A plant extract enriched in esterified dicaffeoylquinic acids reveals high potency to mitigate vascular dark circles

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Abstract

Background: Dark circles, a major cosmetic concern, impact our face looking. They are caused by many etiological factors including excessive pigmentation, thinner and translucent lower eyelid skin, shadowing, periorbital oedema, superficial microvasculature. Using the cutting-edge "Plant Milking technology" on *Ipomea Batatas* plant, we developed a unique root extract enriched in esterified dicaffeoylquinic acids (DCQEs) called IBEE.

Methods: Affimetrix technology was used to decipher the biological effects of IBEE and DCQEs in skin explants at the transcriptomic level. Anti-angiogenesis properties of IBEE and DCQEs were studied in a 3D bioprinted vascularized model co-developed with CTI-Biotech. Clinical study enrolling women suffering from vascular dark circles (7 and 28 days). Skin stiffness was assessed with the Easystiff® (Biomeca). Skin brightness, redness, and vascular network were investigated from pictures taken with the Spectracam® (Newtone technologies). Skin puffiness was assessed by grading.

Results

IBEE thanks to its DCQEs content modulates several genes and growth factors important to improve dark circles. IBEE down-regulates vascular-endothelial signaling pathways and inflammatory responses. In 3D bioprinted vascularized dermis model, IBEE reduces vascular

network. The pre-clinical efficacy observed with IBEE was confirmed at the clinical level. The under-eye area appears brighter, redness fades and dermis is firmer. IBEE efficacy is very fast. Dark circles are improved in only 7 days.

Conclusion

DCQEs found in IBEE are potent molecules acting on dark circles. DCQEs inhibit angiogenesis, inflammation and extracellular matrix degradation. Targeting efficiently in dark circles the formation of new microvessels can contribute to improve dark circles features: color, puffiness and sagging.

Keywords: Dark circles, eye contour, skin vascularization, skin redness, skin stiffness, brightness

Introduction

Dark circles (DCs) a major and common cosmetic concern, impact our face looking and therefore may lower our self-confidence, emotional well-being and overall quality of life. Dark circles are aesthetically unpleasing for many individuals and can occur across all age brackets. Dark circles are associated with numerous extrinsic and lifestyle-related triggers, including illness, allergies, stress, hormonal changes, dehydration, poor nutrition, excessive alcohol consumption and smoking. DCs are associated with tiredness, sleep deprivation and contribute to an appearance of fatigue. DCs play a role in perceived age, affecting emotional well-being and overall quality of life. The anatomy of the eye area is a contributory factor for DC. Indeed, this area is the thinnest skin of the face, and the bone structure and contour of the eye socket can lead to shadowing effects around the eye. A recent work published in 2019, showed that DCs are characterized by an increased dilation of blood vessel and/or an increased number of capillaries providing strong evidence of the vasculature in DCs etiology [1]. Moreover, this area is prone to blood stasis, excessive pigmentation and oedema. Moreover, as the occurrence of dark circles is a clear indicator of aging and of an unbalanced lifestyle (lack of sleep, stressful life…), consumers are looking for efficient cosmetic solutions to attenuate them. Using the cutting-edge "Plant Milking technology", we developed a unique root extract enriched in esterified dicaffeoylquinic acids (DCQEs). We propose here a cosmetic ingredient obtained from *Ipomea Batatas* roots able to act on the biological pathways leading to dark circles appearance.

Materials and Methods

Ipomoea batatas enriched extract

We used Plant Milking Technology based on aeroponic cultivation of plants to developed this active ingredient from *Ipomoea batatas* plants and obtained an extract enriched esterified dicaffeoylquinic acids (DCQEs) named IBEE [2]. After cultivation, the roots are cut, air-dried and then, extracted with a solution of pure 1,3-propanediol. Finally, the extract is filtrated to both remove plant biomass and clarify the final extract. This non-destructive recovery process allows using the same root biomass for successive harvesting dates, in a recyclable way. The dry matter content of IBEE was 8000 ppm. IBEE was tested at 0.125% and 0.75% *in the vitro* experiments and 1% was tested in the clinical investigation.

DCQE quantification and identification in *Ipomoea batatas* root extract

Quantification of DCQE isomers was performed by UHPLC Shimadzu Nexera XR system (Shimadzu, Kyoto, Japan) with a PDA detector SPD-M20A (applied range: 300-350 nm, detection at 330 nm), using Thermo Hypersil GOLD aQ reversed phase column (150x4.6 mm, 3 µm, Fisher Scientific, Illkirch, France), maintained at 40°C during all analyses. The mobile phase was composed of water containing 0.1% vol. of formic acid (A) and pure acetonitrile (B), delivered at 1.75 ml/min by two binary pumps LC-20ADXR with the gradient of B phase as follows: 5-40% (0-9 min); 40-95% (9-9.1 min); hold at 95% (9.1-11.5 min); 95-5% (11.5-11.51), hold at 5% (11.51-14 min). The contents of DCQE isomers (3,5-DCQE, 4,5-DCQE and 3,4-DCQE) in the extract samples were determined using the standards of 3,5-DCQE at 100 mg/L, diluted in DMSO/water, pH 3.0. according to the equation (eq. 1).

$$\text{Content of DCQE}\left[\frac{mg}{L}\right] = \text{FD} \cdot 100 \cdot \frac{\sum\left(A_{3,5-DCQE}+A_{3,4-DCQE}+A_{4,5-DCQE}\right)}{\text{A}^{100\text{mg/L}}_{\text{STD,3,5-DCQE}}}\left[\frac{mg}{L}\right] \text{ (eq. 1)}$$

Where:
FD – dilution factor applied to the sample before analysis;
A_X *– average area of peak of compound X in diluted sample, n=3;*
$A^{100mg/L}_{STD,3,5-DCQE}$ *– average area of peak of 3,5-DCQE standard (100 mg/L), n=2;*

DCQE concentrations were expressed in mg/kg. These values were calculated by dividing the values of DCQE content, expressed in mg/L, by samples densities, expressed in kg/L.

Then, the structural characterization of DCQE isomers was performed by mass spectrometry using UHPLC Shimadzu Nexera X2 system (Shimadzu, Kyoto, Japan) with a PDA detector (applied range: 220-370 nm, detection at 330 nm) coupled to a mass spectrometer LCMS2020 (electrospray ionization in a negative ion mode, m/z 100-1000), using a Kinetex biphenyl (BP) reverse phase column (150 mm x 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA), maintained at 40°C during all analyses. The mobile phase was composed of water containing 0.1% vol. of formic acid (A) and pure acetonitrile (B), delivered at 0.5 ml/min with the gradient of B phase as follows: 5-25% (0-10 min); 25-90% (10-10.5 min); hold at 90% (10.5-12 min); 90-5% (12-12.1 min), hold at 5% (12.1-14.1 min).

