
Microscopic observation of the sections clearly revealed that the **biotinyl-GHK** peptide was localised around the hair.



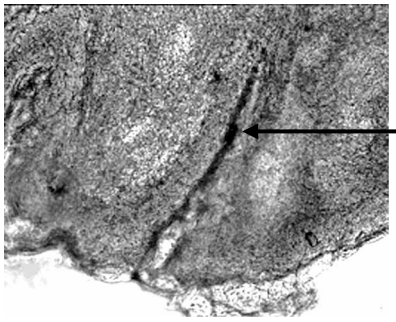
A : Control explant case: no markings around the hair shaft (magnified 20x).



B : Peptide case: clear localisation of the **Biotinyl-GHK** around two adjacent hair shafts (magnified 20x).



C : Peptide case magnified 40x: the peripheral, concentric area around the hair is heavily marked by the presence of **Biotinyl-GHK.**



D : Peptide case : longitudinal section demonstrating the specific localisation of the **Biotinyl-GHK**. We observe good distribution throughout the hair and no marking on the surrounding tissue.

CONCLUSION

Biotinyl-GHK is a peptide that demonstrates specific localisation on the target: the hair follicle. A similar observation of biotinyl-GHK's high affinity for membrane proteins was recently reported by ARUL *et al*, (2005).

➢ Studies of the proliferation of hair follicles maintained in culture

(BIO-EC STUDIES)

PRINCIPLE

For this study, hair follicles and excess tissue prepared for a micro-graft transplantation session were collected and cultured in a medium similar to that used by PHILPOTT *et al,* (1996).

PROTOCOL

These hair follicles were individually incubated for 14 days at 37°C. They were divided into three batches: a control batch in culture medium only and two biotinyl-GHK peptide-treated batches.
The culture media were replenished every two days.

The general morphology was observed at the beginning of the study (T0) and at the end of the testing (T14).
On these dates, a portion of the follicles was frozen for more in-depth immune-histochemical testing at a later date.
Moreover, growth monitoring was performed by regularly taking digital photos, i.e., at T0, T7 and T14.

RESULTS

a. Hair shaft growth

The growth measurements were performed on the exterior portion of the hair shaft (excluding the lower portion of the follicular bulb). The figure 7, next page, illustrates the effect of biotinyl-GHK on the hair shaft growth.

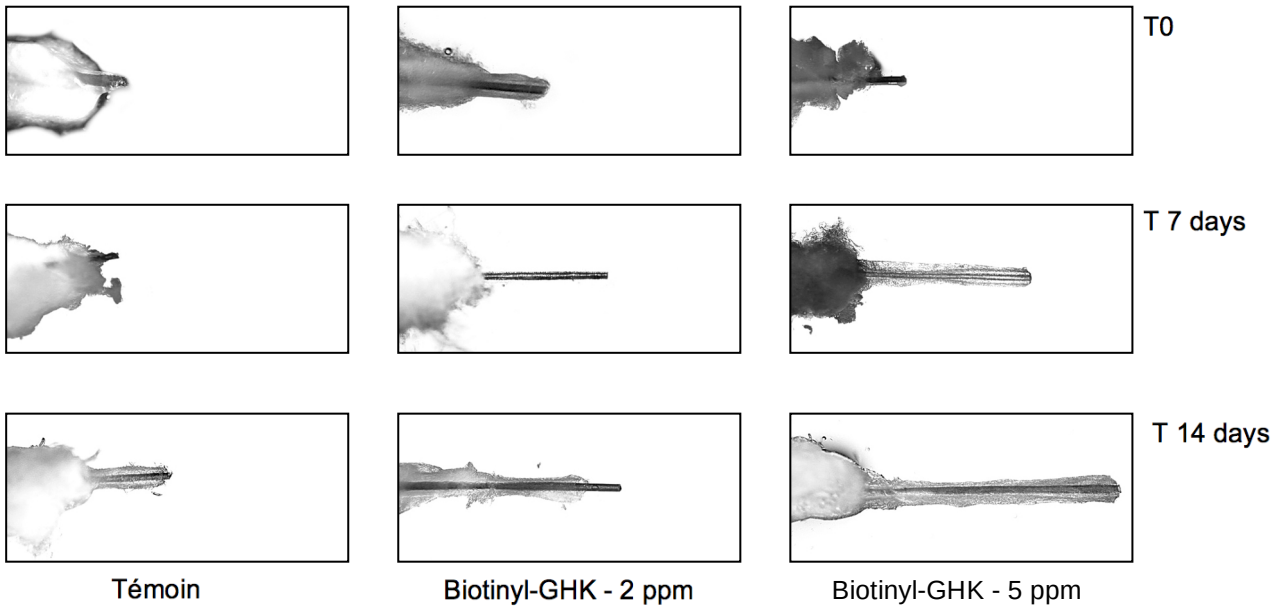


Figure 7: Growth of hair follicles at T0 and T14 days

The results obtained are shown in the following graph:

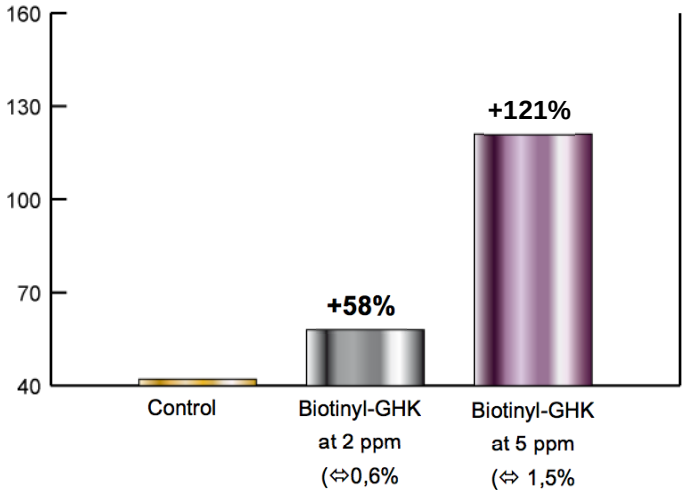


Figure 8: Hair shaft growth after 14 days (%)

CONCLUSION

The control hair shafts grew from T0 to T14 days, which validates the model. The growth observed for the cases in contact with the peptide at 2 ppm (0.6% WIDELASH™) was better than what was observed for the controls. For 5 ppm of biotinyl-GHK (i.e., 1.5% of WIDELASH™), mean growth was +121% compared with T0.

b. Proliferative activity on the epithelial root sheath

PRINCIPLE

In order to determine the cause of the growth observed in isolated follicles, the frozen sections performed on T0 and T14 were processed with an anti-Ki67 antibody. Ki67 is a fragment of nuclear protein present during proliferative phases. It helps reveal cell growth activity. Its precise function is unknown. It has been suggested that it participates in, and may be necessary for, cellular proliferation. It constitutes one of the best markers for following cellular mitosis (RIJEWIJK et al, 1989).

