Linking clinical vascular dark circles reduction to laboratory *in vitro* modelization can be achieved using vascularized 3D Bioprinted skin models.

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Abstract

Background: Translational cosmetics brings laboratory to clinical testing closer together but is an uphill task. Many laboratory tests are not easily relatable to clinical situations, particularly for proof of efficacy. Angiogenesis *in vitro* testing has long been a difficult area for skin biology with few models available. Here, we aimed not only to create a realistic skin angiogenesis model in a full skin thickness setting, but also to create a validated efficacy model to accelerate towards clinical testing. Indeed, development of active ingredients targeting dark circles is a major concern in the cosmetics industry still needing relevant efficacy demonstration models.

Methods: With complex 3D bioprinting we developed a multi-layered vascularized skin model which was made in tandem with a clinical study evaluating identical cosmetics ingredients investigating non-invasive hyperspectral imaging of the under-eye blood vessels (SpectraCam device). Keratinocytes, fibroblasts and Human Dermal Microvascular Endothelial Cells (HDMEC), were expanded from juvenile skin donations, mixed with a bioink into which adhesion proteins were added and cartridged into a pneumatic 3D bioprinting system.

Results: Vascular beds formed inside stimulated models including typical roll formation expected in the capillary system. Vascular inhibition could be achieved sequentially over 14 days, in agreement with the clinical study which showed reduction of the vascular network in dark circle areas with the active ingredient between 7 and 28 days.

Conclusion: This demonstrated that it is now possible to link *in vitro* and *in vivo* efficacy testing, to create more effective cosmetics of the future related to skin vascularization process.

Keywords: (4-6 keywords separated by a semicolon).

Angiogenesis; Clinical; skin model; Efficacy; Dark circles; bioprinting

1. Introduction.

Translational cosmetics testing is the concept of making laboratory *in vitro* testing relate better to *in vivo* testing and user efficacy. Often, however, it is just a theory, with many laboratory tests hardly transferable, nor reliable in delivering true efficacy data. This is particularly a problem with cosmetics ingredients for the future as consumers demand ever more proven solutions. We have aimed to create reliable models for cosmetics testing across many domains, but such innovation is not easy [1]. Skin angiogenesis is one area of much difficulty for development of new products since the creation of any angiogenesis models are known to be extremely difficult and complex [2]. Over and under vascularization of the underlying skin bed can have debilitating effects on sufferers. Such conditions, including rosacea and periorbital odema and dark circles, while not life-threatening can lead to personal embarrassment, mental health issues and depression. Solutions to gently treat these conditions without changing the normal pathobiology of the skin are not easy and hampered by the existence of good *in vitro* models, particularly those which can prove layer penetration from the epidermis to the vascular bed of the skin [3]. Indeed, top-down stimulation of the system without penetration is also important, utilizing the cell-to-cell communication ability of the multilayer skin system. Existing lab models include co-cultures of endothelial cells, keratinocytes and fibroblasts, but this does not well represent the structure and barrier performance of the skin and has major drawbacks in penetration kinetics.

For this reason, we have developed a 3D bioprinted layered full thickness skin model with a vascularized bed within the dermis, as a screening and efficacy tool. At the same time, we have mirrored this promising *in vitro* system with *in vivo* clinical testing to evaluate cross-over efficacy on the angiogenic contribution known in the etiology of vascular dark circles in order to speed up lab to product development.

2. Materials and Methods.

2.1. Cell populations

Epidermal keratinocytes, dermal fibroblasts and Human Dermal Microvascular Endothelial Cells (HDMEC) were expanded from juvenile skin donations <30 mo following ethical

consent under French regulations and authorizations. Cells at early passage 2 or 3 were selected for optimal growth (**Figure 1**.).

