Stimulation of the KLF4 pathway by bee products modulates the progression of hair anagen to telogen molecular switch.

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Abstract

Background: Honey contains many ingredients varying by region highlighting the unique flora and fauna available to bees. Carbohydrate, amino acids, proteins, lipids and vitamins make each Royal Jelly unique and a subject of intense investigation for human benefits as potential medicines and cosmetics.

Methods: We created a scalp / follicular epidermal-hair axis testing system, evaluating bee product extracts. 4 honey and 1 royal jelly previously evaluated to have biological activities were combined with growth medium and applied to scalp for 10 days. Metabolic activity and expression of FGF9, IGF-1, FGF5, KLF4, FGF7, TGF-β, Versican and KGFR known for epidermal/hair follicle kinetics were investigated.

Results: Scalp application did not reduce sample viability, with increases in sample cohesiveness, epidermal thickness and dermal matrices. Melanin promotion was found within hair follicles. Immunofluorescence, was remarkable, showing hair bulb stem cells proliferation promotion (KLF4 expression increase), whilst lower FGF5 and TGF expression, consistent with movement away from telogen. Versican, FGF7, IGF1 were slightly higher in the hair bulb area consistent with anagen phase promotion and hair growth mediation to follicles from fibroblasts. Increased epidermal expression of KGFR, KLF4, FGF7 and TGF-β indicated a higher level of differentiation-maturation cycling / turnover of keratinocytes promoting a thicker more proliferative epidermis, without dysregulation.

Conclusion: We demonstrated this bee product combination has beneficial effects on epidermis and hair follicle and is a good candidate for cosmetics products. Our study

highlights our *ex vivo* model as an accurate approach for testing cosmetic actives dedicated to scalp and hair care.

Keywords: Hair follicle; Anagen; Telogen; Bee products; Stem cells.

1. Introduction.

Medicines containing natural bee products are gaining more and more attention [1]. Numerous studies have now proven the positive benefits of bee products for medical treatments but also as cosmetic ingredients. Each bee product possesses specific components which determine their activities. Honey contains more than 180 ingredients making each honey singular with unique properties. Royal jelly contains carbohydrates and amongst others amino acids, proteins, lipids, vitamins making this bee product a key element [2].

Skin biology has also linked the epidermal-hair axis as an active and effective route for ingredient interaction for regeneration. Here we developed an accurate hair follicle – dermis – epidermis model to effectively evaluate hair phase kinetics through to promotion of coloration in the epidermis and hair itself. Several known molecules, receptors and signal transduction pathways have been identified as being key in the development and growth of hair and particularly hair phases of anagen, catagen and telogen.

Therefore, the aim of this study was to create an accurate scalp and hair model that could allow for screening of any product or active ingredient. Growth of *ex vivo* cultures is known to be difficult due to the issue of keeping skin alive long enough *in vitro* before either the bacterial system overcomes the sample, or necrosis is not held back any longer by the oxygenated growth media. Therefore, such testing systems, although very difficult, are important for validation, efficacy and safety testing in the cosmetics industry.

2. Materials and Methods.

2.1 Sample collection

All tissues used in this study were provided from human skin samples following ethical consent after elective surgery. Skin samples, which were surgical waste, were transported under optimal conditions to ensure and maintain tissue viability. CTIBiotech is also certified by the French Ministry in charge of research for the preparation and conservation of elements derived from the human body.

2.2 Ex vivo model preparation

Explants were produced from scalp skin sample of a 56 year old Caucasian female following planned elective surgery. The sample had a high density of hair follicles (up to 100 hair follicles). Punches of 8mm² (Kai, Laboderm, Asnières-sur-Seine, France) were done on the full skin thickness and placed in 12-well plates (Corning, New-York, USA) according to CTIBiotech optimization protocols and media in order to produce uniform samples for testing (**Figure 1.**).

Samples were placed into skin culture medium for maintenance before the testing phase. The scalp samples taken were classified as non-pathological with no presenting dermatological issues. Hair structure was noted as normal and was confirmed in later histology (**Figure 1.**).