The coupling of the antibody with a peroxidase molecule helps with the in situ identification of proliferating cells and with the microscopic estimate of their numbers at the base of the epithelial root sheath (dark brown colour; area 1).

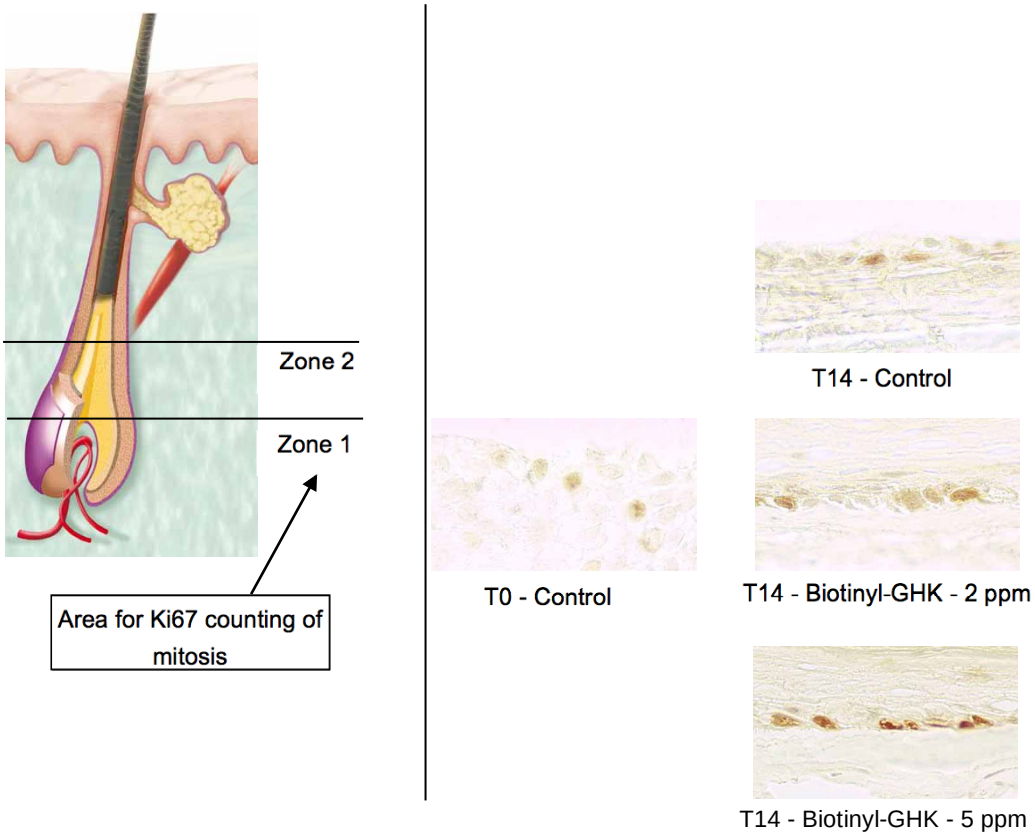


Figure 9 : Observation of mitosis of keratinocytes of the hair bulb

RESULTS

The results indicate that there was a decrease in the number of keratinocytes in mitosis for the control bulb at the 14th culture day, signifying the beginning of cellular senescence.

Biotinyl-GHK at concentrations of 2 ppm and 5 ppm helped maintain the proliferative activity of the epithelial root sheath.

c. Stimulation of protein adherence to the epithelial root sheath and the dermal papilla

PRINCIPLE

A high quality dermal epidermal junction is the result of a dense basal lamina rich in laminin-5 and collagen IV. The keratinocytes of the primary basement layer use this junction for support and adhere to it.

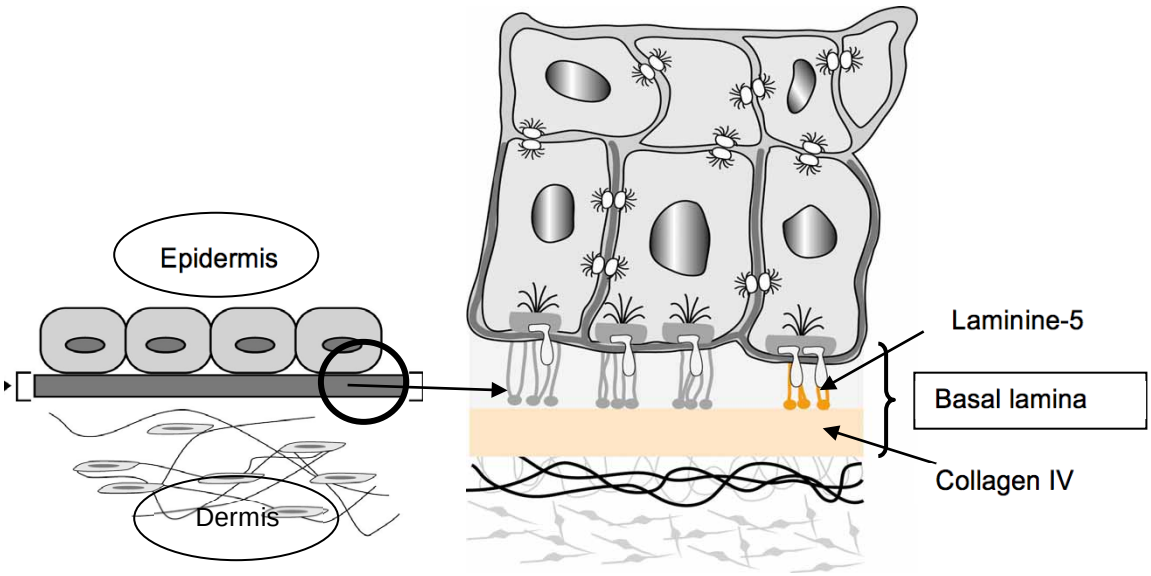


Figure 10: Dermal Epidermal Junction

- The follicles used in the prior study were also used for this evaluation.