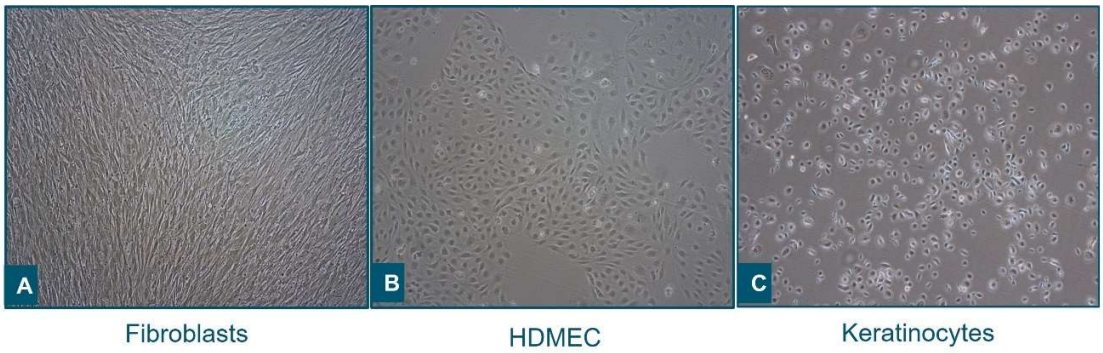

Figure 1*., Growth characteristics of fibroblasts, HDMECs (Human Dermal Microvascular Endothelial Cells) and Keratinocytes before proceeding to printing.*

A: Fibroblasts with extended morphology. B; HDMEC standard physiology during expansion culture before extensive differentiation. C: standard keratinocyte expansion culture. Lense magnification X10.

2.2. 3D bioprinting of cellular models

Cells were mixed with a bioink (CELLINK, Goteborg, Sweden), into which adhesion proteins were added and cartridged into a CELLINK pneumatic 3D bioprinting system which allows for multilayer and multicellular alignment. Software design was used for modelization with Sketchup and SLIC3R slicing software (first described at IFSCC2020, Henry Maso award 2022) and the resultant g-code was transferred to the printing system to control the printer in spatial format [4]. The printer code was designed for full thickness models with a vascularization bed created in the lower quadrant of the dermal structure, with or without a full epidermis to allow for imaging options (**Figure 2**.).

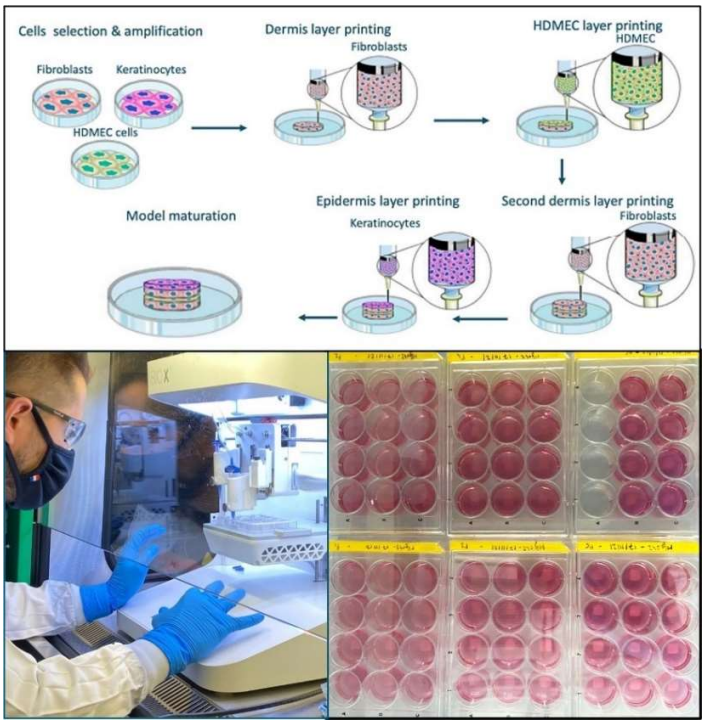

***Figure 2.**, The 3D bioprinting process for vascularized skin models.*

Clockwise: schematic representation of the printing layer procedure; reproducible repetitive models printed: the 3D bioprinting system used.