2.3 Active application to samples

In this study and based on previous lab testing, a combination of four honeys sourced from Ouessant, Corsica, Aland and Ikaria islands plus a royal jelly (together forming product 121) was selected for *ex vivo* testing. Product 121 was resuspended in skin growth medium (CTIBIOTECH, Lyon, France) and prepared daily for use.

In order to assess active effect, product 121 was applied daily for 10 days using a sterile gauze to retain it on top of the biopsies and avoid any systemic application in the medium. No rinsing was carried out during time of culture before a new application of the product. Each condition was performed in triplicates.

2.4 Metabolic activity analysis

To assess metabolic activity, the AlarmarBlue™ Cell Viability reagent (Invitrogen, Carlsbad, Carlifornia, USA) was directly added in each individual well and incubated for 4 hours. Supernatants were collected and transferred to a black-walled 96-well plate. Fluorescence was quantified on a microplate reader (Tecan Spark®) at 550 nm, excitation and 590 nm, emission. This protocol was repeated on day 0, 3, 6 and 10 to follow metabolic activity kinetic.

2.5 Histology coloration

At the end of experiments, explants were rinsed once with PBS 1X (Corning, New-York, USA) and fixed in formaldehyde 4% w/v (Sigma-Aldrich, Saint-Quentin-Fallavier, France), before dehydration in alcohol crescent baths and clarification in xylene. Samples were then embedded in paraffin and sectioned into 5 µm thick slices. Hematoxylin, eosin and saffron coloration (Sigma) were performed on the slide after a rehydration process.

2.6 Immunofluorescent staining

Additionally, skin explants were fixed in OCT Cellpath Embedding Matrix (Dutscher, Issy-les-Moulineaux, France) and stained with antibodies against FGF9, IGF-1, FGF5, KLF4, FGF7, TGF-β, Versican and KGFR receptors (Novotec, Lyon, France). A staining of nuclei was also performed using 1 µg/ml DAPI (Sigma-Aldrich). Staining of samples was grouped where possible to hair phase unless the antibody had cross reactivity.

To maximize data, double tagging was performed, and groups were decided where possible by anagen/telogen/stem cells and other information regarding the hair follicle.
Observations and acquisitions were carried out on Leica (Germany) DMLB fluorescence microscope controlled by image acquisition software (LASv4.2).

3. Results.

3.1 Ex vivo culture parameters and metabolic activity

Samples collected were evaluated as pathologically normal and subsequent histology proved the normal structure of hair bulbs and follicles (**Figure 1.**).

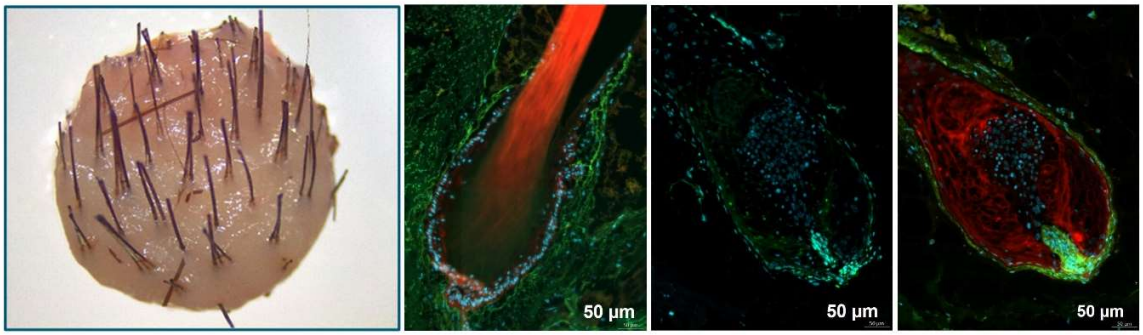

Figure 1., Scalp and hair collected from the donor.

Samples collected from a female donor exhibited normal pathology of scalp skin and normal follicular structure and density with a variety of hair phases.

Overall no difference in viability of the samples was observed between conditions, with a lower viability by Day 10 as expected with human *ex vivo* samples. There was no statistical differences between the control untreated conditions and the treated cultures.

