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"First 3D Facial Hypodermis Donor-derived Tissue Model for holistic assessment and mechanistic evaluation of dermo-cosmetic ingredients"

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1. Introduction

Human skin is a complex, multilayered organ fundamental for barrier function and protection. Comprised of the epidermis, dermis, and hypodermis, these layers interact intricately to maintain skin health and appearance [1]. The process of skin aging is a progressive phenomenon leading to gradual alterations in both the structure and function of the skin, with noticeable visible consequences over time [2,3]

Facial aging involves particularly complex physiological changes. The different layers of facial skin undergo alterations, with the hypodermis playing a critical contributing role [3-5]. Age-related changes in facial tissue include phenomena such as stagnation of fluids and sagging of facial fat pads. These combined alterations lead to the manifestation of various common cosmetic concerns, including dark circles, puffiness, under-eye bags, and a loss of firmness [6,7]. Addressing the visible signs of aging is a primary goal in dermatological research and the development of dermocosmetic ingredients with the potential to mitigate age-related changes. Such ingredients often target diverse mechanisms within the skin, acting as anti-inflammatory agents, vasoconstrictors, antioxidants, agents strengthening the vascular network or promoting lymphatic function, extra-cellular matrix (ECM) remodelling agents or modulators of metabolic function [7,8]. Examples of ingredients relevant to facial cosmetic research include caffeine, enoxolone and N-acetyl cysteine (NAC).

Caffeine is a well-known active ingredient, often included in eye and facial creams as well as in body products, recognized for its potential effects on fat metabolism and microvascular function [9-10]. Enoxolone (also referred to as 18β-Glycyrrhetinic Acid) is a natural extract with documented anti-inflammatory, antioxidant and brightening properties as well as an effect in reducing oedema and swelling [11]. NAC is an antioxidant, playing a crucial role in reducing oxidative stress and modulating inflammation to maintain skin health and preventing premature aging [12].

Several *in vitro* and *ex vivo* models that can replicate basic epidermal and/or dermal anatomy are currently used for dermatological and dermocosmetic research, such as tissue explants, reconstructed human epidermis (RHE), human skin equivalents (HSEs), etc [13]. However, these models are usually derived from human cells and/or tissues from the abdominal area and thus do not properly reproduce facial areas peculiarities in laboratory conditions. Indeed, the facial region shows a particularly complex structure, including heterogeneous hypodermic tissue compartments, that influence processes and characteristics such as skin elasticity, pigmentation and aging [3,14].

Facial hypodermis plays a significant role in facial aging: age-dependent changes in facial fat volume and structure, collagen content and adhesion influence skin mechanical stability and contribute to loss of firmness, wrinkles, and overall skin thinning [3,14-16]. Facial adipocytes and preadipocytes have distinct characteristics, and processes like local inflammation, change in vascularisation, adipocyte differentiation capacity and adipocyte-myofibroblast transition are also involved in the aging of this tissue and adjacent dermis. Moreover, the characteristics and distribution of facial fat pads are key determinants of facial contour and are significantly affected by the aging process [14-16]. Given the increasing use of cosmetic ingredients tailored to the facial area, the availability of laboratory *in vitro* and/or *ex vivo* models to evaluate novel dermocosmetic ingredients in *in vivo*-like relevant conditions is essential.

Most of the available *in vitro* and *ex vivo* skin models do not only lack the hypodermal tissue, but they also lack or only partially reproduce the vascular network of the face, which are very specific and responsible for many aesthetic and cosmetic concerns such as dark circles, fluid stagnation and eye puffiness, loss of firmness [17-19]. The absence of a relevant model capable of mimicking the complex face microenvironment for simultaneous evaluation of the multiple interconnected biological processes involved in facial tissue responses and aging, hinders a deeper understanding and effective evaluation of cosmetic interventions.