Morphologically, at the end of the 14-day culture period, the external sheath of the control had a flattened dermal epidermal junction with a nearly nonexistent basal lamina.

In contrast, when the hair follicle was incubated for 14 days in the presence of biotinyl-GHK, the photos show that the dermal epidermal junction still had a clearly sinusoid appearance. These two elements bear witness to a highly adhesive, living dermal epidermal junction.

- As we saw earlier, laminin-5 and collagen IV are two crucial components for building the basement membrane and the area where the dermis attaches to the epidermis, and in the event that the hair enters the epithelial root sheath and the dermis. These two matricial proteins are also highly present in the dermal papilla (JAHODA et al, 1992) and can be revealed through immunolabelling of the histological sections of cultured hair follicles.

PROTOCOL

The sections frozen on T0 and T14 were labelled using fluorescent antibodies specific for laminin-5 on the one hand and collagen IV on the other. Hence, the richer an area is in one of these elements, the greener it appears. Red counterstaining of the nuclei (using propidium iodide) helped reveal the cells that were present.

The observations were made in an area below the follicle both above and below the bulb (areas 1 and 2, see figure 9)

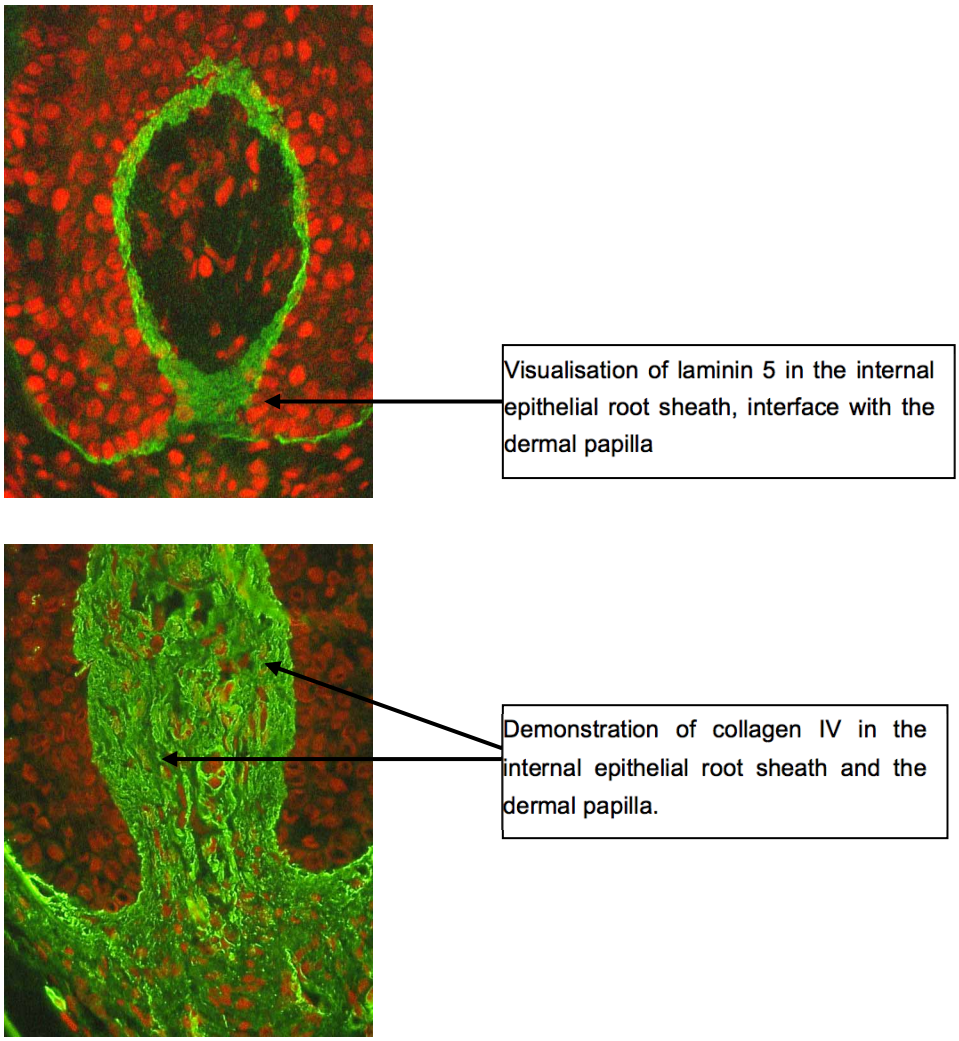
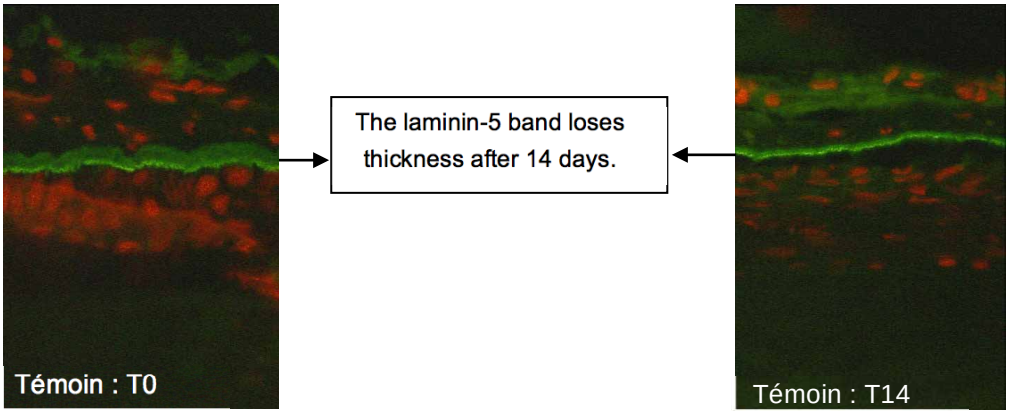


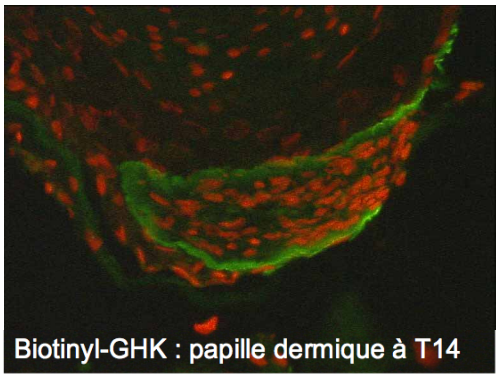
Figure 11: Visualisation of laminin-5 and collagen IV by immunofluorescence at the hair bulb level

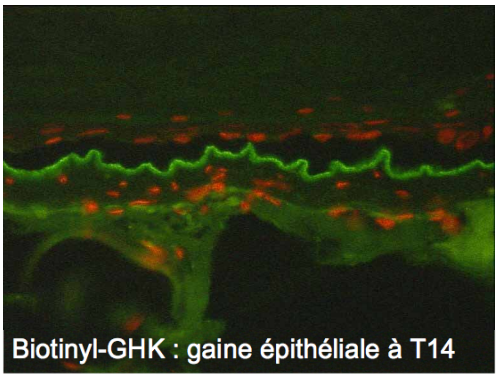
RESULTS

- ***Laminin-5***



These data indicate that the accepted model of the follicle in culture loses laminin-5 in the external epithelial root sheath at T14.