A trend towards increased metabolic activity (with the samples receiving nutrients from the media) was seen at Day 3, while after that a slow decline of activity was observed, which is normal in *ex vivo* tissue cultures (**Figure 2.**).

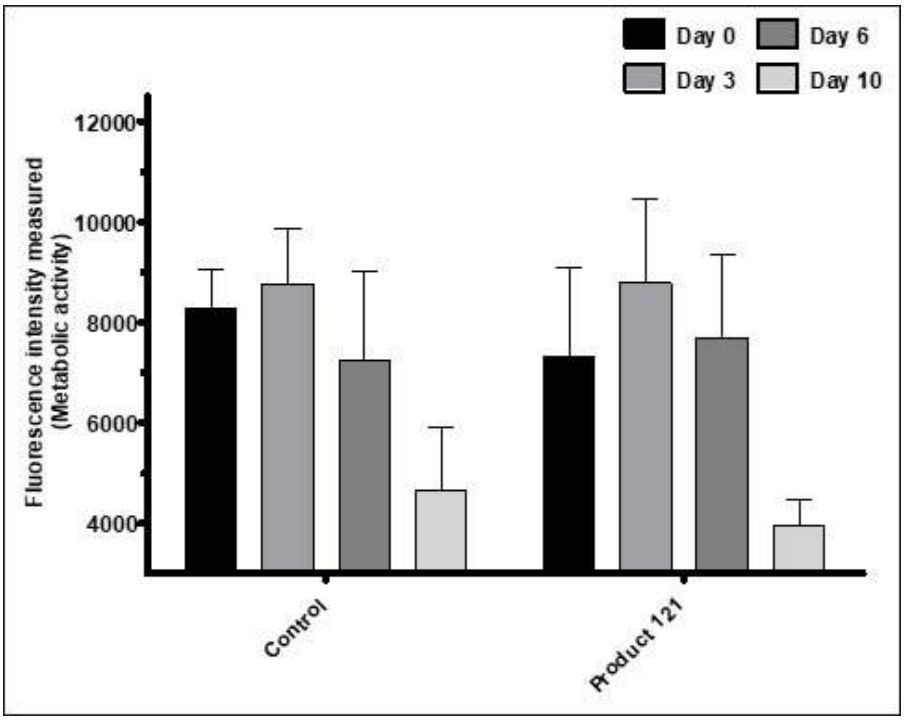

Figure 2., Metabolic analysis of scalp samples over time.

Alamar blue metabolic analysis revealed the time point of development with and without active ingredients over 10 days. No statistical difference in the overall health of the tissues was recorded.

3.2 Structural histology of the ex vivo scalp and hair samples

Structural changes compared to control were not noticed in the dermis, but moisturizing effects were visible in the epidermis with a possible thickening being noticed.

It might be argued that some increase in matrix formation was made in the dermis, but specific staining would be required to prove that but was not carried out in this study (**Figure 3.**).

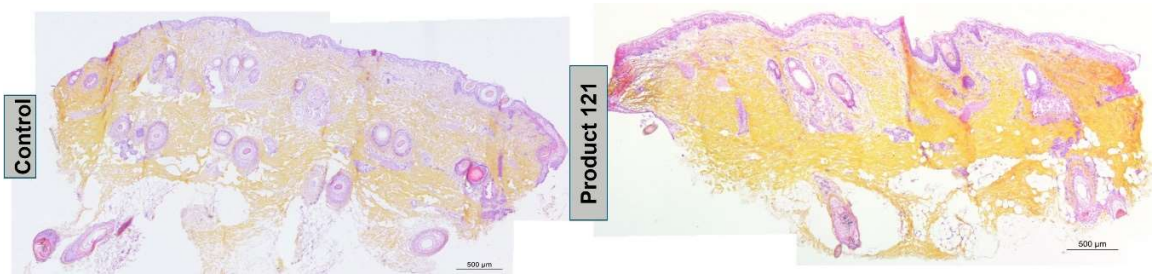

Figure 3., Full scalp histology revealed that active ingredient increased cohesion of epidermis and dermal matrices.