To overcome these challenges and enable a more comprehensive and physiologically relevant assessment of cosmetic interventions targeting the multifaceted aspects of facial aging, we decided to apply a recently described technology to generate a unique 3D *ex vivo* tissue model [20] from donor facial tissues. This tissue model, named ExAdEx (acronym *for Ex vivo Adipocyte Expansion*), can be generated from human hypodermis collected from elective aesthetic surgeries and is designed to preserve the native tissue architecture, including the 3D vascular network and adult cell subpopulations embedded within the donor extracellular matrix, without exogenous scaffolds. This model has been described to be viable for extended periods of time in 3D cell culture conditions [20].

The primary objective of the research presented here was to generate ExAdEx 3D tissue models from human donor hypodermis tissue of the facial area. Later, we used the generated *ex vivo* models to test reference active ingredients and compounds associated with various mechanisms of action (e.g. anti-inflammatory, antioxidant, anti-vascular, pro-vascular, etc.).

2. Materials and Methods

Human Donor Tissue material. Subcutaneous tissue (hypodermis) material used in this study was obtained as anonymised surgical waste leftovers from elective aesthetic surgeries from the submental area (chin), without any alteration of the surgical procedures. Human tissues and derived material were processed under French Ministry of Research declaration number DC-2022-5219. Characteristics of the donors used in this study are shown in Table 1.

Table 1. Characteristics of the donors' tissues used in this study.

# Donor	Gender (m/f)	Age (y)	Body Mass Index
#1	F	61	24.0
#2	F	72	20.5
#3	M	69	22.9

Generation of 3D Human Donor-Derived Models. *Ex vivo* 3D tissue models used in this study (named ExAdEx for the acronym *Ex vivo Adipocyte Expansion*) were generated as described in [20]. Briefly, ExAdEx models were obtained from donor tissues through centrifugation in order to separate the tissue and the cell fractions. Cell fraction containing highly proliferative stem cell population was purified by removing blood and anaesthetic liquid. Meanwhile, the adipose tissue fraction was mechanically dissociated by emulsification through a medical device reducing its size in order to obtain lobular structures. Finally, both cell and tissue fraction were mixed and incubated at 37°C, 5%CO_2 in EGM Plus medium (PromoCell) for a minimum of 2 weeks before analysis or incubation with compounds and active ingredients.

Incubation with compounds and active ingredients. ExAdEx models derived from donor submental tissue and previously cultured for a minimum of 2 weeks were incubated for 7 days in EGM Plus medium (PromoCell) with or without active ingredients: 0.5% caffeine, 300 µM N-Acetyl-Cysteine (NAC), 5 µM enoxolone, 0.5 µM Dexamethasone (DEX). As a provascular control, ExAdEx models were incubated in EGM-2 medium (PromoCell). A complete cell culture medium change was performed every 2-3 days throughout the incubation phase.

Immunostaining. Donor-derived subcutaneous adipose tissues or ExAdEx models were fixed with 4% PFA in PBS for 24 hours at +4°C under agitation. Fixed samples were then incubated with primary antibodies for CD31, collagen 1A1, collagen VI, elastin or laminin overnight at 4°C and the corresponding fluorophore-labelled secondary antibodies were incubated for 2 hours at room temperature. Lipid droplets were stained with Oil Red O (ORO) to visualize adipocytes. Nuclei were stained with DAPI. Samples were visualized on an LSM 780 NLO inverted Axio Observer Z1 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a Plan Apo 25x multi-immersion (oil, glycerol, water) NA 0.8 objective and confocal images of the stained samples were acquired.

RNA Extraction and RT-qPCR. Total RNA was extracted from cultured hypodermis donor-derived 3D ExAdEx models using a commercially available RNA extraction Kit (Zymoclean Gel RNA Recovery kit, Ozyme), according to manufacturer's instructions. RT-qPCR (reverse transcription–polymerase chain reaction analysis and real-time PCR) assay was performed as previously described [18]. Expression of relevant genes for markers of vascular and lymphatic network (*CD31*, *LYVE-1*), adipose progenitor cells (*PDGFRa*, *MSCA1*), adipose tissue function (*Adiponectin*, *Glut4*) and extra-cellular matrix (*Col1A1*, *Laminin A4*) was obtained through real-time PCR assay. The expression level of each gene target relative to expression of the housekeeping gene *TATA box-binding protein* (*TBP*) was calculated using the 2-ΔCt method.