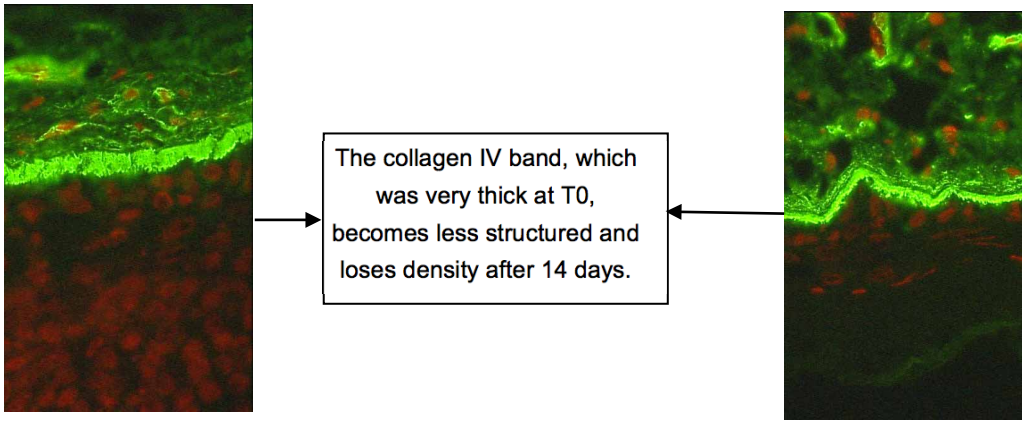


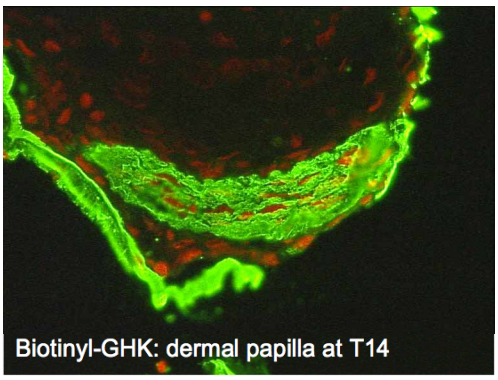


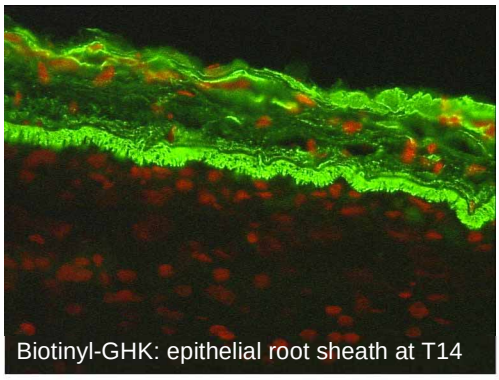
In contact with 2 ppm of biotinyl-GHK, laminin-5 remains very present in the papilla and the external epithelial root sheath after 14 days.

- ***Collagen IV***

As for laminin-5, the papilla of the follicle loses its collagen IV marking at T14.







In the presence of biotinyl-GHK for 14 days, collagen IV remains highly present in the dermal papilla and shows up densely and in a structured manner within the epithelial root sheath. The structure observed is nearly identical to what was seen in the control at T0.

CONCLUSION

The preceding study helped demonstrate the protective effects of the biotinyl-GHK peptide on two essential components of the epithelial root sheath and the dermal papilla: collagen IV and laminin-5.

As for the general morphology, biotinyl-GHK has marked anti-senescence activity on the keratinocytes of the hair follicle (14-day culture), which helps maintain a live epithelial root sheath and keratinocytic

proliferation to form the hair (Ki67 mitosis). It also helps improve the structuring of the adhesion proteins (collagen IV and laminin-5) responsible for communication between the two hair generating compartments.

The close similarity in the in vitro results obtained on the biotinyl-GHK peptide and the complementarity of the properties described for panthenol lead to the belief that there is a combined activity that lengthens and strengthens lashes. However, it was necessary to confirm the concept of lash treatment with an appropriate clinical study.

2.2. *In vivo* studies:

PRINCIPLE

The purpose of the clinical study was to evaluate the following among volunteers:

- Lash lenghtening,
- Lash tickening,
- Reinforcement of lash anchorage (decrease in lash loss during mascara removal).

Lashes were photographed in a precise manner that could be reproduced and at a special angle so that lashes could be viewed perfectly (see figure 12). An image analysis was then performed on these photographs to quantify lash length and diameter in the central segment of the lids of female volunteers. Lash volume was inferred from this data.

Figure 12: Area of measure

Concurrent to this quantitative metrological study, two other evaluations were performed: one clinical evaluation in which trained experts scored photos and another evaluation in which the volunteers themselves evaluated the perceived effects using a multi-item questionnaire.

PROTOCOL

- ***Type of studies and duration***

This was a double-blind study that employed non-invasive methods. 30 female volunteers used two mascaras:
VOLUMISING BLACK MASCARA ref. 18307 (SED 1012913A) containing 2% WIDELASH™ on one eye and placebo VOLUMISING BLACK MASCARA ref. 18309 (SED 1012913B) on the other eye (formulas can be found in the appendix).
The measurements were performed before application (T0) then after 15 and 30 days of daily application. Application during the eight hours preceding a measurement day was not allowed.

The volunteers had to apply each mascara as follows: 3 brush strokes to the outside lashes, 4 brush strokes to the central lashes and 3 brush strokes to the interior lashes. The investigator randomised the products to the right and left eye.

A dermatologist observed and supervised the usual inclusion (criteria, informed consent) and exclusion conditions.

