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"Distinct Body Site-Specific Profiles in 3D-cultured Human Donor Hypodermis-Derived Models Implications for Targeted Skincare Product Development"

Vincent Dani [1], Enora Lecorgne [1], Floricia Crusset [1], Sameh Ben Aicha [2], Bérengère Chignon-Sicard [3], Alain Doglio [4], Christian Dani [5] and Luigi Formicola [1]

[1] EXADEX-INNOV; [2] Institut de Biologie Valrose [3] Polyclinique Saint George; [4] Faculté de Chirurgie Dentaire, Université Côte d'Azur ; [5] Faculté de Médecine Université Côte d'Azur

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1. Introduction

The skin, as the largest and most visible organ of the human body, plays a crucial role not only in protection against the external environment but also in aesthetic perception, and so the development of effective skincare products has become a significant area of research [1]. The efficacy of these products often relies on their ability to interact with specific skin layers and underlying tissues, including the hypodermis, which contains adipose tissue [2]. Differences in skin characteristics depending on gender, age and body region have been reported and have been related to differences in gene expression, functional responses and biomechanical properties, including for the hypodermis [3-6]. Understanding tissue-specific characteristics is paramount for the design and development of targeted and personalized cosmetic skincare formulations.

As the skin ages, several changes occur in the epidermis, dermis, and hypodermis, including collagen content decrease and collagen fibers disorganization, due to reduced collagen synthesis and increased collagenase activity [7-9]. Procollagen synthesis, a precursor to collagen, is significantly decreased in intrinsically aged skin and *in vitro* studies also showed that fibroblasts from older individuals produce less type I procollagen than those from younger individuals [10,11]. Promoting collagen synthesis through dermocosmetic ingredients is thus a viable strategy to delay skin ageing [12]. The hypodermis, or subcutaneous fat tissue, also experiences age-related changes. There is a rearrangement of subcutaneous fat tissue across facial regions, including the periorbital, forehead, malar areas, sub-mental region and jowls. These changes in fat distribution contribute to facial volume loss, sagging, and the formation of wrinkles [13].

In particular, the hypodermis, far from being a passive energy storage depot, is now recognized as a dynamic and complex organ involved in skin aging, and has raised increased interest in dermocosmetic research. Residing under the dermis throughout the body, it exhibits regional variations in cellular composition, gene expression profiles, and physiological properties [14-

17]. The embryonic origins of fat tissue depots can differ depending on their anatomical location, which may contribute to their distinct functional properties [16,17]. In recent years, clinical and preclinical studies have been highlighting these variations within the facial region and when comparing different anatomical locations from the face and lower body parts. For instance, adipose-derived stromal cells (ASCs) from different body sites possess distinct characteristics, including varying potentials for differentiation into different types of adipocytes. Chin-derived ASCs have shown the potential to differentiate into brown-like adipocytes, whereas knee-derived ASCs tend to differentiate into white adipocytes [17,18]. Moreover, it has been shown that adipocytes can behave dynamically in the superficial area of the hypodermis underlying the reticular dermis, where these cells can be involved in diverse processes such as hair follicle cycling, wound healing and inflammatory responses of the skin [19]. Adipocytes has been considered for years as the main responsible for both hypodermis mechanical behaviour and the tissue response to different external factors, however this picture has been strongly challenged in recent years as increasing evidence is being collected as to the importance of other components of this tissue, especially its extra-cellular matrix (ECM) and vascular/lymphatic network [19,20]. Taken together, these data strongly suggest that hypodermis in different body locations is not functionally equivalent and may respond differently to internal and external stimuli, including dermo-cosmetic interventions.

The facial region presents a particularly complex landscape, including heterogeneous subcutaneous adipose tissue compartments that have an effect on processes and characteristics such as skin elasticity, pigmentation and aging [19,21]. For example, the distribution and characteristics of facial fat pads are crucial determinants of facial contour and are significantly affected by the aging process. Changes in facial fat volume and structure contribute to wrinkles, laxity, and overall skin thinning [19,21,22]. Given the increasing use of cosmetic ingredients tailored to the facial area, a detailed understanding of the specific properties of adipose tissue in different facial sub-regions, as well as in comparison to other body sites like the abdomen, is essential. While some studies have investigated the overall biophysical and histological parameters of skin (including the hypodermis) in different facial regions and compared them to other body sites [14,18,22,23], the implications for targeted skincare product development remains less defined.