2.3. Maturation and treatment of 3D bioprinted models

Maturation of the models allowed for development (or not) of the vascular bed and a mature epidermis. To allow for variations in the models, stimulation or inhibition of the vascularization was made by adding anti-angiogenic Endostatin (3 µg/mL) and Pro-angiogenic VEGF (20 ng/mL) and / or an active cosmetic ingredient created with a new form of responsible plant culture technology using 'plant-milking' under aeroponic conditions (Clariant, Toulouse, France) [5]. Additives were added to mature models either topically, or in the media of the support culture as appropriate. Evaluation of the development of the models with the stimulants or inhibitors was continued for up to 2 weeks at which point the models were processed for confocal high-resolution microscopy with anti-CD31 (NB100-2284, Novus), TE-7 (NBP2-50082, Novus) fluorescent labelling. Z-scanning slice analysis and static imaging was recorded. Reconstruction of the images was made on FJI - ImageJ open-source software.

2.4. Clinical *in vivo* study of active ingredients

Concurrently a clinical study was carried out with the same cosmetics ingredient through a double-blind randomized study which enrolled 18 women, aged between 18 to 35 years old who were presenting persistent dark circles. Participants were asked, following consent, to apply a test formulation active at 1% or a placebo treatment, twice daily for 28 days. The clinical efficacy was evaluated by a hyperspectral imaging system using a SpectraCam system (Newtone, QIMA Life Sciences, Lyon, France), which investigated the spectral reflectance of the skin and producing a qualitative map of the underlying blood vessels structures for evaluation [6]. Comparison within the groups and during time periods was made from the testing procedures.

3. Results.

3.1. Printing parameters

Reproducible replicates of the models were successfully achieved following 3D bioprinting of the three cell types used in the study: keratinocytes for epidermis, fibroblasts for dermis and HDMECs for creation of the dermal vascular bed. Generally, on this pneumatic system many replicates could be made per hour and was only limited by cell availability (**Figures 1. and 2**.).

Development of a multilayered skin model required maturation and in the case of the model with an epidermis, also required an airlift sequence to allow for epidermal differentiation and maturation. Confocal imaging of models without a cornified epidermis was considerably more straightforward than those with the epidermis due to the opaque nature of the upper layer and the scanning depth required. However, this was achieved revealing analysis of the multilayered structures (**Figure 3.**).

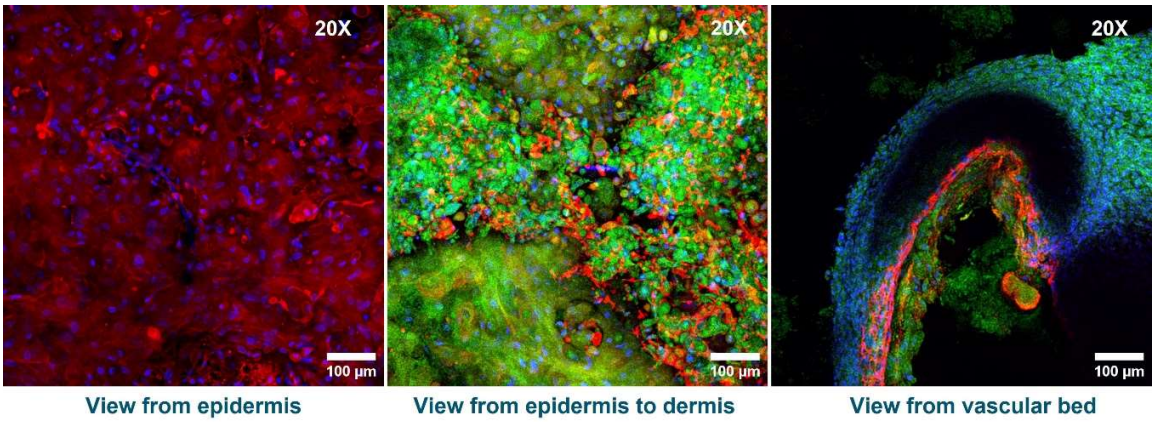

***Figure 3.**, Laser confocal scanning from the top to bottom view in the full thickness vascularized skin model.*

Left to right: view from the epidermis: view from the epidermal to dermal quadrant leading down into the model; view in the vascular bed of a 'roll' structure forming where vascular cells are lining up surrounded by the dermal fibroblasts.

[Red: labelling of epidermis. Green Middle photo: labelling of junction and dermal level. Green vascular bed: labelling of vascular tissues with CD31. Blue in all photos = DAPI nuclear staining.