Statistical analysis. The results were presented as the mean ± SEM. To determine statistical significance, the results were analyzed using GraphPad Prism version 9. Groups were compared using t-test. The statistical significance of the results is denoted as *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

3. Results

3.1. Morphological evaluation of donor hypodermis tissue and donor-tissue derived 3D *ex vivo* models from the submental area

We investigated and compared the morphological characteristics of submental hypodermis tissue and paired donor tissue-derived ExAdEx 3D models by using immunofluorescence and histologic staining techniques. We collected donor tissues right after the surgical procedures and we processed them for immunofluorescence staining rightafter. Additionally, we derived and cultured ExAdEx 3D models from these tissues and processed them after 2 weeks of ex vivo culture.

We chose CD31/PECAM1 as marker for the endothelial vascular network, and Collagen VI, Elastin and Laminin as markers for the ECM. Oil Red O staining specific for lipid droplets was used to visualize adipocytes.

Qualitative analysis of the immunofluorescence technique revealed that the donor tissue collected after surgery and donor tissue-derived ExAdEx models exhibit a comparable vascular network, adipocyte content and structure, as well as ECM proteins organization (***Fig.1***), indicating that these models can be used as a proxy for donor chin hypodermis *ex vivo*.

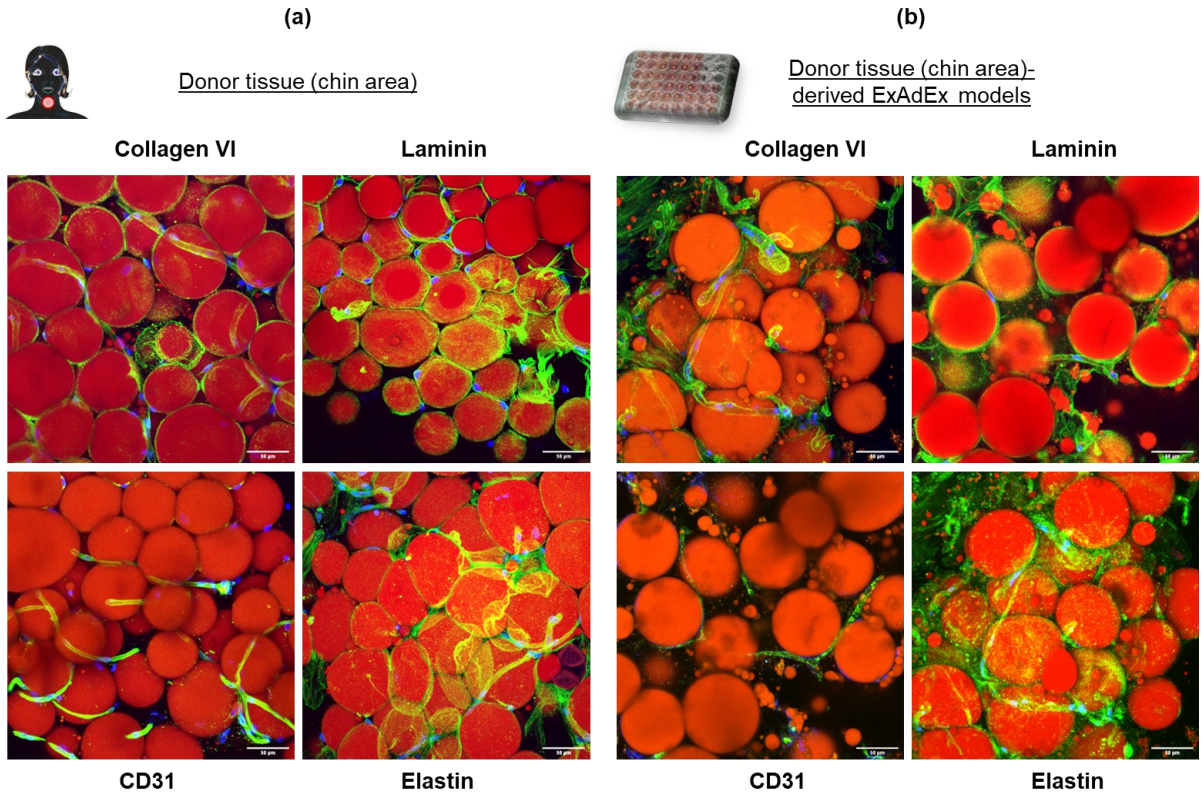