NMR experiments were performed only on the major phytochemical component present in IBEE the 3,5-DCQE using Bruker Avance III 400 spectrometer equipped with a BBFO probe and operating at a 400.13 MHz frequency for 1H experiments. All analyses were performed in the Institute of Jean Barriol, Lorraine University (Vandœuvre-lès-Nancy, France). The

following parameters were used for 1H experiments: 64 scans, number of points: 65536, spectral width: 6000 Hz, repetition time: 1.5s. For these analyses, 5 mg of the sample was dissolved in 600 µL of CD_3OD solvent.

RTqPCR

IBEE biological activities were studied on human skin explants through a full transcriptomic study using Affymetrix technology [3].

Explant treatments, RNA extraction and RNA integrity analysis

Skin explants from an abdominoplasty (woman 39 years old phototype III) were topically treated with a formula cream containing either 0.75% IBEE or 0.75% DCQEs or the vehicle control (80% 1,3 propane diol + 0.5% sodium sulfate). Each condition was done in triplicate. At the end of the skin explants culture, the explants were placed in RNA later solution (RNA protect tissue reagent; Qiagen, ref 76104) and frozen at -80°C. Then, the samples were incubated in Trizol reagent (Invitrogen, ref 15596.018) and then disrupted using a tissue homogenizer (polytron). Chloroform (Merck, ref 102445-1000) was added to tissue homogenates leading to phase separation. The upper phase was taken. The RNA was then precipitated from the aqueous layer using isopropanol and washed with ethanol. After centrifugation, the RNA was resuspended in RNAse-free water for use in downstream applications. The collected RNAs were stored at -80°C until further analysis. The RNA concentration was determined by spectrophotometric measurement. The RNA quality was analyzed by capillarity electrophoresis (Agilent Bioanalyzer 2100 - Agilent RNA 6000 Nano Kit, ref 5067-1511). The integrity of the total RNA was assessed by visualization of intact ribosomal RNA bands.

Data acquisition, data analysis

The amplification of total RNA was performed in 3 steps on 50ng with the Ribo-SPIA technology (Ovation Pico WTA System V2, NuGEN, 3302-12) and amplified samples were purified using the RNA Clean up XP Beads (Agencourt – Beckam Coulter Genomics, ref A29168). For each amplified cDNA, fragmentation and biotin labeling was processed on 5µg with the Encore Biotin Module (NuGEN, ref 4200-12). Hybridization was performed on Human Clariom S arrays (Affymetrix, ref 902916 and 902917). Hybridization, washing and staining were performed according to the Affymetrix user manual. The processing of the raw data was realized with software R (v3.2.3) and the "oligo" package (v1.34.2) of the Bioconductor project (v3.2). The latest version of libraries provided by Affymetrix, constructed on version 19 of the

human genome (UCSC Human genome 19), and the RMA method 4,5 were used to guide and perform the pre-treatments, and the sequences annotations.

3D vascular dermis bio-printing model

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 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.

Maturation of the models for 10 days allowed for development (or not) of the vascular bed and a mature dermis. Along this phase, a viability evaluation was realized by Calcein AM/Ethidium Viability/cytotoxicity assay (Invitrogen Carlsbad CA, ref L3224). The functional vascularized dermis model was validated by adding anti-angiogenic Endostatin (3 µg/mL) and proangiogenic VEGF (20 ng/mL). The vascular network was monitored by HDMEC (CD31 staining) and total cells (nucleus staining with Dapi fluorophore) observation with confocal microscopy (Leica Wetzlar Germany). The non-cytotoxicity of IBEE was checked on NHDF, NHEK and HDMEC cells with Alamar blue assay and Bright field photographies.

The anti-angiogenic effect of IBEE at 0.75% and 0.125% was assessed on this model in presence of VEGF. In addition, DCQE was tested at the same concentrations. The products were applied after the dermis maturation at D0, D1, D2 and D3. At D4, the samples were collected and observed by confocal microscopy.

Clinical evaluation

Concurrently a clinical study was carried out through a double-blind randomized study which enrolled 18 women, aged between 18 to 35 years old who were presenting persistent dark circles (mainly vascular dark circles). Participants were asked, following consent, to apply a test formulation active at 1% or a placebo treatment, twice daily for 28 days on eye contour. 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.

The SpectraCam® is a high-resolution portable system allowing the acquisition of high-resolution skin images (2 MPi) in hyperspectral light (30 wavelengths between 410nm to

700nm), in 2.3 seconds with each window of acquisition every 10nm and width of 10nm. The light emitted by LEDs is transmitted through polarizers and reflected by the skin. A liquid crystal tunable filter let pass a specific wavelength and is used as analyzer. The resulting image is captured by a monochromatic camera [4, 5]. The skin redness (a*) and skin brightness (L*) were measured by analysis of images taken by the SpectraCam® device at D0, D7 and D28. As dark circles were targeted, we focused our investigation on color variation and mechanical properties of this part of the face with a SpectraCam® equipment and a new one the EasyStiff® device respectively. Comparison within the groups and during time periods was made from the testing procedures.

Mechanical properties (EasyStiff® device)

Mechanical properties of the skins were measured on dark circles area with the EasyStiff® device, developed by Biomeca (Lyon, France), based on atomic force microscopy technology (AFM). This device allows the measure of skin stiffness. Measurements were realized at D0, D7 and D28 in triplicate repetitions for each measurement.

Results

DCQE content in IBEE

HPLC analysis identified a family of dicaffeoylquinate esters (DCQEs: 3,5-DCQE, 3,4-DCQE, and 4,5-DCQE as depicted in figure 1. The 3,5-DCQE (peak n°5); 3,4-DCQE (peak n°4); and 4,5-DCQE (peak n°6) and 3-CQE (3-caffeoylquinate ester). The major compound 3,5-DCQE was estimated at 900 ppm (0.9g/kg) in IBEE extract, and its structure was confirmed by NMR analysis (figure not shown). The other compounds were estimated at 90 ppm (0.09 g/kg) respectively for 4,5-DCQE and 210 ppm (0.21 g/kg) for 3,4-DCQE. The total content in DCQEs was estimated at 1200 ppm. The results below confirm the analytic measures performed on each DCQE.

