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## ***“The Aging Process: Unraveling the Connection Between Neuroageing and Skin Sensitivity”***

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### **1. Introduction**

Aging significantly impacts the skin's nervous system through a process known as neuroageing, which alters neuronal function and sensory experiences [1–6]. A key consequence of neuroageing is the reduction in sensory neurons, essential for touch perception [7,8]. As we age, the efficiency of neuronal communication in the skin declines, leading to decreased sensitivity [3,9,10]. While skin hypersensitivity is often studied [11], the issue of diminished sensitivity, including touch and neuronal connections, requires further exploration. The interactions among different skin compartments are not well understood, particularly as the peripheral nervous system deteriorates with age [3,9].

This decline not only impairs neuronal regeneration but also affects skin sensitivity, touch perception, and social interactions [7,8]. Understanding the interplay between aging and neuroageing is crucial for comprehending how these changes influence sensory functions in the skin [12].

### **2. Materials and Methods**

#### *2.1. Neurite growth study in human sensory neurons in co-culture with keratinocytes*

##### *2.1.1. Cell culture*

The sensory neurons are derived from hiPS cells (human induced Pluripotent Stem cells) which are themselves obtained from human fibroblasts. The cells were seeded and maintained for 6 days in culture inducing their differentiation (M1). The culture medium was changed every 2 days. After 8 days of culture, the medium was replaced by a mixture of maturation medium for sensory neurons (M2) and growth medium for keratinocytes. The keratinocytes (from 23 years old and 58 years old donors) were seeded in the plates above the layer of differentiated hiPS. After 9 days of culture, the culture medium was replaced by a culture medium without growth factors containing or not the positive reference NGF at 50 ng/mL. After 4 days of incubation, the cells were fixed in a 2% PFA (Paraformaldehyde) solution in culture medium.

##### *2.1.2. $\beta$ -tubulin immunostaining*

The cultures were then washed twice in PBS then permeabilized and the non-specific sites were blocked. The cells were incubated in the presence of an anti- $\beta$ -tubulin primary antibody.

This antibody was revealed by a secondary antibody coupled to a fluorochrome. The nuclei were labelled with Hoechst's solution, a nuclear fluorescent marker. Photographs were taken and a count of sensory neurons and a measurement of their neurite length were performed and compared to the control condition.

## *2.2. CGRP release study in human sensory neurons in co-culture with keratinocytes*

The sensory neurons are derived from hiPS cells (human induced Pluripotent Stem cells) which are themselves obtained from human fibroblasts. The cells were seeded and maintained for 6 days in culture inducing their differentiation (M1). The culture medium was changed every 2 days. After 9 days of culture, the M1 medium was replaced by a mixture of maturation medium for sensory neurons (M2) and growth medium for keratinocytes. Then, after 13 days of culture, keratinocytes (from 23 years old and 58 years old donors) were seeded in the plates above the layer of differentiated hiPS. Finally, after 9 days of co-culture, the cells were incubated for 4 hours with or without the presence of oxytocin at 0.2mg/mL. Then, the cells were activated by capsaicin at 10 $\mu$ M. 30 minutes later, the supernatants were collected and stored at -80°C then thawed and a dosage of the quantity of CGRP neuropeptide released was carried out by ELISA.

## *2.3. Aged re-innervated skin explants*

### *2.3.1. Isolation and differentiation of primary sensory neurons (PSN)*

Human Skin-derived precursor cells (SKPs) were obtained from abdomen of healthy donors who had undergone plastic surgery. After 1 month of culture, a differentiation (10 days of culture) step was initiated to obtain a neuronal network (confidential). The primary sensory neurons (PSN) were pre-cultured *in vitro* at 2 different concentrations of NGF to impact the neuronal development.

### *2.3.2. Skin explants and PSN co-culture*

Primary sensory neurons (PSN) were seeded in precoated insert to obtain a neuronal network and culture in maturation medium (confidential). Then, after 3 days of culture, skin explants obtained from abdomen of healthy female donor who had undergone plastic surgery, in accordance with the Declaration of Helsinki. (from a 55 and 60 years old donors) were placed on 24-well insert in survival with BEM culture medium (Bio-EC's Explants Medium) for 6 days, allowing innervation. The innervation is modulated thanks to 2 different concentrations of NGF. After incubation, half-skin explants were firstly fixed in PFA 4% and successively in sucrose at 30% and then snap-frozen in cold isopentane. The other half was fixed in a formaline solution.