Figure 1. Comparison between donor submental (chin) hypodermis and paired 3D ExAdEx models. Immunofluorescence for CD31, Collagen VI, Elastin, Laminin on donor hypodermis tissue material collected from aesthetic surgeries from the chin area (a) and paired 3D ExAdEx models cultured for 2 weeks *ex vivo* (b). Representative confocal images are shown: adipocytes are stained in red (ORO), nuclei in blue (DAPI) and each protein of interest reported on the top of each column is stained in green.

3.2. 3D *ex vivo* models derived from donor submental hypodermis are competent for vascular stimulation

To assess the relevance of 3D ExAdEx tissue models in evaluating tissue responses to active ingredients with regards to vascularization and ECM remodelling, we incubated ExAdEx models derived from donor submental hypodermis tissue (chin area) with different compounds and active ingredients for 7 days. We then performed immunostaining and labelled vascular network through CD31 and ECM through collagen 1A1. Among the compounds tested, we chose the synthetic glucocorticoid dexamethasone (DEX) as a molecule known for its anti-angiogenic and anti-vascular potential [21]. As a provascular stimuli, we used a cell culture medium supplemented with growth factors including vascular endothelial growth factor (VEGF).

As expected, DEX induced a visible and strong reduction in endothelial cell network compared to non-treated negative control (***Fig.2a,b***). In addition, the use of a cell culture medium rich in provascular and proangiogenic growth factors resulted in a massive increase of the vascular network compared to non-treated negative control (***Fig.2a,c***). These data demonstrate the ability of chin tissue donor-derived ExAdEx models to respond to pro-vascular and anti-vascular stimuli, confirming previous studies from our group performed on abdominal hypodermis donor tissue derived models [20].