3,5-DCQE: 1H NMR (400 MHz, CD_3OD) pale-yellowish powder; MS: *m/z* 573 $[M-H]^-$; UV: λ_{max} 239, 295, 327 nm; ^1H NMR (400 MHz, CD_3OD, an annotation of atoms was performed) δ 7.62 (d, *J* = 15.9 Hz, 1H, H12 or H12'), 7.54 (d, *J* = 15.9 Hz, 1H, H12 or H12'), 7.05 (dd, *J* = 3.2, 2.0 Hz, 2H, H15 and H15'), 6.96 (dt, *J* = 8.2, 2.0 Hz, 2H, H18 and H18'), 6.78 (dd, *J* = 8.2, 3.2 Hz, 2H, H19 and H19'), 6.33 (d, *J* = 15.9 Hz, 1H, H13 or H13'), 6.22 (d, *J* = 15.9 Hz, 1H, H13 or H13'), 5.40 (dt, *J* = 7.6, 3.2 Hz, 1H, H3), 5.30 (td, *J* = 6.5, 3.9 Hz, 1H, H5), 4.31 – 4.19 (m, 1H, H8), 4.19 – 4.07 (m, 1H, H8), 3.97 (dd, *J* = 6.5, 3.2 Hz, 1H), 3.64 – 3.56 (m, 2H, H10), 2.37 – 2.27 (m, 2H, H2 or H6), 2.21 – 2.11 (m, 2H, H2 or H6), 1.84 (q, *J* = 6.3 Hz, 2H, H9).

4,5-DCQE and 3,4-DCQE: no NMR data available, MS: *m/z* 573 $[M-H]^-$; UV: λ_{max} 239, 295, 327 nm.

The major compound 3,5-DCQE was used in the experimental assay to demonstrate if the biological activity provided by IBEE is effectively due to its DCQE content.

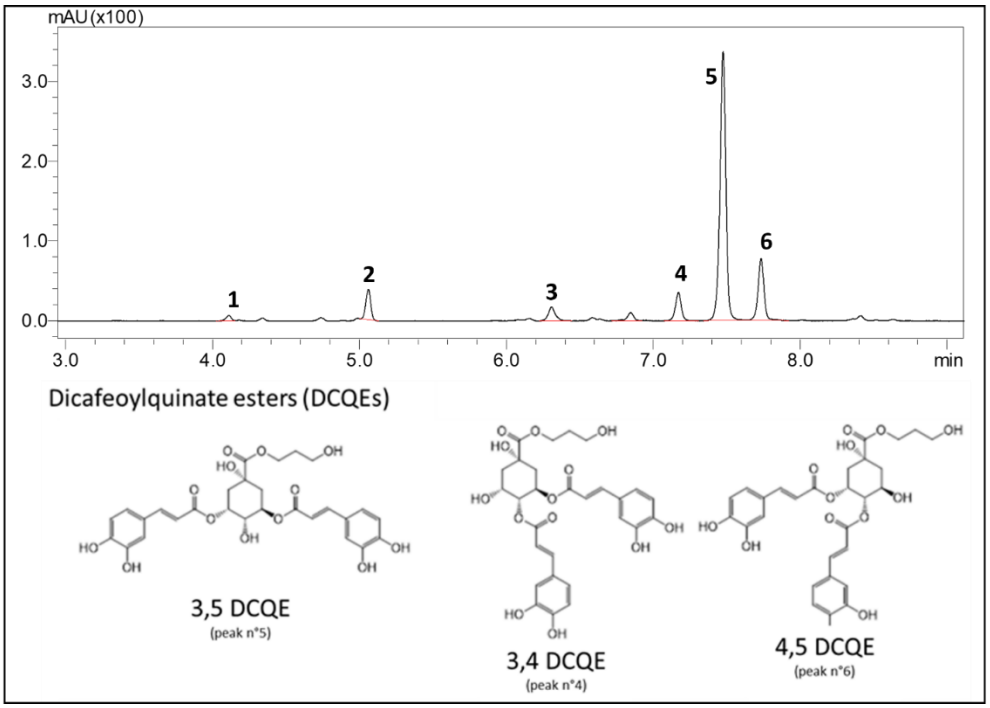

Figure 1: Phytochemical profile of IBEE – HPLC analysis

IBEE regulates genes involved in dark circles etiology

The dark circles result from the induction of several biological pathways such as inflammation, extracellular matrix decrease and vascular network alteration. Interestingly, we found in the transcriptomic study several genes belonging to these different pathways down regulated.

Regulation of inflammation process

In figure 2, a statistically significant down regulation of the transcription of several genes involved in inflammatory process was observed in IBEE condition. For example, IBEE mitigates the lipidic inflammatory mediators by inhibiting the arachidonic acid pathway [6]. ALOXE3 (Arachidonate Lipoxygenase 3), ALOX12B (Arachidonate 12 Lipoxygenase 12R type), LTC4S (Leukotriene C4 Synthase) are three enzymes implicated in lipoxins and leukotriene synthesis known to be potent lipidic inflammatory mediators [6], TBXA2R the receptor of thromboxane A2 is a key element of the signal transduction of a lipidic inflammatory mediator leading to inflammation [6]. Moreover, IBEE inhibits the transcription of NFkB2, a subunit of NFκB, a central transcriptional nuclear factor [7, 8] implicated in inflammation cell signal transduction. NFκB is the master transcriptional regulator of potent proinflammatory inflammatory factors such as IL-1 [9], TNFα [10, 11], COX2 [12], iNOS [13] or adhesion molecules such as ICAM1 (Intercellular Adhesion Molecule 1), [14]. IBEE decreases also ICAM-1 expression, a key adhesion molecule for leukocytes recruitment during inflammatory process [14, 15]. This cell recruitment also known as diapedesis phenomenon sustains inflammation. Moreover, ICAM-1 is also implicated in vascular leakage [14]. IBEE reduces also LITAF (Lipopolysaccharides Induced TNF Factor) expression. LITAF activation is induced by LPS [16], a bacterial wall component. Following cellular activation by LPS, LITAF translocated into the nucleus which leads to the upregulation of the potent TNF-α pro inflammatory transcription factor [11, 16, 16].Thus, down regulation of these numerous and pleiotropic pro inflammatory mediators will reduce local inflammation. As inflammation is a major trigger of hyperpigmentation, redness and edema at the dark circles area, reducing inflammation in this sensitive area might improve dark circles visibility.