- ***Tolerance***

Neither the investigators nor the volunteers reported any intolerance problems.

2.2.1. Evaluations

a. Photo measurements

Once the head was correctly positioned on an appropriate mechanism, the lashes were frontally photographed using a Reflex Nikon D300 (12.3 megapixel) digital camera with a "macro" (60 mm F/2.8 GED) lens and a flash. The images were analysed with special software. The calibrated lash images helped determine lash length in mm. The portion of the lids with the lashes was divided into 5 segments, which were numbered from S1 to S5. The lengths and diameters were measured on the lashes of segments S2, S3 and S4 (5 lashes per segment) on each side

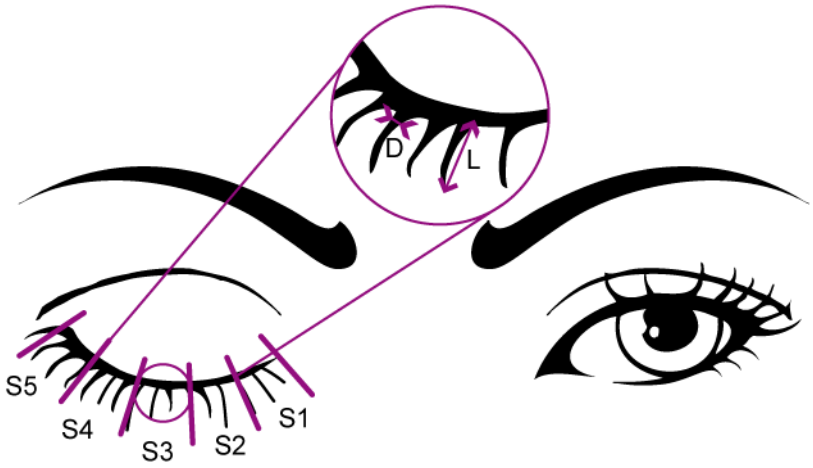


Figure 13: Measurement of lash length (L) and diameter (D)

b. Expert clinical evaluation

Three trained experts analysed the photos of the lashes of the 30 volunteers and evaluated the outcome.

c. Lash count evaluation

In order to evaluate the strengthening effect, two methods were used: self-evaluation (see below) and counting. The volunteers were invited to perform a daily rating of the number of lashes lost on each eye after makeup removal.

d. Self-evaluation by volunteers

Several self-evaluation questions were posed to the volunteers after 30 days:

1) "After mascara use, my lashes are..."
   a) **Longer**
   b) **Thicker**
   c) **Better nourished**
   d) **Strengthened**

With each statement, the volunteers needed to state:
Totally agree; Agree; Disagree; Totally disagree.

2) "In general, how would you rate the lash lengthening effect"?
   Excellent; Very good; Good; Fair; Poor.
3) "In general, how would you rate the lash thickening effect?"
   Excellent; Very good; Good; Fair; Poor.
4) Regarding the strengthening/anchorage effect:
   "The product helps prevent lash breakage/loss?"
   Totally agree; Agree; Disagree; Totally disagree

2.2.2. Results

➢ ***Measurement of lengthening increase***

a. Photo measurement

Table 1:
Variation in lash length after application of WIDELASH™ 2%

n=30	WIDELASH™			Placebo		
	T0	**T15 days**	**T30 days**	**T0**	**T15 days**	**T30 days**
Mean (mm) ± SEM	6.051 ± 0.126	6.690 ± 0.124	7.081 ± 0.126	6.047 ± 0.133	6.296 ± 0.131	6.433 ± 0.131
Difference TXj – T0 → **Max**	-	**0.639** 1.796	**1.030** 2.108	-	0.249 0.559	0.387 0.984
Variation (%) Significance → **Max**		**+10.56%** *p<0.001* +32%	**+17.02%** *p<0.001* +43%		+4.12% *p<0.001*	+6.38% *p<0.001*
Increase **Significance** ***versus* placebo**		**X 2.6** ***p<0.001***	**X 2.7** ***p<0.001***			

These results are shown in the following graph:

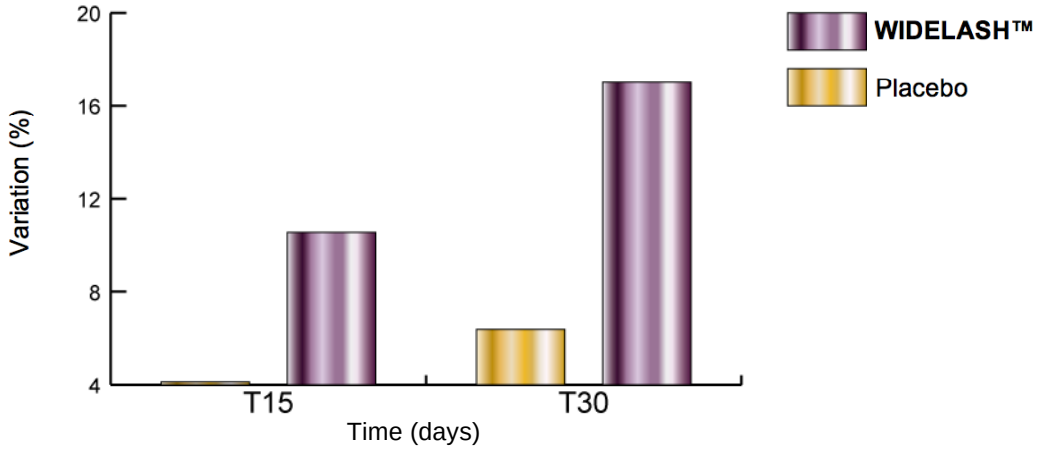


Figure 14: Variation in lash length compared with T0 (in %) (n=30)

The measurement of the increase in lash length shows that the mean length of the placebo lashes increased significantly by 4% and 6% at 15 days and 30 days respectively. This effect can be explained by natural lash growth.
At the same time, 2% WIDELASH™ stimulated natural lash growth by 10% and 17% at 15 days and 30 days respectively. Thanks to the biotinyl-GHK peptide, which stimulates keratinocyte proliferation in the hair bulb, WIDELASH™ helps lashes grow 2.6 times more than the mascara placebo at both study evaluation dates.

This difference in growth obtained with the two mascaras is significant in favour of WIDELASH™ ($p<0.001$).

This effect is illustrated by the following images.