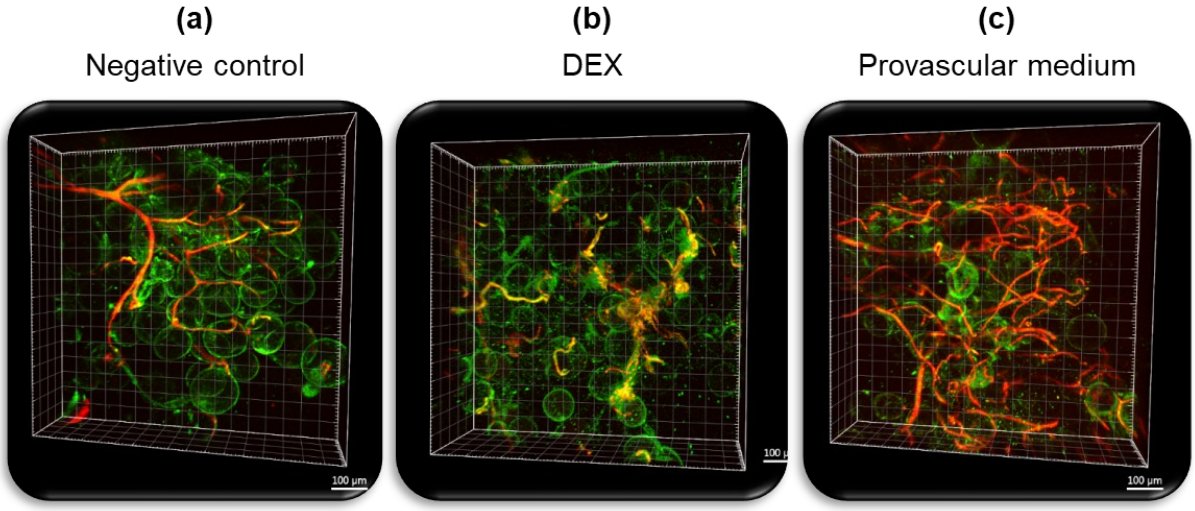

Figure 2. 3D structural effect of anti-vascular and pro-vascular treatment in 3D donor chin hypodermis-derived ExAdEx models. Representative 3D confocal images of immunofluorescence for CD31 (red) and Collagen 1A1 (green) on 3D ExAdEx models derived from submental hypodermis and incubated for 7 days without (a) or with anti-vascular compound (DEX) (b) or pro-vascular medium (c).

Among the active ingredients tested, we included caffeine for its extensive use in cosmetic research and described effects on vascularisation, fat cells and ECM [9,10], and NAC for its well-described antioxidant properties [11]. Qualitative analysis on immunofluorescence staining showed no evident visible differences in terms of vascular network and ECM structure compared to non-treated negative control (***Fig.3***). We therefore decided to further investigate the effects of these active ingredients on cultured ExAdEx models derived from chin hypodermis tissue through molecular analysis (RT-qPCR).

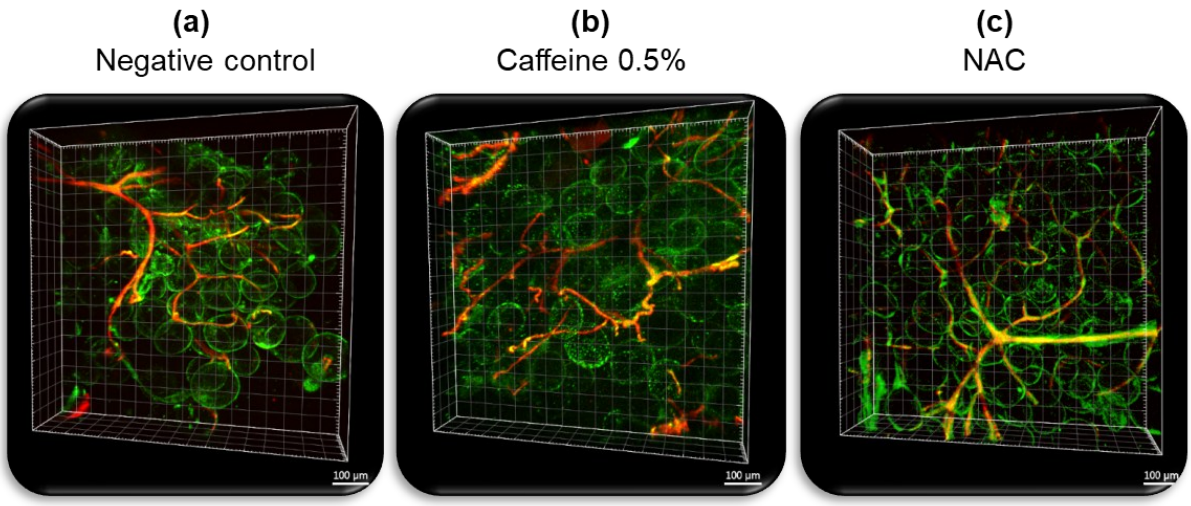

Figure 3. 3D structural effect of caffeine and NAC treatment in 3D donor chin hypodermis-derived ExAdEx models. Representative 3D confocal images of immunofluorescence for CD31 (red) and Collagen 1A1 (green) on 3D ExAdEx models derived from submental hypodermis and incubated for 7days without (a) or with 0.5% caffeine (b) or N-Acetylcysteine (c).

3.3. Different active ingredients and compounds elicit different responses in gene expression in submental hypodermis donor-tissue derived 3D ex vivo models

To better characterize the modifications induced by different treatments on 3D ExAdEx *ex vivo* models derived from donor chin hypodermis, we performed a transcriptomic analysis on donor submental tissue-derived ExAdEx models that had been incubated with or without different compounds and active ingredients for 7 days. Among the compounds and active ingredients tested we included caffeine, enoxolone, N-acetyl cysteine (NAC) and dexamethasone (DEX). For comparison, we used paired 3D ExAdEx *ex vivo* models derived from donor abdominal hypodermis.