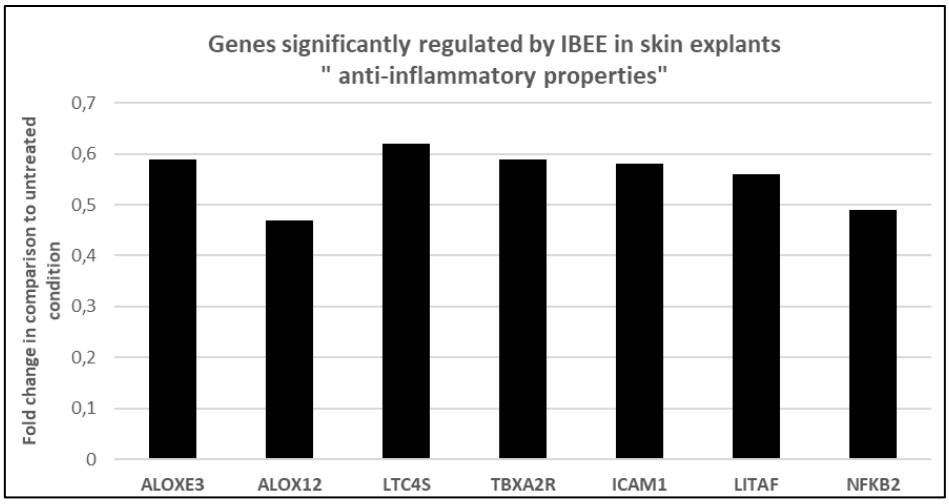

Figure 2: Genes regulated in human skin explants after 24 hours of topical treatment with Ipomea batatas root extract (IBEE). ALOXE3 (Arachidonate Lipoxygenase 3), ALOX12B (Arachidonate 12 Lipoxygenase 12R type), TBXA2R (Tromboxane A2 Receptor), LTC4S (Leukotriene C4 Synthase) genes implicated on arachidonic acid metabolism. ICAM-1 (Intercellular Adhesion Molecule 1), LITAF (Lipopolysaccharides Induced TNF Factor), NFkB2 (Nuclear Factor kappa B subunit 2).

Regulation of dermal extracellular degradation

In figure 3, a statistically significant down regulation of the transcription of several genes involved in extra-cellular matrix remodeling was observed in IBEE condition. IBEE decreases the transcription of proteolytic enzymes such as ADAM15, implicated in adhesion molecules degradation and inflammation [17], ELANE (elastase Neutrophil Expressed) a serine protease degrading elastin [18], MMP1 (Matrix MetalloPeptidase 1) a collagen endopeptidase [19–21], MMP3 (Matrix MetalloPeptidase 3) a collagenase and elastase [20–23], MMP10 (Matrix MetalloPeptidase 10) a collagenase and elastase [20, 21], MMP19 (Matrix MetalloPeptidase 19) a gelatinase [20, 21, 24]. The down regulation of these enzymes will likely prevent the dermal thinning and attenuates the visibility of underlying vascular network of dark circles.

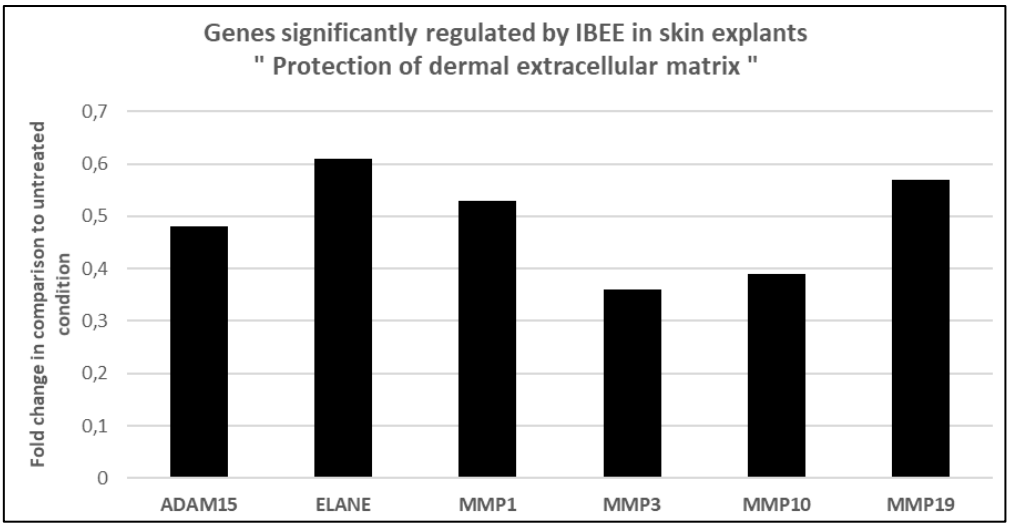

Figure 3: Genes regulated in human skin explants after 24 hours of topical treatment with *Ipomea batatas* root extract (IBEE). ADAM15 (ADAM metallopeptidase Domain 15), ELANE (elastase Neutrophil Expressed), MMP1 (Matrix MetalloPeptidase 1), MMP3 (Matrix MetalloPeptidase 3), MMP10 (Matrix MetalloPeptidase 10), MMP19 (Matrix MetalloPeptidase 19).

Regulation of vascularization process

In figure 3, a statistically significant down regulation of the transcription of several genes involved in vascularization and lymphatic processes was observed in IBEE condition. For example, NRP2 (neuropilin 2), PGF (placental Growth Factor) and VEGFB (Vascular Endothelial Growth Factor B), are three ligands of vascular receptors (VEGFR3 and VEGR2) [25–27]. SOX18 (SOX18 SRY-box Transcription Factor 18) is a key transcription factor implicated in the synthesis of proteins responsible for vascular and lymphatic remodeling [28] and TGFB1 (TGFB1 (Transforming Growth Factor β 1) one of the growth factor implicated in vascularization process [25], Moreover, we found that IBEE inhibits the transcription of ACTB (actin β), a highly conserved protein implicated in cell motility and structure of endothelial one [29]. During the vascularization process, endothelial needs to migrate to create a new vessel. We found that 3,5 DCQE inhibits also SOX18 and ACTB.

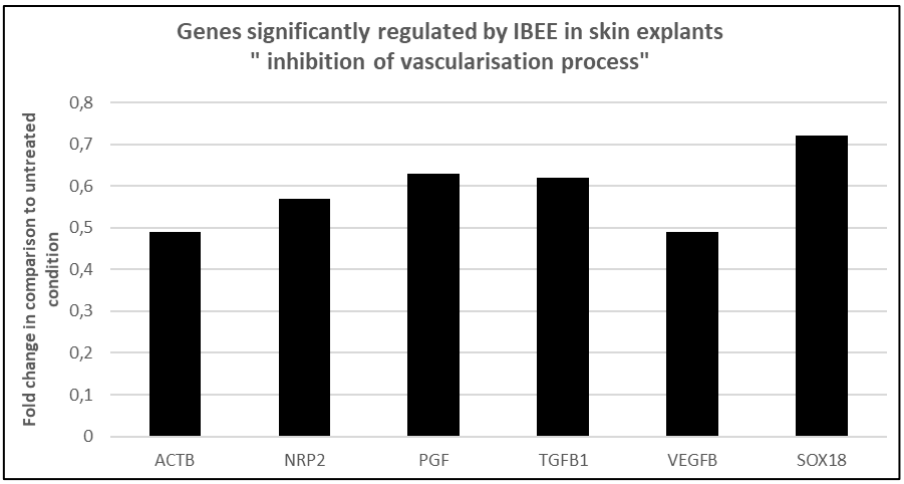

Figure 3: Genes regulated in human skin explants after 24 hours of topical treatment with Ipomea batatas root extract (IBEE). ACTB (actin β), NRP2 (neuropilin 2), PGF (placental Growth Factor), TGFB1 (Transforming Growth Factor b1), VEGFB (Vascular Endothelial Growth Factor B), SOX18 (SRY-box Transcription Factor 18).