### *2.3.3. PGP9.5 immunostaining*

PGP9.5 immunostaining has been performed on 30  $\mu$ m-thick frozen skin sections with a polyclonal anti-PGP9.5 antibody diluted in PBS, BSA 1%, Triton® X-100 at 0.3% overnight at room temperature and revealed by Alexa Fluor 488.

The immunostaining was assessed by counting the number of nerve fibers in the epidermis and expressed as number of nerve fibers per cm of epidermis.

### *2.3.4. TRPV1, ZO1, Ki67 and P53 immunostainings*

4  $\mu$ m-thick sections were prepared using a microtome. The sections were then de-wrinkled and mounted on slides, placing one vertical section per slide (4 fragments in one block). After drying for 1 hour in an oven at 65°C, immunofluorescent staining was performed using a Discovery Ultra-VENTANA automated system (Roche®).

Following deparaffinization of the slides at 75°C for 7 minutes, the slides were incubated with an antigen retrieval solution for 40 minutes at 95°C. After rinsing, the primary antibody was applied and incubated for 60 minutes at 37°C. After another wash, a secondary antibody (Anti-Rabbit or Anti-Mouse) was applied and incubated for 16 minutes, followed by incubation with the Rhodamine kit (Roche®) for 20 minutes. The slides were rinsed again, counterstained with DAPI for 4 minutes, and then mounted with the aqueous mounting medium Fluoromount-G. After checking, the slides were digitized at 20x resolution using a Hamamatsu slide scanner (S60/C13210 - NANOZOOMER) equipped with a fluorescence unit and filters for DAPI ( $\lambda=350$  nm) and TRITC ( $\lambda=555$  nm).

For each section, the epidermis and dermis (ROI) were first segmented using an automatic pixel classification method (QuPath software). For slides stained with TRPV1, and ZO1, the area occupied by the staining in the epidermis was calculated, along with the mean intensity of the epidermis. For Ki67 and p53 slides, nuclei were segmented with thresholding on DAPI level, then the number of nuclei positive for nuclear staining was calculated.

## 2.4. Clinical study

### 2.4.1. Panel description

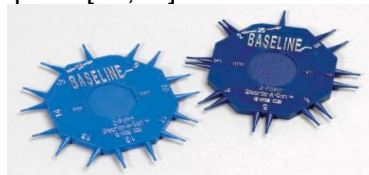
A total of 123 volunteers (aged between 18 and 74 years, mean age:  $43.3 \pm 16.6$  years) were involved in the pilot study. The measurements were taken without the application of any product.

### 2.4.2. Ethics statement

All participants received both verbal and written information regarding the study, which emphasized that participation was voluntary. They were informed that they could withdraw from the study at any time and for any reason. Participants were encouraged to ask questions about the study and were given ample time to consider their involvement before providing consent. Written informed consent was obtained from each subject prior to the initiation of any study-related procedures.

### 2.4.3. Sensory evaluation of touch with Static Two-Point Discrimination test

The test for the discrimination of two static points involves determining the smallest distance at which a subject can perceive the difference between two static points applied using a specialized tool designed for this purpose [13,14].



The test involves consecutively applying two tips with a decreasing gap. The technician narrows the gap until the subject hesitates. At this point, the technician conducts a series of 10 tests at the spacing where the subject showed hesitation. During this series, one point is applied 5 times and two points are applied 5 times, in random order. If the subject provides at least 7 correct responses, the value is validated, and the technician can perform the same test with a smaller deviation of one millimeter until the test yields a negative result. The final result is the last distance at which the subject achieved at least 7 validated responses.

Subjects had their eyes closed to prevent them from seeing the technician's manipulations. This test was conducted on the index finger of the most frequently used hand of each panelist

at D0, D28, and D56 for the group aged between 50-75 years old and only at D0 on the group aged between 18-30 years old.