The molecular signature of these ExAdEx models was analysed using RT-qPCR to assess the expression of key markers associated to adipose tissue function and metabolism (*Adiponectin, Glut4*), vascular and lymphatic network (*CD31, Lyve-1*), adipose progenitor cells (*PDGFRα, MSCA1*) and ECM structure (*Col1A1, LamininA4*).

Data revealed that the compounds and active ingredients tested displayed a different pattern in ExAdEx models derived from chin area and models derived from abdominal area (***Fig.4***).

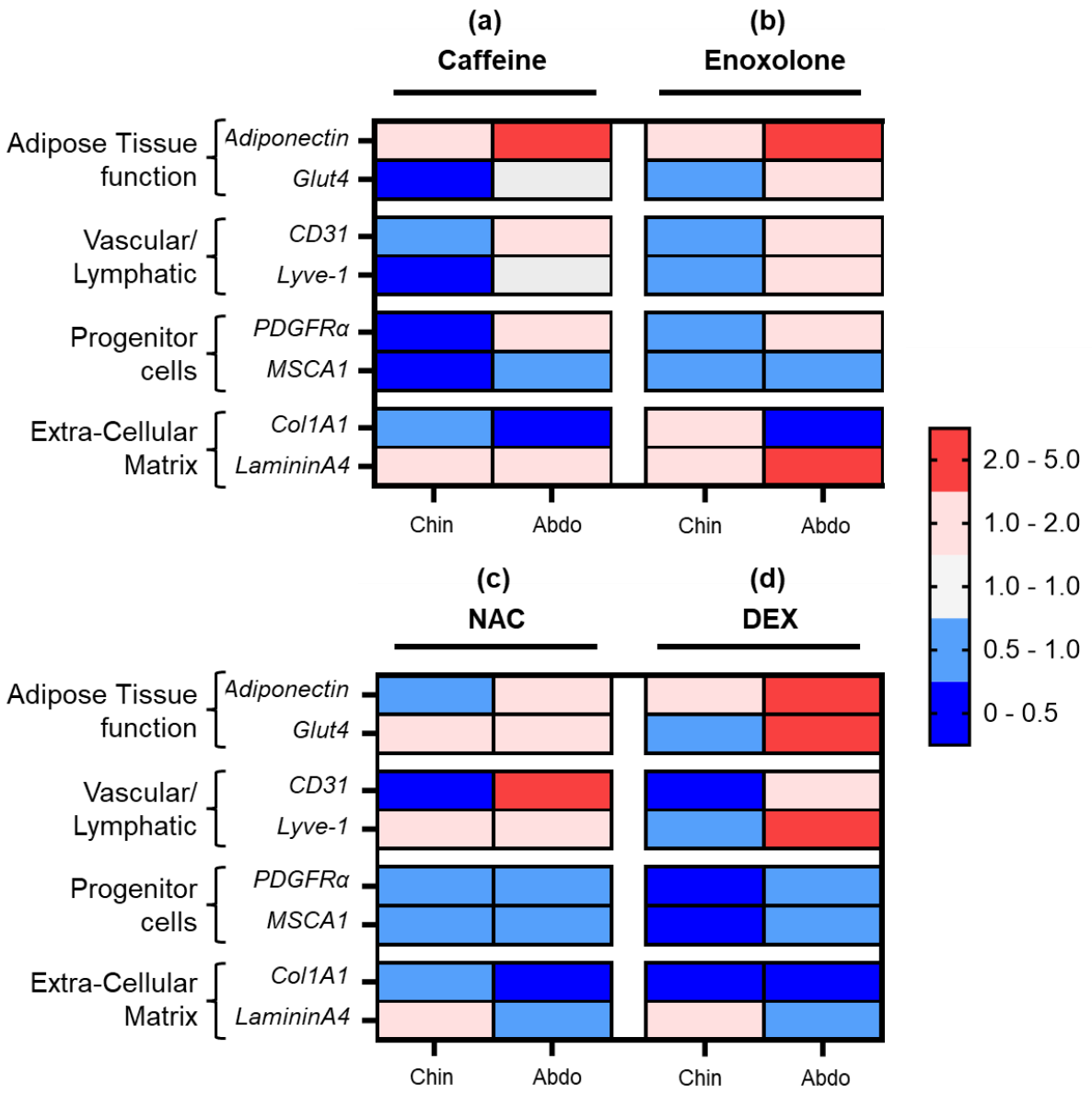

Figure 4. Heatmap presenting the expression levels of various markers in ExAdEx models derived from donor chin or abdominal hypodermis. 3D ExAdEx models derived from submental (Chin) or abdominal (Abdo) hypodermis were incubated for 7 days with 0.5% caffeine, enoxolone, NAC or DEX. The higher the expression level, the darker the color (red, upregulated; blue, downregulated), as compared to non-treated negative control. Value equal to 1 means no difference vs non-treated negative control.

Caffeine (***Fig.4a***) induced a mild upregulation of metabolic marker *Adiponectin* and a decrease in glucose transporter marker *Glut4* in ExAdEx chin treated models compared to non-treated controls, whereas the upregulation in *Adiponectin* was much more pronounced in ExAdEx abdominal treated models and *Glut4* expression showed no major difference in these models compared to non-treated controls. On the other hand, caffeine had an opposite effect in ExAdEx chin and abdominal models with regards to the vascular and lymphatic network markers, with a downregulation of *CD31* and *Lyve-1* markers in ExAdEx chin treated models compared to non-treated controls, in contrast to ExAdEx abdominal models where a mild upregulation of these markers was observed in response to treatment. Caffeine induced a strong

downregulation in progenitor cell markers *PDGFRα* and *MSCA1* in ExAdEx chin treated models compared to non-treated controls, whereas the effect on these markers was less pronounced or opposite in ExAdEx abdominal treated models. Finally, caffeine displayed a similar pattern with regards to ECM markers *Collagen I* and *Laminin* in both types of models.