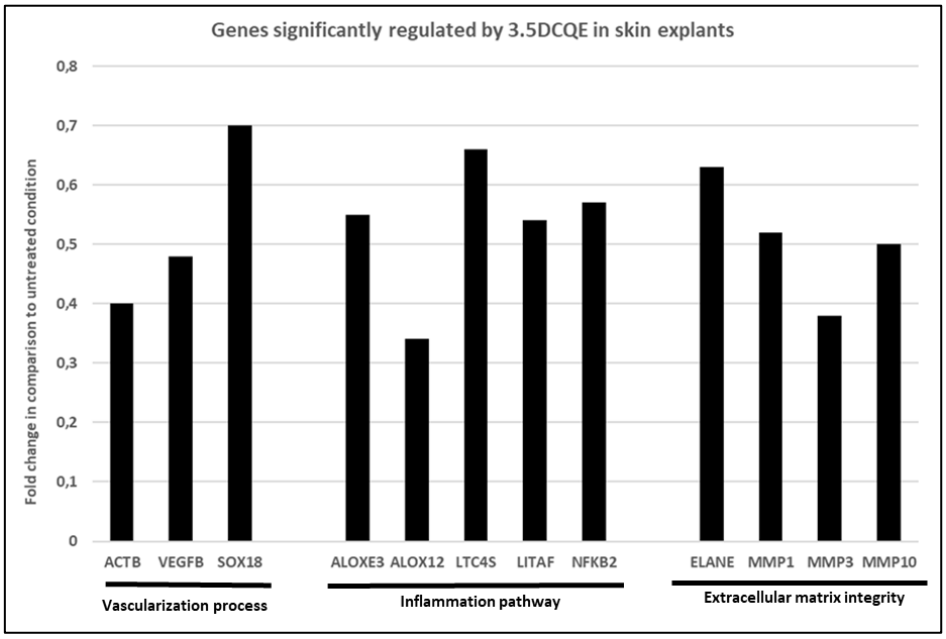

Figure 4: Genes regulated in human skin explants after 24 hours of topical treatment with 3,5-DCQE tested at the same concentration found in IBEE extract.

IBEE impact on Vascular network induced on 3D dermis model

We first checked by the alamar cytotoxicity assay, that IBEE is not cytotoxic for HDMEC and NHDF (figure 5).

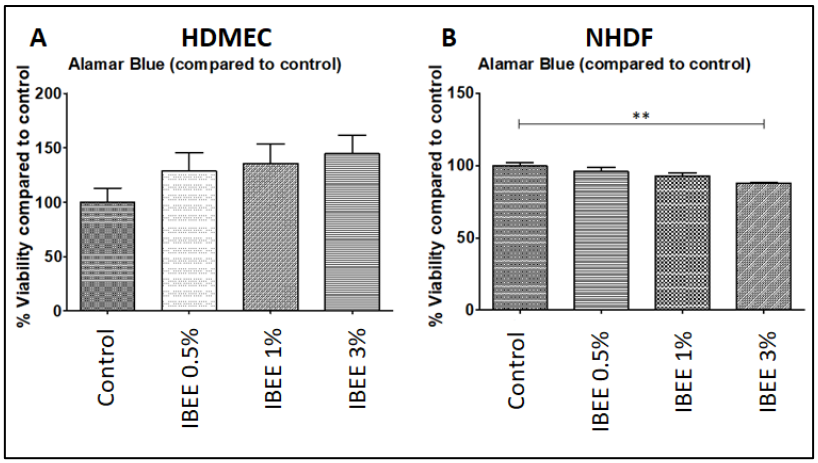

Figure 5: IBEE cytotoxicity evaluation on HDMEC (A) and NHDF (B) (Alarm assay)

The figure below shows the validation of the vascularized dermis model. The figure 5A represents the bioprinted model after 10 days of maturation just before the addition of VEGF. In figure 6A, the green staining represents HDMEC cells and the blue le nuclei of all cells (NHDF and HDMEC). This picture shows a homogenous repartition of both cell types. Without VEGFA stimulus, the HDMECs slowly proliferate, some rare and random structures are made. The figure 6B, shows the condition representing the bio-printed dermis model treated with VEGFA an pro-angiogenic factor. The figure 5B confirms the functional validation of this newly developed model. Indeed, under VEGF, the HDMECs showed an organised tubular network looking like vascularisation. The figure 6C (anti-angiogenic condition), confirms this functional validation. Indeed, under VEGF and in presence of endostatin, less vascularization is observed (figure 6C). IBEE (0.75% or 0.125%) or 3,5 DCQE (tested at the same concentration found in IBEE 0.75%) are efficient to inhibit vascularization process (figure 6D and E). The effect is dose depend as we can see a stronger inhibition in the highest IBEE concentration tested. We demonstrate also that this inhibition is probably due to its 3,5 DCQE content (figure 6F).