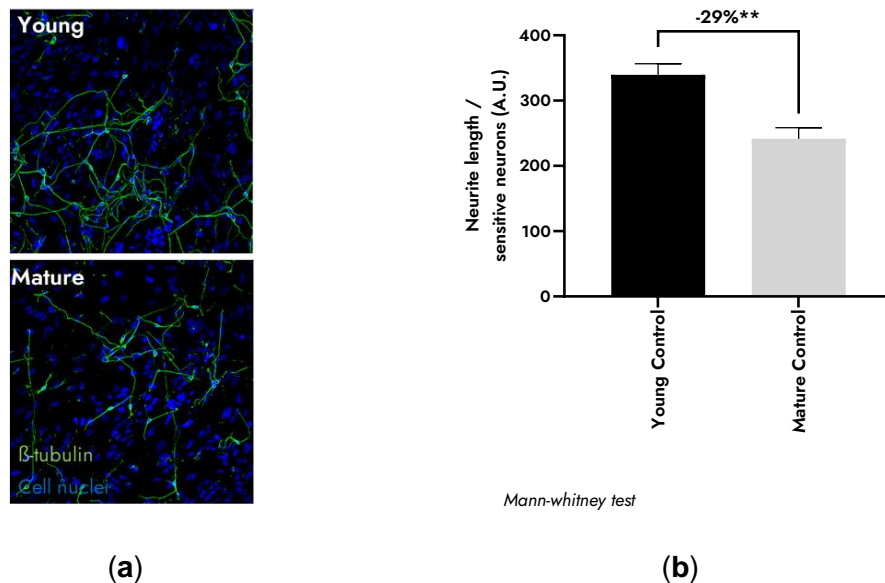
#### 2.4.4. Data analysis

We analyzed age and grade differences between groups with high and low sensitivity using ANOVA, followed by Tukey's post-hoc test for detailed comparisons to identify key differences. This approach helped us pinpoint where the key differences lay. All the data analysis was performed using the statistical package R 4.2.1 (<https://www.r-project.org/>).

### 3. Results

#### 3.1. Neurite outgrowth

The main goal of this study was to evaluate the impact of age in neurite outgrowth. Keratinocytes from two donors (aged of 23 and 58 years-old) were co-cultured with sensory neurons derived from iPS. Neurite length was evaluated using immunostaining against beta-tubulin.



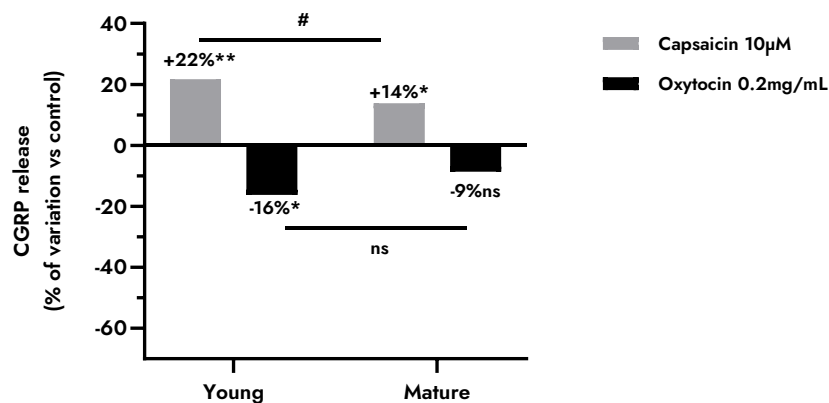
**Figure 1.** (a) Neurite immunostaining (beta-tubulin in green, nuclei in blue) in a co-culture of sensory neurons and keratinocytes. (b) quantification of neurite length. Statistical analysis with Mann-Whitney test with \*\*  $p < 0.01$ .

In Figure 1, the results allowed us to evidence that age has an impact on neurite outgrowth since we demonstrated a significant difference between the “young condition” and “mature condition” of 29%\*\*.

#### 3.2. Sensitivity (CGRP release)

A second point of investigation was to determine whether an aged condition modified the environment sensing of the sensory neurons.

For that, a co-culture of sensory neurons derived from iPS and keratinocytes from two donors (aged of 23 and 58 years-old) was used. CGRP release was monitored after incubation with relaxant stimulus (oxytocin) and noxious stimulus (capsaicin). The release of CGRP evidenced the reactivity/sensitivity of the sensory neurons toward its environment.



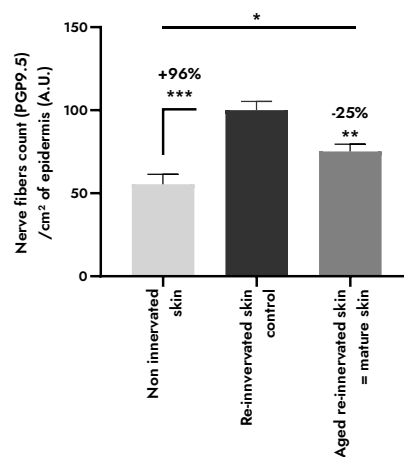
**Figure 2.** CGRP quantification with ELISA with or without capsaicin and oxytocin stimuli. Results are expressed in % of variation vs control condition. Statistical analysis with Mann-Whitney test with #  $p < 0.1$ , \*  $p < 0.5$  and \*\*  $p < 0.01$ .