Enoxolone (***Fig. 4b***) showed an overall similar mechanism of action as caffeine both in ExAdEx chin and ExAdEx abdominal treated models. Enoxolone induced an upregulation of metabolic marker *Adiponectin* and a downregulation of *Glut4* expression in ExAdEx chin treated models compared to non-treated controls, whereas both *Adiponectin* and *Glut4* were upregulated in ExAdEx abdominal treated models compared to non-treated controls. Enoxolone showed an opposite effect in ExAdEx chin and abdominal treated models compared to non-treated controls with regards to the vascular marker *CD31* and lymphatic marker Lyve-1 (downregulation in ExAdEx chin models vs upregulation in ExAdEx abdominal models). Enoxolone induced a downregulation in progenitor cell markers *PDGFRα* and *MSCA1* in ExAdEx chin treated models compared to non-treated controls, whereas the effect on *MSCA1* marker was the opposite in ExAdEx abdominal treated models. Finally, enoxolone showed an upregulation of both ECM markers *Collagen I* and *Laminin* in ExAdEx chin treated models compared to non-treated controls, whereas a downregulation of *Collagen I* and an upregulation of *Laminin* was observed in ExAdEx abdominal treated models.

N-acetyl cysteine (NAC) (***Fig.4c***) showed a reduction in *Adiponectin* expression in ExAdEx chin treated models and an upregulation in ExAdEx abdominal treated models compared to non-treated controls. *Glut4* expression was found upregulated in both types of models compared to respective non-treated controls. NAC strongly downregulated vascular marker *CD31* in ExAdEx chin treated models, whereas it strongly upregulated this marker in ExAdEx abdominal models. Lymphatic marker *Lyve-1* was upregulated in both types of models. NAC exhibited a similar pattern in both types of models with regards to progenitor cell markers *PDGFRα* and *MSCA1* (downregulation as compared to non-treated controls). With regards to ECM markers, NAC induced downregulation of *Collagen I* expression in both types of models, whereas *Laminin* was found to be upregulated in ExAdEx chin treated models and downregulated in ExAdEx abdominal treated models compared to non-treated controls.