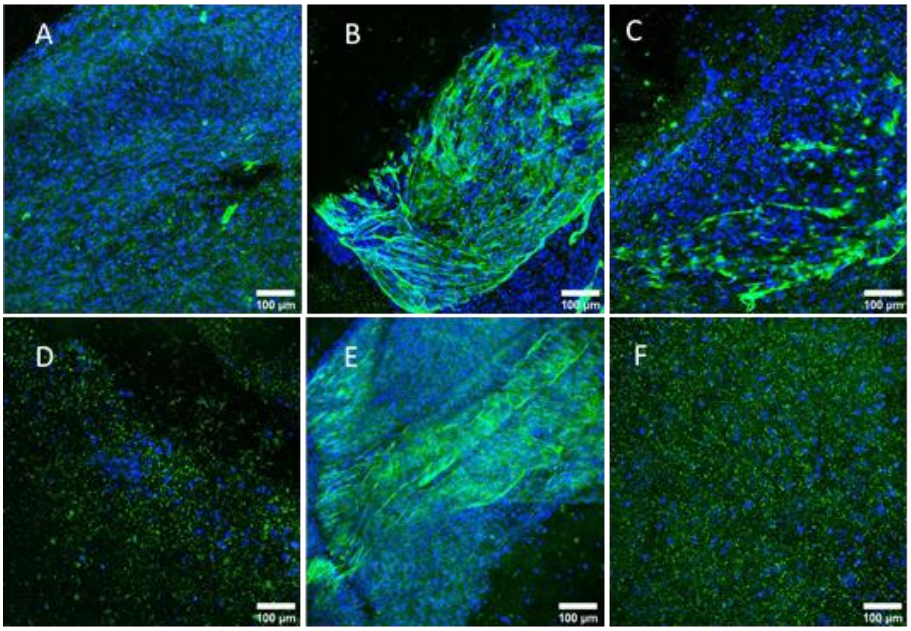

Figure 6: 3D bio-printed vascularized dermis observed by confocal microscopy before (A) and after VEGFA 20ng/ml (B), after VEGFA 20ng/ml + Endostatin 3 µg/mL (C), after VEGFA 20ng/ml + IBEE 0.75% (D), after VEGFA 20ng/ml + IBEE 0.125% (E), after VEGFA 20ng/ml + 3.5 DCQE (equivalent content as in IBEE 0.75%) (F). Cell nuclei (DAPI staining, blue) and HDMEC cells (CD31 staining, green).

IBEE improves clinical outcomes of dark circles

IBEE decreases the visibility of dark circles through the reduction of skin redness, reduction of vascular network and improvement of skin brightness

The analysis of both parameters a* and L* in the dark circles area clear show that IBEE is efficient to improve dark circles color (figures 7 and 8). Indeed, dark circles appears often dark due to a local hyperpigmentation and redness. The decrease of a* parameter known to be associated with skin redness and inflammation and the increase of L* parameter known to be correlated to a brighter skin confirm this effect. In addition, the redness observed in dark circles is also due to the development of a vascular network in this sensitive area (figure 9). After 7 days, the dark circles redness is significantly less visible after 1% IBEE treatment and this effect is maintained at D28 (Figure 7). In contrast and as expected in placebo group there is no skin colors improvement, and the vascular network tends to increase. The reduction of redness observed in IBEE group can be attributed to its inhibitory effect on vascular network observed *in vitro* and at the clinical level (figure 9). In addition, the skin brightness improvement (increase in L*) is achieved very quickly (D7) and this effect is maintained after 28 days of IBEE use.

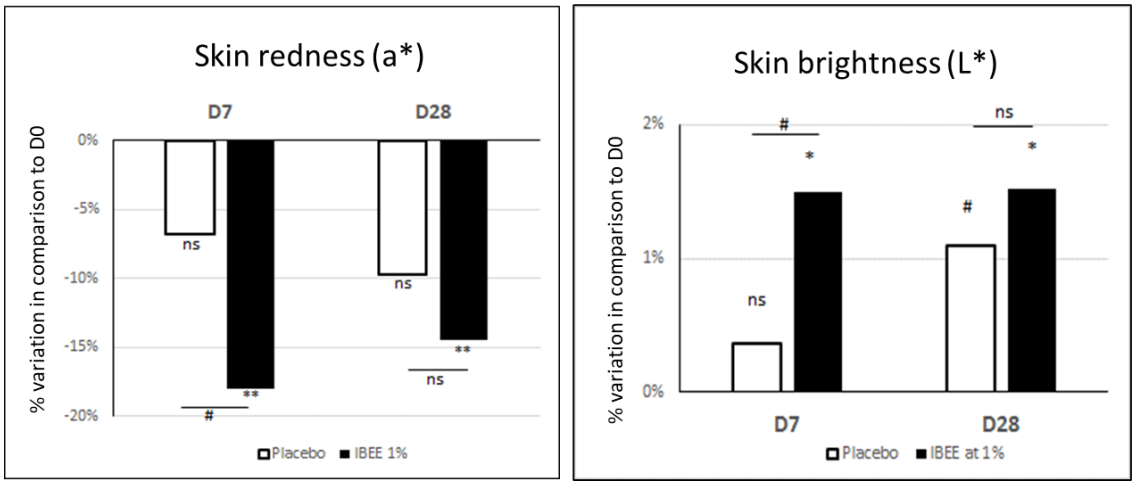

Figure 7: Skin color parameters measured at the dark circles area before and after IBEE twice daily application in comparison to placebo. Skin redness (a* parameter) and skin brightness are calculated by image analysis (Newtone technologies, Lyon). Statistical thresholds: ns= non-significant; $^{\#}p \leq 0.1$; * $p \leq 0.05$; **$p \leq 0.01$. (n=18 volunteers).

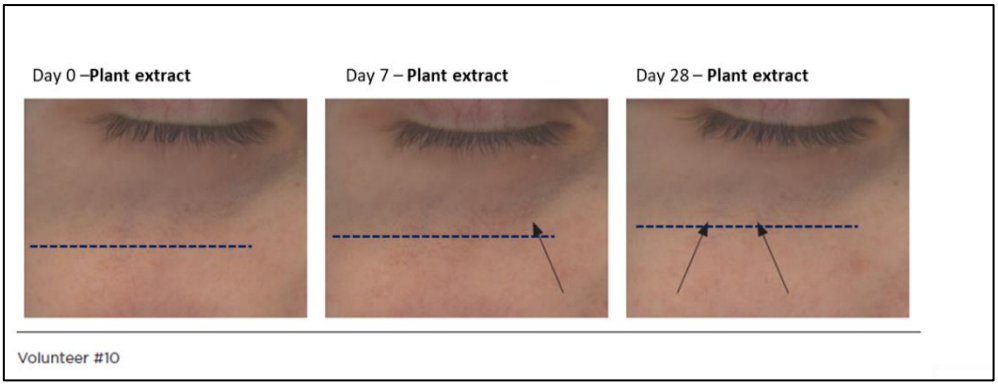

Figure 8: Illustrative pictures taken with the SpectraCam® device (Newtone technologies, Lyon) showing dark circles improvement after 7 and 28 days of IBEE use.