Full scalp histology (HES) was carried out on full samples to investigate overall structure and response of samples with and without active ingredient (photo scale bars 500 µm).

High resolution imaging was made of the histological samples. Interestingly, an increase in melanin production was seen in the samples treated with bee products. This could be observed in the areas where melanocytes normally reside and upwards towards the forming follicle (**Figure 4.**).

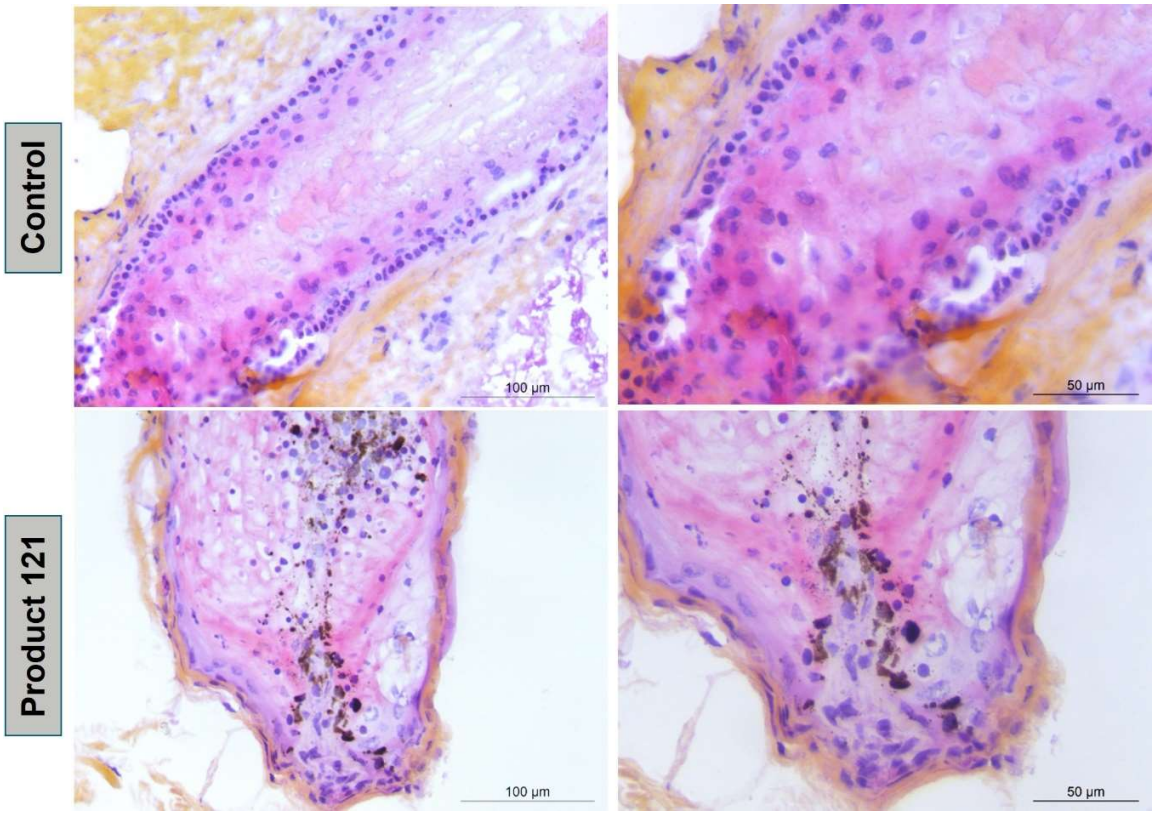

***Figure 4.**, Follicular analysis following active ingredient treatment revealed upregulation of melanin expression* (scale bars 50 – 100µm).

Histological examination of samples with and without treatment were processed for histology (HES) and analyzed by high resolution microscopy. Increased melanin expression was noted in samples which were treated with the product 121.

3.3 Fluorescent imaging reveals regulation in the hair bulb system

High resolution fluorescence imaging after labelling with markers relevant to the hair, epidermis and dermis were made and the differences between untreated controls and samples treated with bee products is summarized on **Table 1**.