Dexamethasone (DEX) (***Fig.4d***) induced an upregulation in *Adiponectin* expression in both types of models, whereas *Glut4* expression was found downregulated in ExAdEx chin treated models and upregulated in ExAdEx abdominal treated models. DEX exhibited a dramatically different pattern in both types of models with regards to vascular and lymphatic markers, with a strong downregulation in ExAdEx chin treated models and upregulation in ExAdEx abdominal treated models compared to non-treated control. DEX induced downregulation in progenitor cell markers in both types of models. With regards to ECM markers, DEX induced downregulation of *Collagen I* expression in both types of models, whereas *Laminin* was found to be upregulated in ExAdEx chin treated models and downregulated in ExAdEx abdominal treated models compared to non-treated controls.

These data highlight the fact that intrinsic morphological and molecular differences in hypodermis tissue from the facial (chin) area and abdominal area result in different responses to the test of dermocosmetic active ingredients, suggesting the importance of selecting the most relevant *ex vivo* models when developing active ingredients or cosmetic formulations depending on the final use by consumers and professionals.

4. Discussion

The *ex vivo* 3D tissue model generated from facial hypodermis through the technology named ExAdEx and previously described in [20] represents a significant advancement in dermocosmetic research, particularly for evaluating the efficacy of active ingredients targeting facial aging. This model addresses several limitations of existing 2D *in vitro* and *ex vivo* (explants) models, which often fail to accurately replicate the complex structure and physiology of facial skin, especially the vascular content and the hypodermis. By preserving the native tissue architecture, including the 3D vascular network and adult cell subpopulations [20], this model provides a more physiologically relevant environment for testing dermocosmetic ingredients.

Our study demonstrated that the ExAdEx model derived from submental hypodermis tissue is competent for vascular stimulation and ECM remodelling, as evidenced by its response to various active ingredients and compounds. The model's ability to respond to pro-vascular and anti-vascular stimuli, such as dexamethasone and provascular growth factors, highlights its potential for evaluating the vascular effects of cosmetic ingredients. Furthermore, the differential gene expression patterns observed in response to the active ingredients caffeine, enoxolone and NAC confirm previous study performed by our group and others for the determination of a molecular signature of facial hypodermis tissue [20-24] and underscore the model's sensitivity to various mechanisms of action, including anti-inflammatory, antioxidant, vascular/lymphatic function, ECM remodelling and metabolic effects.

The comparison between ExAdEx models derived from chin and abdominal hypodermis revealed intrinsic morphological and molecular differences, resulting in distinct responses to the same active ingredients and confirming previous research by us and others indicating regional variations in adipose tissue characteristics and functions [14,15,20,22-24]. These finding emphasizes the importance of selecting the most relevant *ex vivo* models for developing active ingredients or cosmetic formulations tailored to specific facial and/or body areas and applications. The facial region's unique structure and aging processes necessitate specialized models to generate physiologically relevant data and enhance our understanding of facial skin aging.

The ExAdEx model's ability to simultaneously evaluate multiple tissue components, including the vascular network, adipocytes, and ECM, offers a holistic approach to dermocosmetic research. This multi-parametric evaluation is crucial for generating comprehensive data prior to *in vivo* studies and for substantiating cosmetic claims related to vasculature and age-related loss of facial firmness.

5. Conclusion

In conclusion, the 3D tissue models derived from facial hypodermis described in this study provide a versatile and comprehensive tool for facial dermocosmetic research. These models are particularly suited for evaluating claims related to vasculature, such as dark circles and eye puffiness, and age-related loss of facial firmness, including deep tissue loosening and under-eye sagging. By enabling multi-parametric evaluation of various tissue components simultaneously in a single model, they represent a valuable asset for generating physiologically relevant data and enhancing our understanding of the biological mechanisms involved in facial skin aging and stress. Future research should focus on further validating these models and exploring their potential applications in the development of innovative dermocosmetic ingredients and formulations.

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