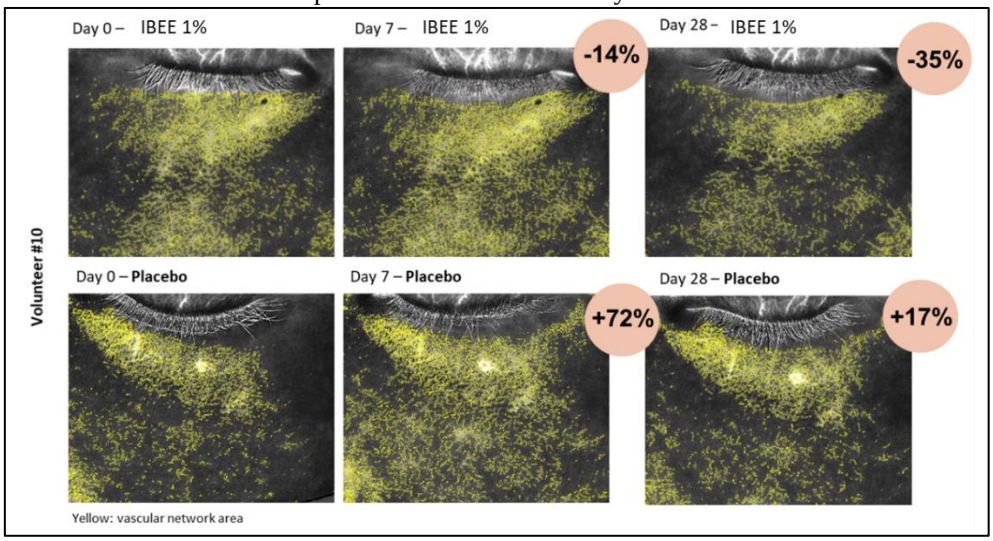

Figure 9: Illustrative pictures taken with the SpectraCam® device (Newtone technologies, Lyon) showing the vascular network reduction in plant extract treated dark circles (yellow masks highlight the vascular network).

Mechanical properties (EasyStiff® device)

The eyelid skin is known to be the thinnest skin in the face [30]. This typical characteristic makes this skin very weak mechanically (a low firmness or stiffness). Interestingly, we observed that IBEE reinforces after 7 days the skin stiffness mechanical property of this very fragile skin area (figure 10). This effect is improved with the time of IBEE use. This result might be explained by the effect of IBEE on extracellular matrix integrity. Indeed, we demonstrated *in vitro* that IBEE inhibits several enzymes mainly MMPs responsible in collagen degradation.

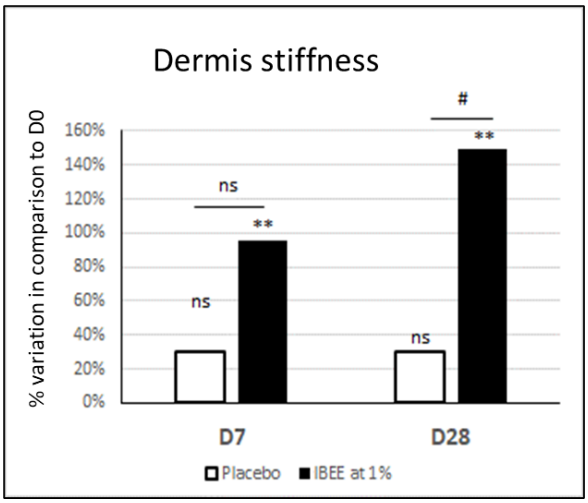

Figure 10: Skin mechanical properties measured at the dark circles area before and after IBEE twice daily application in comparison to placebo. Dermis stiffness was measured with Easystiff® device (Biomeca, Lyon). Statistical thresholds signification: ns= non-significant; # p≤ 0.1; **p≤ 0.01. (n=18 volunteers).

Discussion

Dark circles, a major cosmetic concern, impact our face looking. They are caused by many etiological factors including excessive pigmentation, thinner and translucent lower eyelid skin, shadowing, periorbital oedema, development of a microvasculature network and vasodilatation of this vascular network. Vascular endothelium is known to play a critical role in a number of physiological and pathological processes, such as hemostasis, inflammation, wound healing and angiogenesis. Angiogenesis continues the growth of the vasculature by processes of sprouting and splitting. Inflammation in this fragile eye area, contributes also to these vascular changes [1], skin hyperpigmentation, skin redness, and decreases the skin brightness. In addition to the role of hypervascularization in dark circles development. It has been described that hypervascularization can affect the skin and is an aggravating factor of some medical pathologies (ulcers and cancers) and dermo-cosmetics troubles such as rosacea [1, 30, 31].

Ipomea Batatas plant extract (IBEE) was developed by "Plant Milking technology" a cutting-edge process dedicated to the development of unique, natural and efficient active ingredient for cosmetic applications. We found that when Ipomea Batatas plants are cultivated in aeroponic condition, the roots can be enriched in DCQEs. Thanks to HPLC coupled to mass spectrometry we identify and quantify several DCQE molecules and among them the major DCQE was the 3,5-DCQE. NMR allows the exact identification of 3,5-DCQE. The *in vitro* studies demonstrate that IBEE is able to mitigate dark circles by acting on different biological pathway involved in dark circles etiology: inhibition of vascularization process, inhibition of inflammation, protection of extracellular matrix against enzymatic degradation. We demonstrate also that this activity is due to its major phytochemical compound 3,5-DCQE.

To demonstrate, clearly the efficiency of IBEE to reduce vascular network growth, the use of a suitable *in vitro* model able to stimulate or mitigate the angiogenesis process without changing the normal skin physiology was a pre-requisite for this project. To reach this goal, we developed a reliable 3D bioprinted vascularized dermis model. This model was successfully developed and allowed to reinforce the scientific knowledge found at the transcriptional level.
The vascular network inhibitory effect observed *in vitro* by IBEE was confirmed by the clinical investigation performed on 18 women having mainly vascular dark circles. The results show that skin redness (a* parameter) and skin brightness (L* parameter) were significantly improved. The use of SpectraCam a multispectral imaging device allows the visualization of vascular network along with the use of IBEE. Interestingly, we found that in IBEE group, after 7 and 28 days of product use, the vascular network is reduced while in placebo group this network tend to increase confirming the worsening of the dark circle's visibility. Moreover, volunteers skin stiffness or rigidity was evaluated for the first time by an equipment based on AFM technology showing a skin stiffness increase by 1% IBEE treatment. This result confirms the protective extracellular matrix effect of IBEE against enzymatic degradation responsible for the skin fragility. Altogether the results show that IBEE can improve dark circles aspect a major cosmetic concern.

Conclusion

An innovative and sustainable approach, plant milking, was used to obtain a family of natural compound DCQEs from *Ipomoea batatas* roots. Our *in vitro* and *in vivo* investigations highlight the strong biological activities of IBEE to improve dark circles etiology. Indeed, *in vitro* studies such as transcriptomic and 3D bioprinted vascular dermis model showed that IBEE and its

major phytochemical compound 3,5-DCQE can effectively improve dark circles. Clinical study confirmed the promising *in vitro* results and clearly state that IBEE at 1% reduces dark circles visibility by reducing skin redness and increasing skin brightness. In addition, this fragile skin is reinforced as confirmed by the improvement of eyelid stiffness. The efficiency was achieved very quicky, just after 7 days and the results were maintained after 28 days of use.