Volunteer 1 : T0 - Placebo

Volunteer 1 : T30 - Placebo

Volunteer 1 : T0 - **WIDELASH™**

Volunteer 1 : T30 - **WIDELASH™**

Volunteer 2 : T0 - Placebo

Volunteer 2 : T30 - Placebo

Volunteer 2 : T0 - **WIDELASH™**

Volunteer 2 : T30 - **WIDELASH™**

b. Expert clinical evaluation

The evaluation by the 3 experts, who analysed the lash photos of the 30 volunteers, helped demonstrate that the placebo mascara was not perceived as a mascara that promotes lash growth (80% negative opinions; p<0.001*); concurrently, for the experts, 86% of the volunteers found that the 2% WIDELASH™ mascara had a significant effect (p<0.001) compared with the placebo, which had had a moderate to clear effect (43% and 44%).

* Chi² test

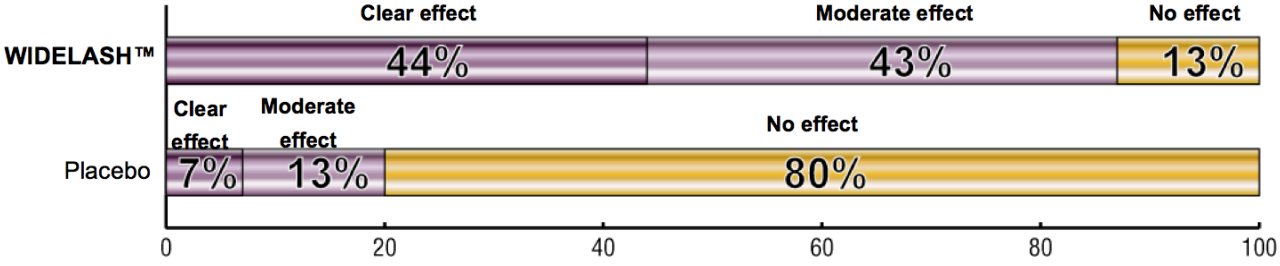


Figure 15: Blind evaluation performed by 3 trained experts on lash lengthening in photos of 30 volunteers

c. Self-evaluation

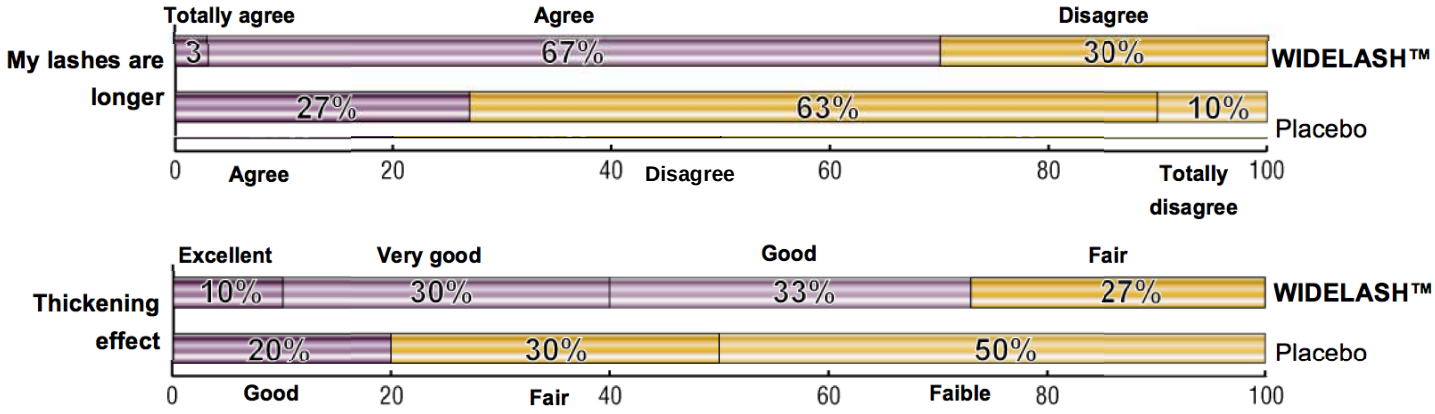


Figure 16: Self-evaluation performed by 30 volunteers on the thickening effect of mascara containing 2% WIDELASH™

The volunteers clearly noticed the benefits on their eyelashes with the mascara containing WIDELASH™ in comparison with the placebo mascara.

At the same time as the eyelash growth study, the diameter of the lashes was also studied.

➢ ***Measurement of lash diameter increase***

d. Photo measurements

The device used to measure the length of the lashes was also used to measure their diameter.

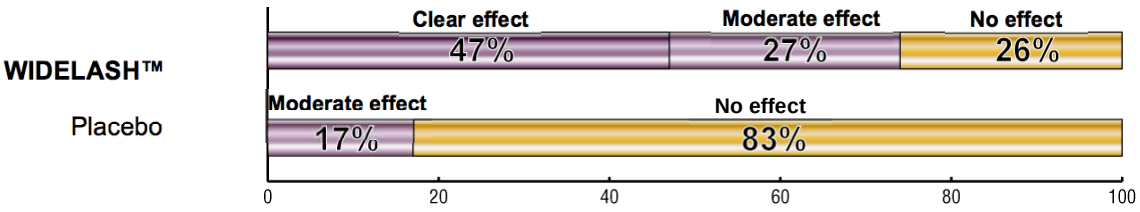
Table 2:
Variation in lash diameter after applying WIDELASH™ 2%

n=30	WIDELASH™			Placebo		
	T0	T15 days	T0	T15 days	T0	T15 days
Mean (mm) ± SEM	76.1 ± 2.0	85.2 ± 2.6	76.1 ± 2.0	85.2 ± 2.6	76.1 ± 2.0	85.2 ± 2.6
Difference TXj – T0 → **Max**	-	**9.0** 25.3	-	**9.0** 25.3	-	**9.0** 25.3
Variation (%) Significance → **Max**		**+11.96%** *p<0.001* **+33%**		**+11.96%** *p<0.001* **+33%**		**+11.96%** *p<0.001* **+33%**
Increase Significance *versus* placebo		**X 2.9** ***p<0.001***		**X 2.9** ***p<0.001***		**X 2.9** ***p<0.001***

The study of the variation of the lash diameter shows that with the placebo, their mean diameter increases by 4% and 7% at 15 and 30 days respectively. In parallel, the mascara with 2% WIDELASH™ increased the lash diameter by 12% and 19% at 15 and 30 days respectively.
WIDELASH™ thus increases by a factor of 2.9 in comparison with placebo, the diameter of the lashes after two study periods. This difference between both mascaras is significant and in favour of WIDELASH™ (p <0.001).

e. Expert clinical evaluation

The evaluation by the 3 experts of the lash thickening of the 30 volunteers helped demonstrate the placebo mascara was not perceived as a mascara that promotes an increase in lash diameter (83% negative opinions); concurrently, for the experts, 74% of the volunteers found that the 2% WIDELASH™ mascara had a moderate to clear effect (27% and 47%; figure 17 below). This perception was significant compared with the placebo (p<0.001*).