In these experimental conditions, we can observe that capsaicin stress induced a significant release of CGRP in the two conditions but in higher manner than the mature culture. The presence of oxytocin was able to significantly reduced the stress in the young culture but not in the mature culture.

These results allowed us to evidence that modulation is less important when co-culture is in “aged/mature” environment. Indeed, the total amplitude of variation is different ( $\Delta 38$  vs  $23$ ).

### 3.3. Design of the aged-innervated model

In order to have a biobimetic model of a poor innervated skin, we modulated the NGF concentration of a published re-innervated skin explants model.



One-Way ANOVA followed by Tukey's test

**Figure 3.** Quantification of nerve fibers (PGP9.5 immunostaining) per cm² of epidermis in non innervated skin explants, re-innervated skin explants and the aged re-innervated skin explants. Statistical analysis with One-way ANOVA followed by Tukey's test with \*  $p < 0.5$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

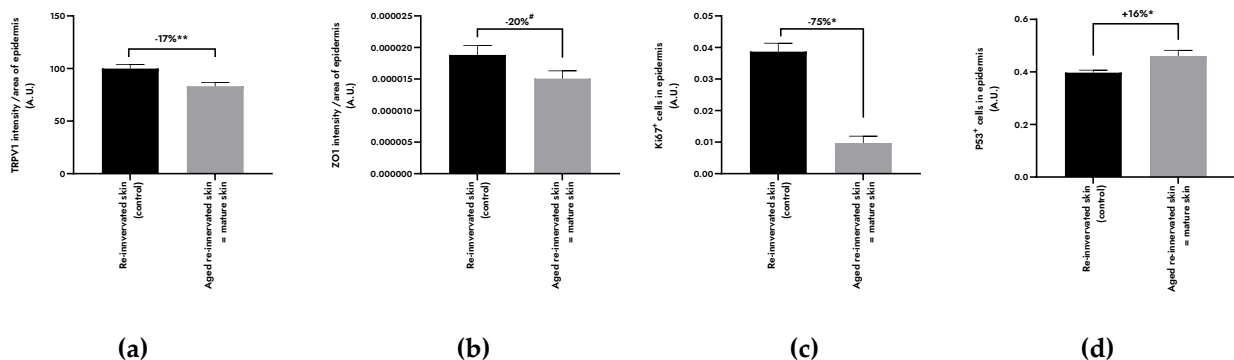
In Figure 3, we observed that the number of nerve fibers was significantly increased by +96%\*\*\* in the re-innervated skin explants in comparison with the non-innervated skin. These results showed that re-innervation was successful.

Comparatively, in the “mature condition” in which we decreased the concentration of NGF to mimic the age impact, we can observe that the number of nerve fibers was decreased by -25%\*\* but still higher than the non-innervated skin explants.

These results validate the model mimicking a poor innervation observed in mature skin.

### 3.4. Molecular characterization of the aged re-innervated model

Immunostainings were performed in the aged re-innervated model in order to characterize its molecular profile.



**Figure 4.** (a) Quantification of TRPV1 intensity in epidermis. (b) Quantification of ZO1 intensity in epidermis. (c) Quantification of Ki67 positive cells in epidermis. (d) Quantification of P53 positive cells in epidermis. Statistical analysis with Mann-Whitney with #  $p < 0.1$ , \*  $p < 0.5$  and \*\*  $p < 0.01$ .

4 targets were identified: TRPV1 for the skin sensitivity/environment sensing, ZO1 for barrier function, ki67 for cell proliferation and P53 for apoptosis.

The results of the quantification showed that TRPV1's and ZO1's intensities were decreased by -17%\*\* and -20%# respectively, evidencing a decrease of sensitivity and a defect in skin barrier function.

Number of ki67 positive cells was decreased by -75%\*, indicating that the poor re-innervation has an effect on cell proliferation. Finally, P53 positive cells was increased by +16%\*, indicating an increase of cell apoptosis.