3.2. Stimulation of vascularization in the skin models

In models where no stimulation was added vascular cells did not grow well, but conversely did not specifically die off during the time point of the experiments (data not shown). However, when VEGF stimulation was made, the vascular cells were stimulated not only to multiply but also to form interconnections and even started the procedures of capillary formation (**Figure 4.**).

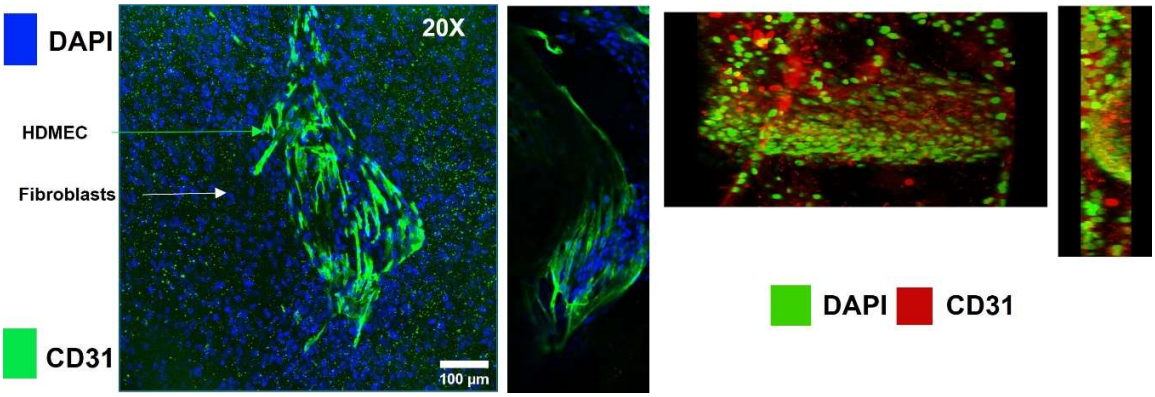

***Figure 4.**, Vascular bed formation was seen upon stimulation with pro-angiogenic VEGF.* Left two panels: following some days stimulation hitherto separated more rounded HDMEC cells expanded in numbers and connections between each other, causing longer extensions to connect. Right 2 panels: longer incubation allowed the 'roll' structures of capillary formation to appear and were imaged deep in the dermal areas.

3.3. Screening parameters and active ingredient testing

Testing over several days of the pro- and anti- angiogenic factors was joined with screening of the plant extract product (referred to as total extract) and the major phytochemical compound (referred to as purified extract), at different concentrations. Competition challenge between the products and the pro-angiogenic stimulation made it possible to screen for the plant-derived product efficacy.

Reduction or inhibition of pro-angiogenic factors resulted in the sequential reduction of the vascular bed over 14 days, whilst continual supplementation maintains the model and cellular interconnections, whilst the clinical study similarly demonstrated after 28 days of use the reduction of vascular network in dark circle area thanks to the active ingredient.