* Chi² test

Figure 17: Blind evaluation performed by three trained experts on increased lash diameter in photos of 30 volunteers

f. Self-evaluation

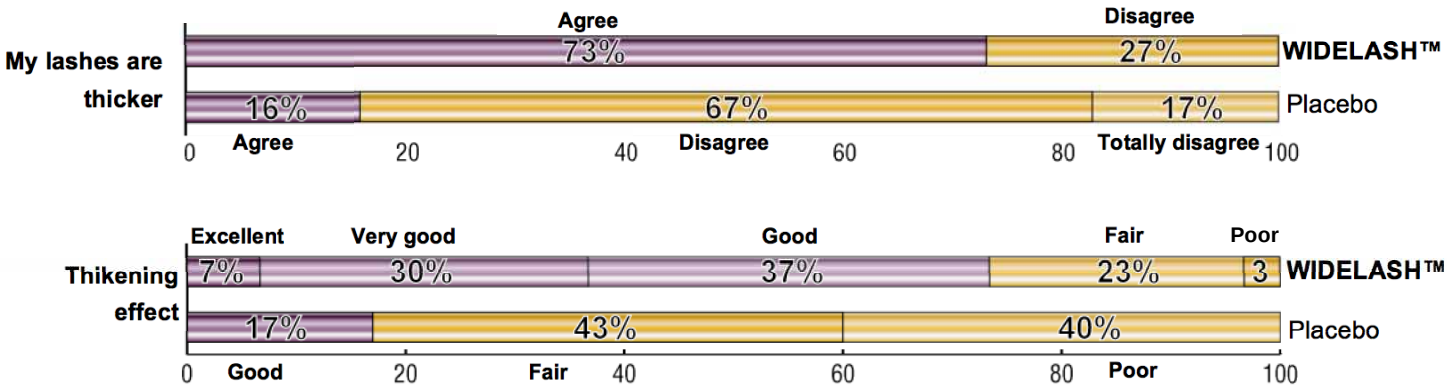


Figure 18: Self-evaluation performed by 30 volunteers on the thickening effect of mascara containing 2% WIDELASH™

The volunteers clearly noticed the benefits on their eyelashes with the mascara containing WIDELASH™ in comparison with the placebo mascara.

➢ ***Lash volume evaluation***

The length and diameter data helped to infer the increase in volume due to the application of the product or the placebo.

A lash can be compared to a cone, the volume of which is calculated using the formula provided in the appendix

Table 3:
Variation in lash volume after applying WIDELASH™ 2%

n=30	WIDELASH™			Placebo		
	T0	T15 days	T0	T15 days	T0	T15 days
Mean (mm) ± SEM	9.35 ± 0.51	13.06 ± 0.84	9.35 ± 0.51	13.06 ± 0.84	9.35 ± 0.51	13.06 ± 0.84
Difference TXj – T0 → **Max**	-	**3.71** 10.11	-	**3.71** 10.11	-	**3.71** 10.11
Variation (%) Significance → **Max**		**+39.7%** *p<0.001* +97%		**+39.7%** *p<0.001* +97%		**+39.7%** *p<0.001* +97%
Increase Significance *versus* placebo		**X 3** ***p<0.001***		**X 3** ***p<0.001***		**X 3** ***p<0.001***

The evaluation of the change in lash volume demonstrates that the volume of the placebo lashes increased by 13% and 22% at 15 and 30 days respectively. Concurrently, mascara with 2% de WIDELASH™ increased lash diameter by 40% and 67% at 15 and 30 days respectively.

WIDELASH™ therefore helped increase volume by a factor of 3 compared with the placebo lashes at both evaluation dates.

This difference obtained with the two mascaras is significant in favour of WIDELASH™ ($p<0.001$).

➢ ***Evaluation of lash anchorage***

In order to evaluate the strengthening effect, the volunteers counted the number of lashes lost during daily eye makeup removal

g. Counting by volunteers

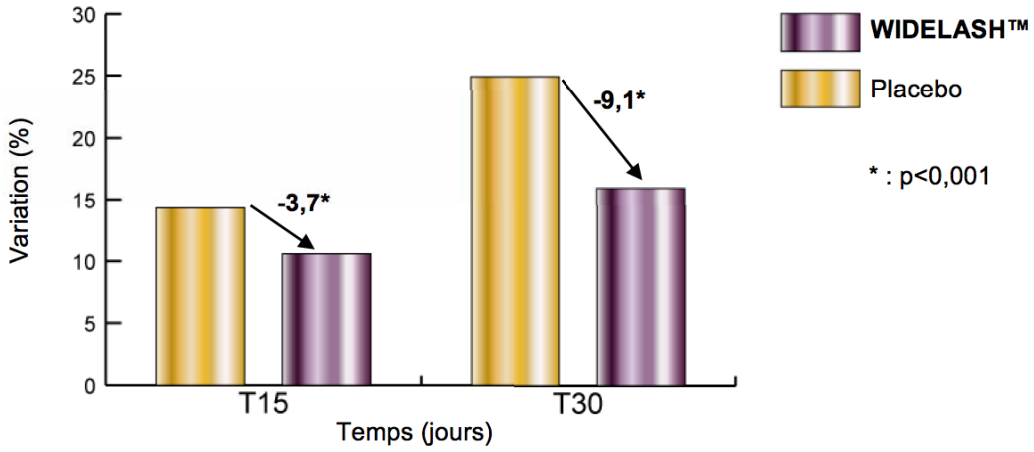


Figure 19: Variation of lashes lost

Above figure shows that using mascara with 2% WIDELASH™ helps reduce lash loss when removing makeup. The mean loss reduction was 4 lashes (at 15 days) and 9 lashes (at 30 days) compared with the placebo mascara.

h. Self-evaluation

The fortifying effect of WIDELASH™ was estimated with the volunteers after an average of 3 assertions. The 30 volunteers rated their own perceived effects following the mascara use.