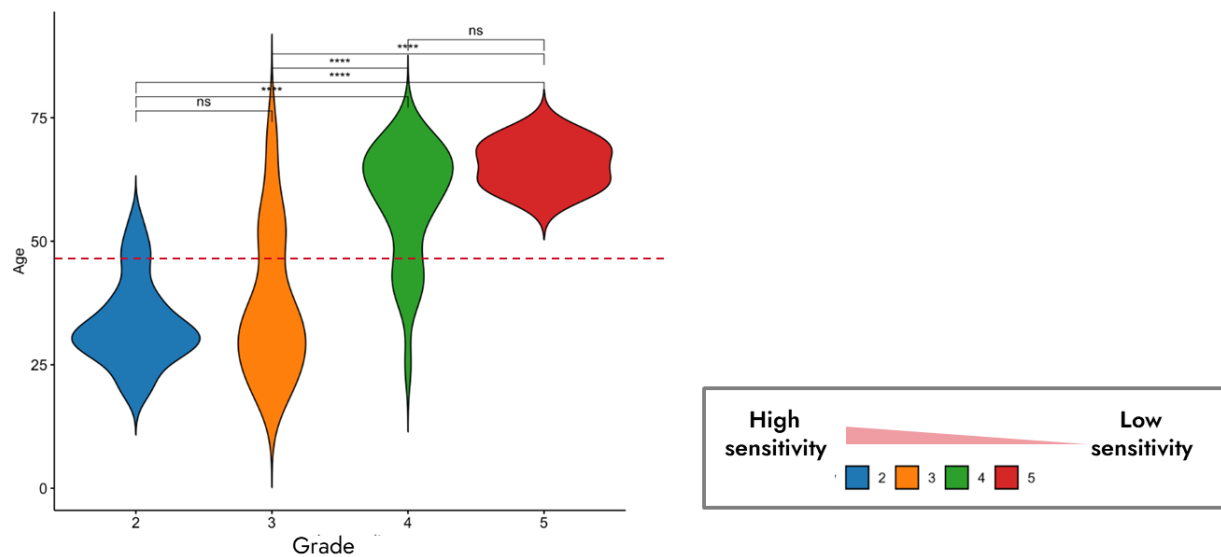
The results in these two last markers showed that cell renewal was affected by a poor innervation.

### 3.5. Touch sensitivity assessment in vivo – pilot study

More than 120 male and women volunteers aged from 18 to 74 years old were recruited.

A non-invasive method (2 point discrimination test) was used to determine the effect of age on difital pressure perception and to assess the loss of environment sensing. A grade score was defined and it has been firstly analysed by age.

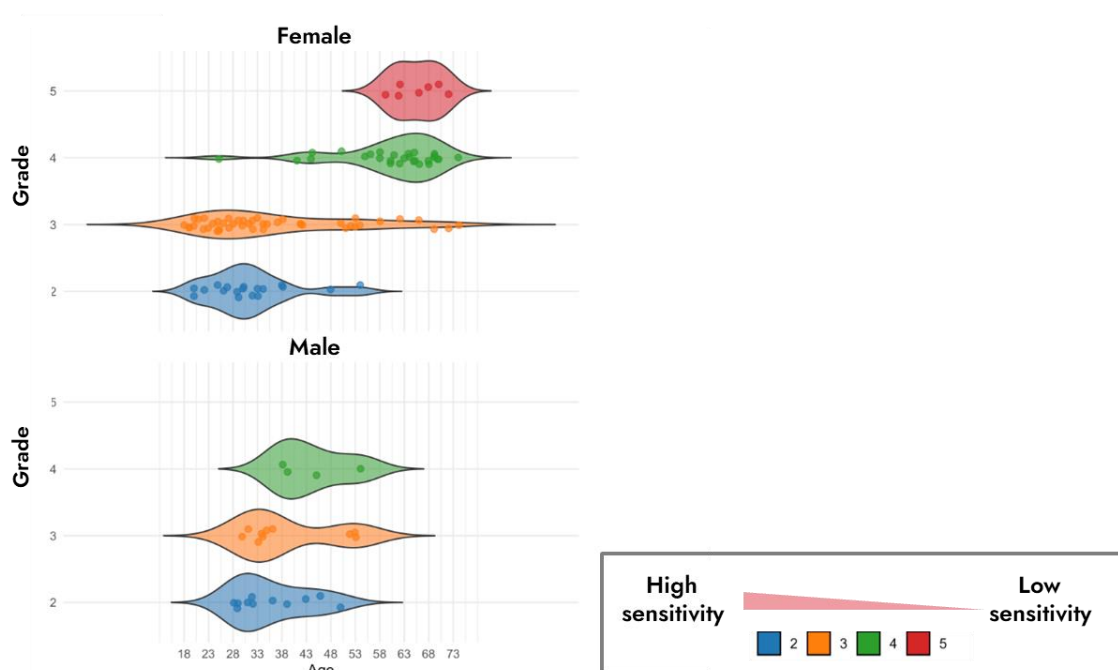
#### 3.5.1. Age comparison



**Figure 8.** Violin plot comparing sensitivity grade and age. Statistical analysis with ANOVA followed by Tukey's test with \*\*\*\*  $p < 0.001$ .