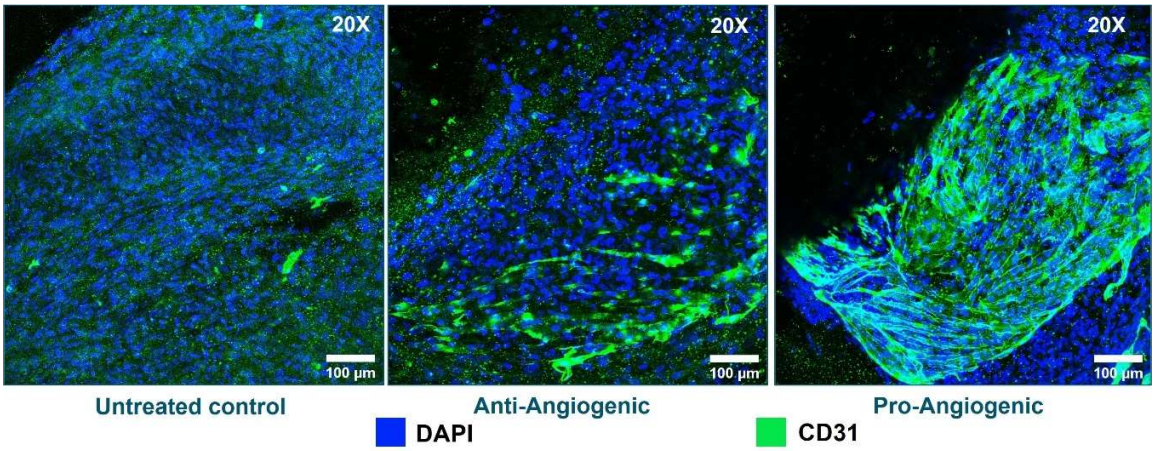

***Figure 5.**, Control conditions for screening testing.*

From left to right: untreated control with diffuse HDMEC growth; endostatin inhibitory effect brought by accelerated endothelial growth; Pro-angiogenic stimulation from VEGF allows for promotion of vasculature development in the dermal section of the skin.

Without specific stimulus, the HDMECs only slowly multiplied. In the normal situation in the human body, angiogenesis is carefully controlled. If it were not, then the capillaries inside organs, including skin, would overrun the system. Even turnover of the endothelial system is an active and ongoing process. In the untreated control without cytokine involvement, the cells do not much develop (**Figure 5**.). In this situation, endothelial cells do not produce extensions between each other and communicate to form capillaries.

However, endostatin has an effect on receptors and signal transduction pathways within the cells, causing a move towards terminal differentiation. Whilst in this situation there may be less cell division, cellular processes are not inhibited completely, and the cells will attempt to move to the terminal end of differentiation and later die off. Without positive stimulation endothelial cells normally die. On the other hand, when the cells receive VEGF cytokine support, the cells not only multiply but form structures within the skin and start to communicate in a more controlled manner leading to the early formation of capillary structures (**Figure 5**.)

Since the reason for this study was to create a 3D bioprinted vascularized model for screening of actives and ingredients, we used the test products created from plant aeroponic technology.

In addition, as this technology allows for the enrichment of specific phytochemical compounds, we tested the printed models with both the total extract and the 'purified ingredient'.

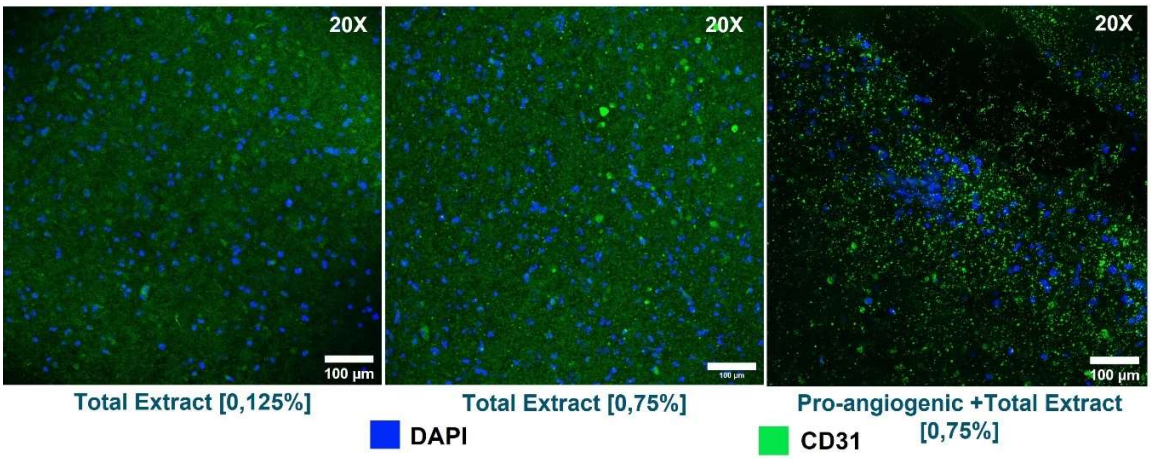

***Figure 6.**, Screening of aeroponic-derived total extract product on 3D bioprinted vascularised models.*

From left to right: Extract at 0.125% and 0.75% percent slowed down cellular production in the vascular layer without affecting their viability. However, when pro-angiogenic VEGF was put in competition with the Total Extract at 0.75%, the development of vascular structures was not seen.

Validation of the 3D bioprinted model was first proven with Total Plant Extract at two different concentrations. A concentration effect could be seen between 0.125% and 0.75%, but also against the control with no stimulation (**Figure 6.**).