References

1. Mac-Mary S., Solinis I. Z., Predine O., Sainthillier J.-M., Sladen C., Bell M., O'Mahony M. (2019) Identification Of Three Key Factors Contributing To The Aetiology Of Dark Circles By Clinical And Instrumental Assessments Of The Infraorbital Region. Clin Cosmet Investig Dermatol 12:919–929
2. Chajra H, Salwinski A, Guillaumin A et al. (2020) Plant Milking Technology-An Innovative and Sustainable Process to Produce Highly Active Extracts from Plant Roots. Molecules 25. https://doi.org/10.3390/molecules25184162
3. Lowe R. Shirley N., Bleackley M., Dolan S., Shafee T. Transcriptomics technologies. PLoS Comput Biol 13(5):e1005457
4. Seroul P, Hébert M, Cherel M et al. (2016) Model-based Skin Pigment Cartography by High-Resolution Hyperspectral Imaging. jist 60:60404-1-60404-7. https://doi.org/10.2352/J.ImagingSci.Technol.2016.60.6.060404
5. Nkengne A, Robic J, Seroul P et al. (2018) SpectraCam® : A new polarized hyperspectral imaging system for repeatable and reproducible in vivo skin quantification of melanin, total hemoglobin, and oxygen saturation. Skin Res Technol 24(1):99–107
6. Wang B., Wu L., Chen J., Dong L., Chen C., Wen Z., Hu J., Fleming I., Wang D. W. (2021) Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. Signal Transduct Target Ther 6:94. https://doi.org/10.1038/s41392-020-00443-w
7. Lawrence T (2009) The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 1:a001651. https://doi.org/10.1101/cshperspect.a001651
8. Liu T, Zhang L, Joo D et al. NF-κB signaling in inflammation. Signal Transduct Target Ther 2
9. Di Paolo NC, Shayakhmetov DM (2016) Interleukin 1α and the inflammatory process. Nat Immunol 17(8):906–913
10. Alam MS, Otsuka S, Wong N et al. (2021) TNF plays a crucial role in inflammation by signaling via T cell TNFR2. Proc Natl Acad Sci U S A 118. https://doi.org/10.1073/pnas.2109972118
11. Zelová H. HJ (2013) TNF-α signalling and inflammation: interactions between old acquaintances. Inflamm Res 62(7):641–651
12. Yao C. NS Prostaglandin-cytokine crosstalk in chronic inflammation. British journal of pharmacology 176(3):337–354
13. Cinelli MA, Do HT, Miley GP et al. Inducible nitric oxide synthase: Regulation, structure, and inhibition. Med Res Rev 40(1):158–189
14. Wee H., Oh H.-M. Jo J.-H., Jun C.-D. (2009) ICAM-1/LFA-1 interaction contributes to the induction of endothelial cell-cell separation: implication for enhanced leukocyte diapedesis. Exp Mol Med 41:341–348. https://doi.org/10.3858/emm.2009.41.5.038
15. Bui T. M., Wiesolek H. L., Sumagin R. (2020) ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. J Leukoc Biol 108:787–799. https://doi.org/10.1002/JLB.2MR0220-549R

16. Bushell K. N., Leeman S. E., Gillespie E., Gower A. C., Reed K. L., Stucchi A. F., Becker J. M., Amar S. (2011) LITAF mediation of increased TNF-α secretion from inflamed colonic lamina propria macrophages. PLoS ONE 6:e25849. https://doi.org/10.1371/journal.pone.0025849
17. Charrier-Hisamuddin L., Laboisse C. L., Merlin D. (2008) ADAM-15: a metalloprotease that mediates inflammation. FASEB j. 22(3):641–653
18. Korkmaz B., Horwitz M. S., Jenne D. E., Gauthier F. (2010) Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev 62:726–759. https://doi.org/10.1124/pr.110.002733
19. Pardo A. SM (2005) MMP-1: the elder of the family. Int J Biochem Cell Biol 37:283–288. https://doi.org/10.1016/j.biocel.2004.06.017
20. Zitka O., Kukacka J., KrizkovaS., HuskaD., Adam V., Masarik M., Prusa R. Kizek R. (2010) Matrix Metalloproteinases
21. Nagase H., Visse R., Murphy G. (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69(3):562–573
22. Manka SW, Bihan D, Farndale RW (2019) Structural studies of the MMP-3 interaction with triple-helical collagen introduce new roles for the enzyme in tissue remodelling. Sci Rep 9:18785. https://doi.org/10.1038/s41598-019-55266-9
23. Okada Y, Nagase H, Harris ED (1986) A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization. Journal of biological chemistry 261:14245–14255. https://doi.org/10.1016/S0021-9258(18)67011-5
24. Stracke J. O., Fosang A. J., Last K., Mercuri F. A., Pendas A. M., Llano E., Perris R., Di Cesare P. E. , Murphy G., Knauper V. Matrix metalloproteinases 19 and 20 cleave aggrecan and cartilage oligomeric matrix protein (COMP) 2000
25. Kofler N, Simons M (2016) The expanding role of neuropilin: regulation of transforming growth factor-β and platelet-derived growth factor signaling in the vasculature. Curr Opin Hematol 23:260–267. https://doi.org/10.1097/MOH.0000000000000233
26. Favier B, Alam A, Barron P et al. (2006) Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. Blood 108:1243–1250. https://doi.org/10.1182/blood-2005-11-4447
27. Alghamdi A. A. A., Benwell C. J., Atkinson S. J., Lambert J., Johnson R. T., Robinson S. D. (2020) NRP2 as an Emerging Angiogenic Player; Promoting Endothelial Cell Adhesion and Migration by Regulating Recycling of α5 Integrin. Front Cell Dev Biol 8:395. https://doi.org/10.3389/fcell.2020.00395
28. Olbromski M, Podhorska-Okołów M, Dzięgiel P (2018) Role of the SOX18 protein in neoplastic processes. Oncol Lett 16:1383–1389. https://doi.org/10.3892/ol.2018.8819
29. Dugina VB, Shagieva GS, Shakhov AS et al. The Cytoplasmic Actins in the Regulation of Endothelial Cell Function. Int J Mol Sci 22(15)
30. Ohshima H. & Takiwaki H. (2008) Evaluation of dark circles of the lower eyelid: comparison between reflectance meters and image processing and involvement of dermal thickness in appearance. Skin Res Technol 14:2150034. https://doi.org/10.1142/S0217751X21500342
31. Sarkar R., Ranjan R., Garg S.,Garg V. J., Sonthalia S., Bansal S. (2016) Periorbital Hyperpigmentation: A Comprehensive Review. J Clin Aesthet Dermatol.