Age is a significant predictor of grade score. Older people tend to have higher grade (low sensitivity, grade 5) than younger people (high sensitivity, 2). A clear shift seems to be drawn above 50 years old.

### 3.5.1. Gender comparison



**Figure 9.** Violin plot comparing sensitivity grade and age in female and male.

**Table 1.** Logistic ordinal regression. Statistical analysis with ANOVA followed by Tukey's test with \*\*\*\*  $p < 0.001$

Mean age	Coefficient of regression	SD	p value
Age * Female	0.0908	0.0139	<0.0001
Age * Male	0.0637	0.0175	<0.0001

Age is a significant predictor of grade score. Older men and women are more likely to have higher score, but the effect of age is less pronounced for male than for female, since the coefficient of the regression is inferior in male (respectively 0.0637 and 0.0908).

A second comparison was performed by analysing the mean marginal estimate (when the median is equal) in order to confirm the impact of gender in an identical age population.

**Table 2.** Comparison of the mean marginal estimate. Statistical analysis with ANOVA followed by Tukey's test with \*\*\*\*  $p < 0.001$

Mean age	Gender	Emmean	SD	p value
43.28	Female	-0.8416	0.2161	<0.0001
43.28	Male	-2.0175	0.4918	<0.0001
Age * gender				0.0215

The mean marginal estimate (Emmean) for men (-2.0175) is lower than that for female (-0.8416). This suggests that at an average age of 43.3, male have a lower score than female. This difference is significant, as there is a statistically significant difference between men and women at the average age of 43.3 years ( $p = 0.0215$ ).

#### 4. Discussion

The results obtained with the co-culture (decrease of neurite outgrowth and loss of sensitivity) allowed to confirm the previous works and *in vivo* observations described in the literature [7,8,10]. Indeed, keratinocytes and sensory neurons have intricate relationship and communications in epidermis [2,6,12,15], leading to the confirmation of the hypothesis that aged keratinocytes may have an effect on sensory neurons that contributes to the nerve shrinking. The co-culture with aged keratinocytes also demonstrated that age impacts the environment sensing. Based on these works confirming previous works and the statement that there was no existing model allowing to assess the effect of a poor innervation [16], we decided to design a model mimicking the neuro-ageing. The model of aged re-innervated skin explants confirmed that a lower innervation reduced the TRPV1 expression, a possible explanation for this reduction of environment sensing. In parallel, the molecular characterization allowed us to confirm that, with ageing, skin homeostasis is impacted: cell proliferation (Ki67) is significantly decreased, apoptosis (P53) is significantly increased, leading to a defect barrier function (ZO1). It is interesting since some of these markers (TRPV1, Ki67 and ZO1) were significantly modulated in works presenting the beneficial effects of skin re-innervation [17–19], confirming that presence of peripheral nerve is important for skin homeostasis [20,21]. Other markers should be further investigated to explore other pathways and compartments of the skin. Nevertheless, the design of this model will help us to screen active ingredients and find a cosmetic solution for this newly-identified ageing phenotype. Finally, the *in vivo* pilot study allowed us to confirm that sensitivity is decreased in mature people [3–5,9] and with that, loss of emotional touch and pleasantness [7,8,22]. It also gave the opportunity to explore the differences between men and women, parameter that was not yet demonstrated before [5].

## 5. Conclusion

Neuroageing is poorly studied today because of experimental limitations and a lack of interest. Nevertheless, thanks to the results, it is clear today that nervous system is implicated in the ageing process by being intricately correlated to the homeostasis of the skin. The study of this process called “neuroageing” is definitively a must have for a cosmetic point of view when we approach aging process in a holistic manner. The clinic pilot study confirmed that touch perception is decreased with ageing, allowing to identify the right population (women, > 45 years old) for further investigation. The innovative design of an aged re-innervated model of skin explants and the identification of the population of interested will help to screen cosmetic ingredients that will tackle this new ageing phenotype and bring back emotional touch and pleasantness.

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