Further to this, stimulation of the skin models, by VEGF which had created vasculature and capillary formation (**Figure 5**.), was inhibited by addition of the Total Extract at 0.75% (**Figure 6.**, end panel), demonstrating an active effect. Inhibition of development and cellular multiplication could be seen.

Since plant Total Extract had an effect on the cells, it was hypothesized that a purified ingredient would also have an effect. This testing is demonstrated below in **Figure 7**.

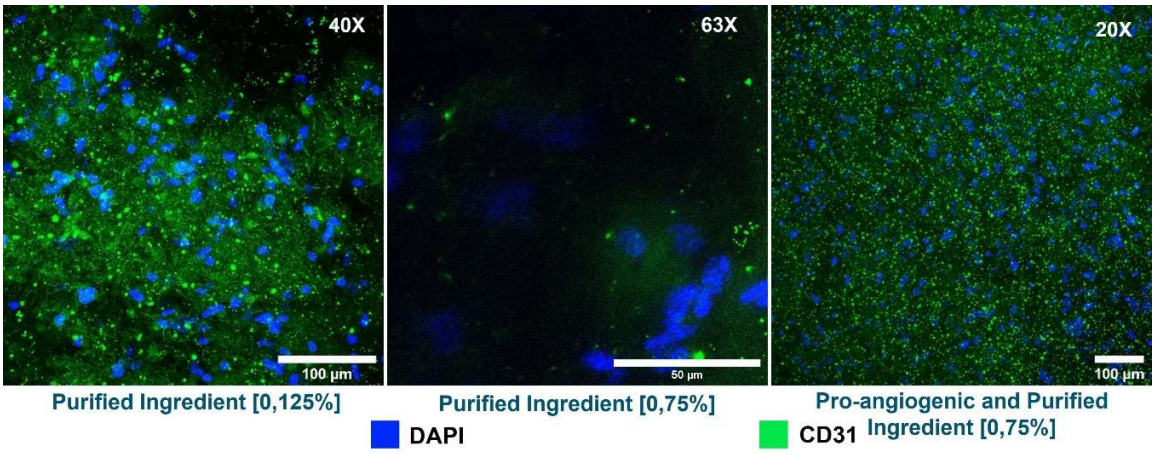

***Figure 7.**, Screening of a plant purified ingredient on vascularization of a 3D bioprinted skin model.*

From left to right: first two panels showed a strong inhibitory effect of the purified ingredient with a concentration dose effect. Final right panel shows that addition of pro-angiogenic VEGF was not able to overcome the effect of the ingredient.

Inhibition of the cell multiplication without any cytotoxicity is seen with addition of the purified ingredient with a strong dose-response effect and even stronger than the Total Extract seen in **Figure 6**. The effect at the higher percentage of purified ingredient was significant with a very low cell production and maintenance within the cultured model (**Figure 7**.).

Challenge of the model with purified ingredient at 0.75% was not fully reversed by the addition of pro-angiogenic VEGF and indeed was more like the untreated control.

3.4. Clinical screening testing

Concomitant to this study on 3D bioprinted skin models, a clinical study was carried out evaluating the effect of the active ingredient on women. By Day 28 it was evaluated using advanced skin-scanning technologies that a reduction of the vascular area network could be achieved under the eye in the dark circles area, demonstrating an important co-relation to the laboratory study. In contrast, in the placebo control group, the vascular network was not improved (data to be presented at podium, IFSCC 2022, London).

4. Discussion.

Plant based active ingredients are increasingly important particularly if they are both responsibly sourced and the climate impact of harvesting the ingredients is a net-positive in terms of oxygen production and carbon dioxide reduction. Aeroponic technologies are an important development towards such production of active ingredients [7]. Here in this study, we have evaluated these next-generation ingredients in an advanced 3D bioprinted skin model created specifically for this purpose. Vascularized models have historically been very basic. Often normal co-culture systems fail to realistically give an idea of the interaction between a product and a vascular bed [8]. For this reason, we have advanced our existing basic 3D bioprinted models towards more developed and functional models. In this case we employed human dermal endothelial cells to create a vascular bed. However, such experiments must take into account the normal growth characteristics in the human body to be useful. In order to do this, we rigorously employed the use of controls with and without stimulation or inhibition of the vascular bed within the skin model. Ultimately the aim was to create a useful screening *in vitro* model which could better predict – and justify – moving to the clinical testing stage, which is hugely expensive as part of the product development chain.