	CONTROLS			PRODUCT 121		
	E	D	Bulb	E	D	Bulb
FGF5	✓	low	low	✓	low	↘
KGFR	✓	×	low	↗	×	↗(matrix)
DAPI	✓	✓	✓	✓	✓	↑
KLF4	✓	low	✓	↗	low	↑
FGF9	×	low	×	×	low	×
DAPI	✓	✓	✓	✓	✓	↑ 2/3
Versican	×	✓	×	×	↓	↗(papilla)
FGF7	✓	×	✓	↑	×	↑(matrix)
DAPI	✓	✓	✓	✓	✓	↑(papilla)
TGFb	✓	low	✓	↗	low	↘
DAPI	✓	✓	✓	✓	✓	✓
IGF1	low	low	low	low	low	↗
DAPI	✓	✓	✓	✓	✓	↑ 2/3

Table 1., Fluorescent marker evaluation summary.

Following histological processing markers for FGF5, KGFR, KLF4, FGF9, Versican, FGF7, TGF β and IGF-1 were evaluated in groups as appropriate for hair phasing. Code: ✓ - expressed / or same as control Low – low expression, but there. × - not expressed ↗ - slightly higher ↘ - slightly lower

To understand the role of these molecules or receptors, it is also important to view their location. Relevant markings where there were differences of note to the controls are given on **Figures 5 and 6**.

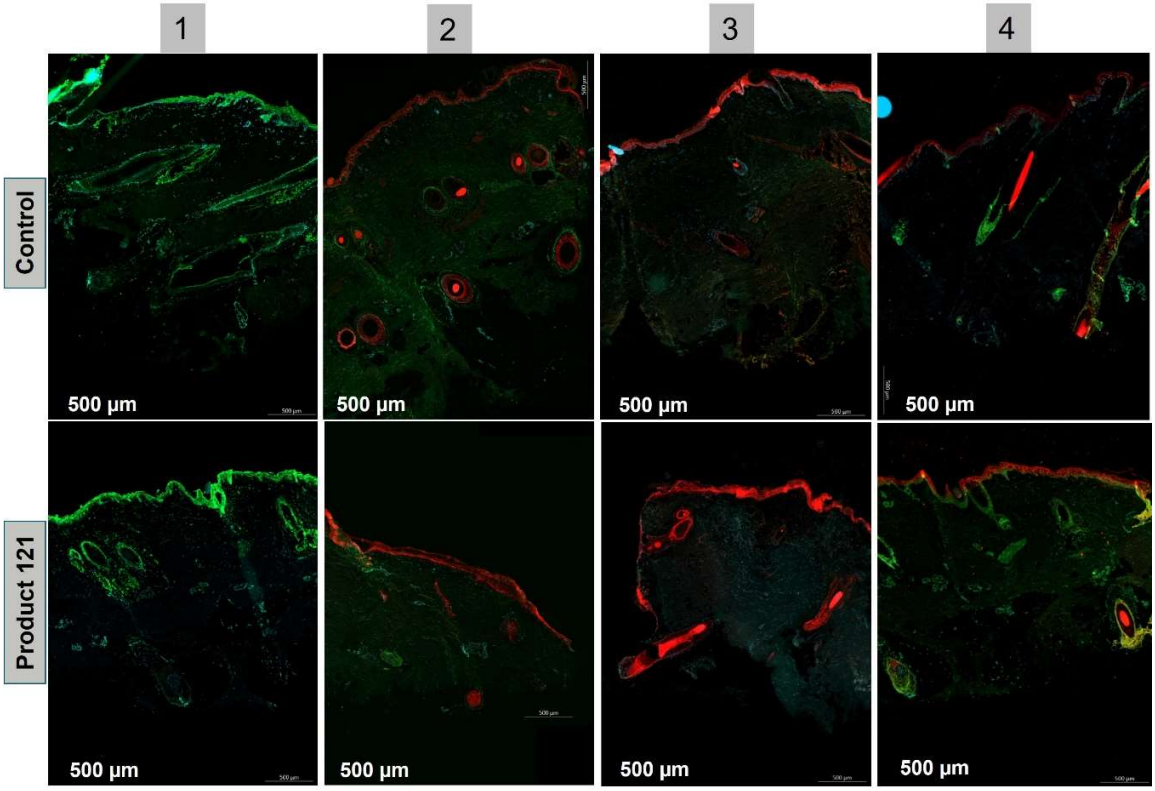

Figure 5., Epidermal expression of hair related markers was modulated by the active ingredient.