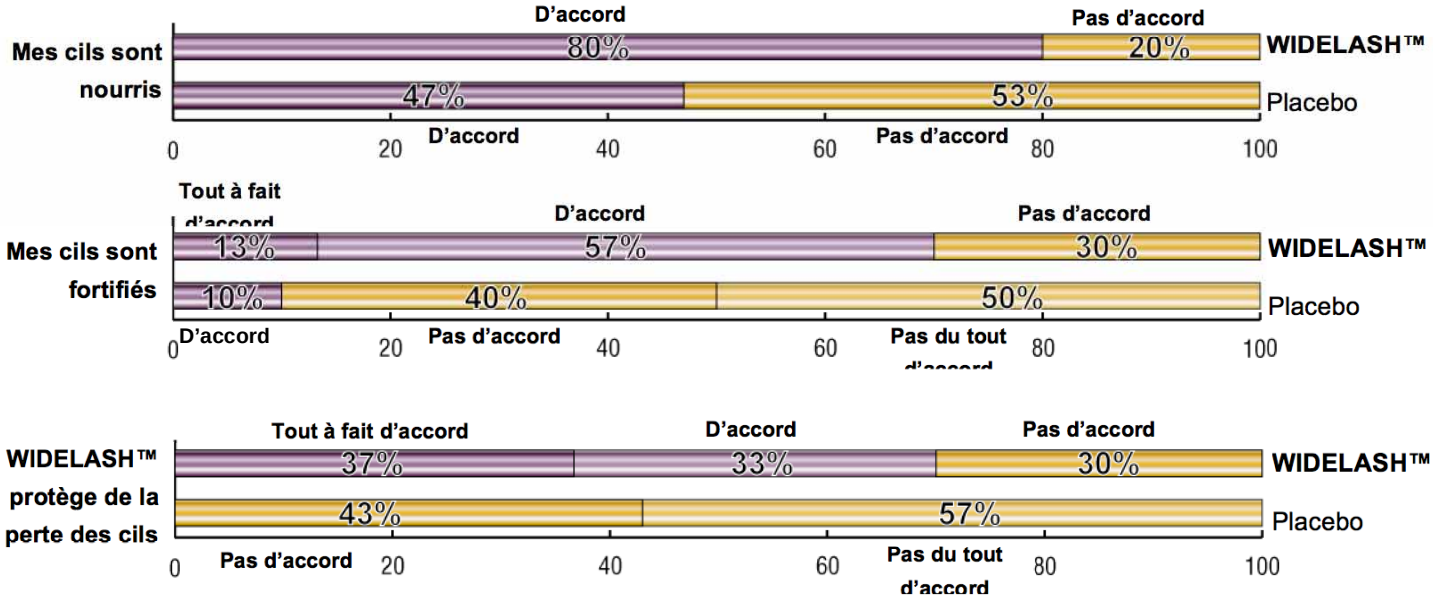


Figure 20: Self-evaluation performed by 30 volunteers on the strengthening effect of mascara containing 2% WIDELASH™

80% of the volunteers think that their lashes were nourished thanks to the mascara containing **WIDELASH™** while the opinions remained mixed against the placebo. The difference is made clear with the assertion "my lashes are strengthened" whereas 70% of the volunteers agree with this assertion only 10% were for the placebo. Finally, 70% find that **WIDELASH™** prevents eyelash loss and none of the panelists were for the placebo.

The beneficial effect of **WIDELASH™** to strengthen lashes is very clearly perceived by the volunteers, contrary to the placebo.

3. CONCLUSION

WIDELASH™ is an active ingredient that improves the appearance of lashes. The in vitro studies identify a specific mechanism of action on the hair bulb.

First of all, this specificity was demonstrated by the substantivity of the main ingredient, the biotinyl-GHK, around and on the lengh of the hair follicle.
On the other hand, a test of the proliferative activity on the sections of the lower part of the follicles showed that the biotinyl-GHK supports the mitotic activity on the root sheath keratinocytes and the hair shaft generators. This result was confirmed by measures of hair follicle growth maintained in culture, amounting to 58%/T0 with 2 ppm of biotinyl-GHK and 121%/T0 with 5 ppm of biotinyl-GHK.
The integrity of the hair also ensures anchorage to the root sheath. Morphological observations of hair follicles incubated for 14 days revealed that the biotinyl-GHK is capable of strongly stimulating the synthesis of two anchoring proteins, collagen IV and laminin-5, and their organisation at the root sheath junction/dermal papilla
The in vitro results strengthened **WIDELASH™**'s positioning and the benefits shown after a double-blind clinical study on 30 female volunteers.

This study clearly shows that after a one month eyelash treatment using a mascara with 2% **WIDELASH™** leads to a significant increase in lash length ($p < 0.001$) 17% which accords with the clinical evaluation by the trained experts who confirm a visible to very visible effect on 87% of the panel.
In parallel, the lashes treated with the placebo mascara did not grow as much as the ones with the mascara containing **WIDELASH™,** making them unnoticeable to the naked eye (no effect 80%).
A great majority of the volunteers noticed that their lashes were longer (70%) and the thickness was good to very good (73% opinions).

This study also showed, with complementary methods, that lashes were thicker thanks to 2% **WIDELASH™** (+19%) against placebo (7%). A significant difference of ($p<0.001$). The trained experts were able to notice this improvement (noted in 74% of the cases, the placebo showing no effect in 83% of the cases). To sum up, the volunteers indicated that their lashes were thicker for 73% of them, an overall phenomenon being good to excellent in 74% of the cases.

This data allowed us to calculate the lash volume which clearly increases in just 15 days with 2% of **WIDELASH™** (+40%) and even more after 30 days (67%). The observations made by the experts and the volunteers are thus confirmed.

The fortifying effect of **WIDELASH™** was appreciated by a regular counting of lashes, the number of lashes lost during make-up removal were less important with **WIDELASH™** than with the placebo (mean difference of 4 lashes (15 days) to 9 lashes (30 days). 70% of the volunteers noted that the mascara containing 2% **WIDELASH™** strengthened their lashes, and helped to prevent lash loss and breakage.
The same volunteers noted that the mascara containing **WIDELASH™** nourished the lashes (80% opinions).

By formulating with **WIDELASH™,** it is possible to claim a volumising and protective effect for lashes on the basis of clinical proofs confirmed by a study carried out according to the Gold Standard: controlled, randomised and double-blinded studies against placebo.

It is recommended to formulate **WIDELASH™** at 2%.

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