VEGF is considered to be an important part of the endothelial control process while endostatin has long been investigated for the ability to be anti-angiogenic [9]. However, the two molecules are not opposite and equal in their roles. The molecules are quite specific in their signal transduction pathways and well known in medical science. VEGF acts via a cellular receptor which sets off a defined signal transduction pathway inside the cell. Such pathways can be fast or slow, depending on the state of the cell at the time. Ultimately, it stimulates endothelial cells to grow and eventually make small blood vessels. On the other hand Endostatin inhibits the angiogenesis – but in a slightly different way – it binds to both the VEGFR-1 and VEGFR-2 receptors and blocks the interactions of VEGF to Flt-1 and Flk-1 to prevent VEGF-induced tyrosine phosphorylation of VEGFR-1 and VEGFR-2 and all the later signaling downstream transductions. Further since receptor kinetics are involved,

competition is important through concentration effects. In our studies, we were able to show that endostatin becomes involved in the terminal differentiation process without much promotion of proliferation, unlike VEGF. Reaction of these two control molecules was important to define and prove the model was able to react appropriately as a screening tool, and this was achieved (**Figures 5**.).

Reproducibility was also important since it was not our attempt to create a 'research-grade' a few times, but rather a repetitive screening system. 3D bioprinting whilst not new, is still a relatively young science, particularly applied to skin [10]. The concept is to put the cellular components into a 3-dimensional area and keep them there long enough that they can mature and develop into a useful system. This is an advantage that organoids and spheroids cannot achieve. Further, basic organoid / spheroid systems are limited in overall cellularity and time of growth [11, 12]. In our system we successfully achieved a full-thickness epidermis-dermis model with a vascular bed which could be reproduced many times in an hour and matured into multiple models which allow for realistic and longitudinal screening (**Figure 2.**). Since cosmetics testing, in efficacy and importantly safety, need to become more rigorous, longitudinal studies become increasingly important. Further, only longitudinal studies can relate well to the clinical donor testing phase. In order to justify clinical testing, which is a laborious and expensive phase in cosmetics ingredients development we believe that our models contribute to the next level of laboratory testing, moving more in sync with clinical testing, for everyone's benefit.

Competition testing (test inhibitory ingredients versus known promotor molecule VEGF) was carried out here as part of the proof-screening process. Since VEGF is a strong stimulator, the ability of any ingredient to reduce the baseline effect was evaluated. This data (**Figures 6. and 7**.), was extremely important in the testing phase. Interestingly, both the Total Extract and the Purified Ingredient had an effect on the vascularization of the models. Thus, we can assume that the activity found with the plant extract is mainly due to its major phytochemical compound (referred here as purified ingredient). Cost is also an issue in this development chain, but important because the dose of any ingredient to be added to the final cosmetic has many decision-making factors and the development of new botanicals with effective proof is a popular area of development [13, 14].

Since vascularized models have been rare and often not very reproducible, we believe that the future uses of these models will have many avenues for effective screening, not least skin penetration, and barrier testing, since regulations for penetration of cosmetics are increasing. We continue to develop our vascular bioprinted skin towards perfusive models which will allow a new form of barrier testing never before achieved. Convergences of technologies have shown in this study that it is possible to effectively innovate in efficacy testing and advance technologies for the cosmetics industries.

5. Conclusion.

Modelization in the laboratory is an expensive part of ingredients development, but an essential step for safety and efficacy. We have successfully achieved the 3D bioprinting of vascularized skin models which were not only reproducibly, but also reactive to stimulation and inhibition. Further, they were successfully demonstrated as a useful screening model for advanced botanical cosmetics development. Our models allow now a better translation between the lab and clinical testing to evaluate deep bed effects at the stratified skin levels of both epidermis and dermis, which should help to create safer more effective cosmetics of the future.

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Conflict of Interest Statement.

NONE.

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