TGF β, FGF7, KLF4 and KGFR were increased in expression in samples treated with active ingredient. The higher level of differentiation-maturation cycling and turnover of keratinocytes created a thicker more proliferative epidermis without dysregulation. Left to right: Panels 1, TGF-β (green); Panels 2, Versican (green), FGF7 (red); Panels 3, FGF9 (green) KLF4 (red); Panels 4, FGF5 (green), KGFR (red). Scale bars 500 µm.

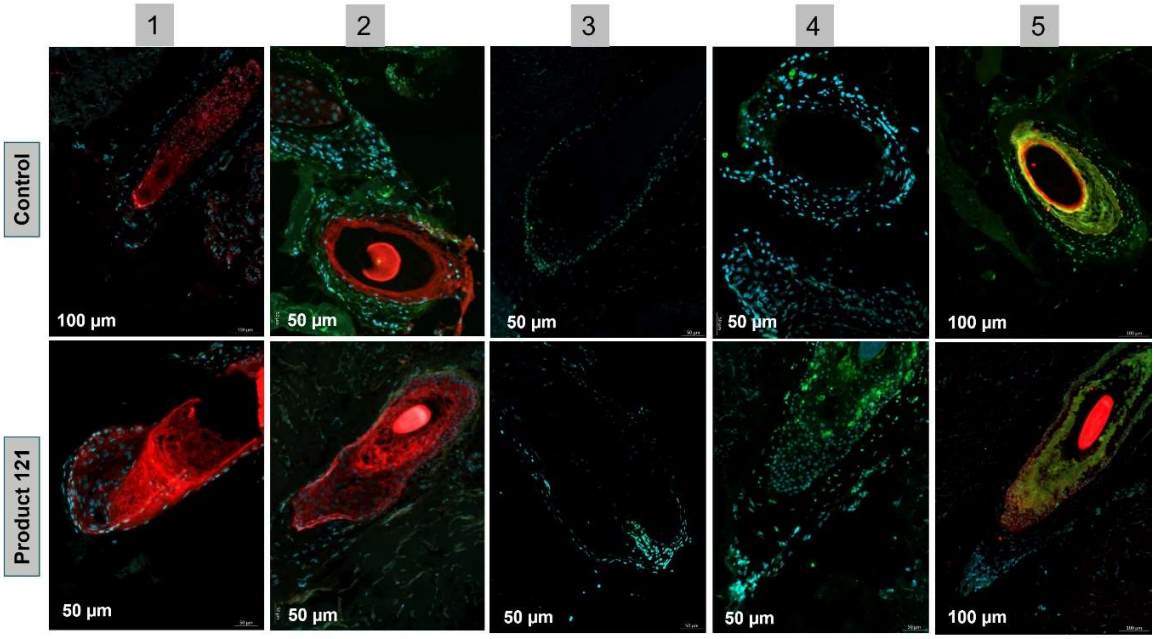

Figure 6., Modulation of hair bulb and structure was stimulated with the active ingredient.

Left to right: Panels 1, FGF9 (green), KLF4 (red; Panels 2, Versican (green), FGF7 (red); Panels 3, IGF-1 (green); Panels 4, TGF-β (green; Panels 5, FGF5 (green), KGFR (red). Scale bars 50 to 100 µm.

Promotion of hair bulb stem cells was shown with KLF4 elevation. Anagen phase was promoted in the hair bulb where FGF7 and Versican were increased. IGF-1was slightly increased. TGF- β and FGF were lower consistent with a move away from telogen phase.

4. Discussion.

Ex vivo skin and scalp testing are hard to perform because of the microbiome of the skin and particularly in the case of scalp, the removal of the underlying blood vessel system removes the immunological protection normally found. Therefore, the time of *ex vivo* cultures is extremely limited. Despite this, the maintenance media that we have created allows for healthy cultures over several days.

Further to this, as we have seen in our laboratory, if a product is toxic, an *ex vivo* sample will respond to it very quickly, particularly with discoloration. However, in this study, the bee products employed did not reveal any particular negative effects. This was confirmed in the histological evaluations (**Figures 3.** and **4.**) and in the metabolic analysis (**Figure 2.**). Positive hydration effects were observed with an overall better sample quality in the dermis and particular the epidermis when bee products were applied. Whilst normal physiology was noticed, what was surprising was the increase in melanin production in the hair follicles and this warrants further investigation in a new study.

Immunofluorescence in this study was a difficult and laborious task, particularly because expression of some of the markers is very specific and required much microscopy to confirm location. The markers were chosen for their known roles in the skin. FGF9: Stem cell marker expressed in derma papilla [3]. IGF-1: Marker of the anagen phase of the hair follicle expressed in derma papilla. FGF5: Marker of the telogen phase of the hair follicle expressed in derma papilla. KLF4: Stem cell marker expressed especially in hair stem cells. FGF7: Marker of the anagen phase of the hair follicle expressed in derma papilla. KGFR: FGF7 receptor. TGF-β: Marker of the telogen phase of the hair follicle expressed in derma papilla: crucial for hair follicle downgrowth. Versican: Marker of the anagen phase of the hair follicle expressed in derma papilla [4]. The expression of the markers (summarized on **Table 1.**), showed, despite the short time period, differences between the controls and the treated samples. It could be concluded that the bee products had an effect on the cell proliferation of the hair bulb area.

This is also in agreement with KLF4 which is expressed in the stem cells area of the hair bulb, which was slightly increased.

FGF5 and TGF-β being lower when bee products were applied also is interesting, since the expression is often more in telogen phase, so if the products were promoting cell division in the hair bulb (which it seemed they were) then FGF5 would be expected to be lower [5].

In agreement with the above is that Versican, FGF7 and IGF-1 were slightly higher in the hair bulb area indicating higher activity [6]. All of these considerations suggest that the bee products were promoting the healthy growth of the hair.

Although the focus of the study was on the hair, the response of the epidermis was interesting because of the increase in expression of KGFR, KLF4, FGF7 and TGF-β [7].

KLF4 is a transcription factor required for establishing the barrier function of the skin [8]. As such the increase of this marker in the epidermis indicates that differentiation and maturation transfer has been promoted, which is generally a good sign after product application with bee products [9]. KGF receptor is essential for the normal turnover of keratinocytes and the abnormal expression of this receptor is implicated in many skin diseases, but at lower levels is important when skin is damaged [10]. The small increase here could be beneficial and might account for the slightly thickened epidermis, but more testing would be required to confirm that. FGF7 promotes a thicker and more proliferative epidermis in skin [11]. It has been understood for some time that TGF-β is essential in normal skin homeostasis and wound healing [12]. Certainly, the response here with an increase in expression would suggest an activation of that pathway. Whether this activated the skin to become thicker with more barrier function seems a possibility, but more analysis would be needed. Therefore, taken together (bearing in mind the short time of the *ex vivo* culture) the increase in these markers would seem to indicate a beneficial effect of bee products on the epidermis of the skin samples.

5. Conclusion.

Ex vivo scalp containing hair samples were treated with a combination of four honey sourced from Ouessant, Corsica, Aland and Ikaria islands and royal jelly. We demonstrated that this association had a promoting effect on epidermal keratinocyte kinetics, melanin production in the hair follicle and exert a stimulation of the stem cell compartment of the hair bulb, suggesting that this combination of bee products was specific in its actions. Therefore, these active ingredients are good candidates for further investigation and cosmetic industry formulations dedicated to scalp and hair care.

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Conflict of Interest Statement. NONE.

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