Our study aims to address this gap by investigating the distinct profiles of donor subcutaneous adipose tissue and by using a recently described technology that allows to derive and culture *ex vivo* tissue models from human material to better characterize physiological responses [24]. These models, named ExAdEx (for *Ex vivo Adipocyte Expansion*) are generated by the emulsification of lipoaspirates and allow endogenous donor-derived adipose progenitor cells to expand *ex vivo* within the cellular complexity and the 3D vascularized extracellular matrix of the native tissue [24]. Use of this model allowed us to overcome the common limitations of the different adipose tissue substitutes engineered *in vitro*, such as the loss of the native 3D tissue structure, reduction of viability and loss of vascularisation [25] and to preserve the donor tissue at its closest.

The primary objective of the research presented here was to provide a deeper understanding of the inherent differences in hypodermal tissue from distinct body sites, such as the chin and the abdominal area (the latter used as a representative of a non-facial site with abundant subcutaneous fat), and to explore the implications of these differences for the development of more targeted and personalized skincare products.

2. Materials and Methods

Human Adipose Tissue material. Subcutaneous adipose tissue (hypodermis) material used in this study was obtained as anonymised surgical waste leftovers from elective aesthetic surgeries from the submental area (chin) and abdominal area, 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	M	69	22.6
2	F	65	24.2
3	F	61	24.2
4	F	61	23.9
5	F	72	20.6

Morphological analysis. Donor-derived subcutaneous adipose tissues 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 and collagen IV 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. Collagen deposition (collagen I/III) was assessed by Picro-sirius Red solution (Sigma). 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. Quantitative analysis of samples was performed with ImageJ software.

RNA Extraction and RT-qPCR. Total RNA was extracted from donor-derived subcutaneous adipose tissues 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 [24]. Expression of relevant genes for markers of vascular and lymphatic network (*CD31*, *LYVE-1*), adipocytes and adipose progenitor cells (*PLN1*, *PDGFR*α, *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.

Generation of 3D Human Donor Hypodermis-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 [24]. 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.

Metabolic Activity. An XTT colorimetric test was performed to assess viability and metabolic activity, using a commercially available XTT Assay Kit (Roche), according to manufacturer's instruction. The XTT reagent was added to ExAdEx models in culture for 5 hours and supernatants were transferred to a new plate for absorbance reading at 450nm using the Biorad iMarkTM microplate reader.

Lipolysis. ExAdEx models were washed once with PBS and maintained for 24 hours in RPMI supplemented with 2% fatty acid free BSA, 5 µM triascin C and 1 ng/mL ascorbic acid. Lipolysis was stimulated with 1 µM isoproterenol (isoP) for 24 hours. Glycerol release into the culture medium was determined as an index of lipolysis according to the manufacturer's instructions using a Glycerol Detection Assay (Promega)

ELISA assay. Conditioned media were collected 24 hours after the last medium change from either subcutaneous adipose tissue cultured for 24 hours or ExAdEx models cultured for a minimum of 2 weeks. Adiponectin and pro-collagen 1α levels were determined using commercial ELISA kits (R&D Systems) on conditioned media samples stored at -80°C, according to the manufacturer's instructions.

Incubation with Dexamethasone (DEX) and TGFβ1. ExAdEx models were incubated for 7 days in EGM Plus medium (Promocell) with or without 0.5 µM dexamethasone (DEX) from day 0 to day 2, and/or 5 µg/ml TGFβ1 from day 2 to day 7. A complete cell culture medium change was performed every 2-3 days throughout the incubation phase in control conditions.

Statistical analysis. The results were presented as the mean ± SEM. To determine statistical significance, the results were analysed 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-derived hypodermis from facial and lower body depots

We investigated the morphological characteristics of hypodermis tissue by using immunofluorescence and histological staining techniques on paired subcutaneous adipose tissue obtained from the abdominal and chin area. We chose CD31/PECAM1 as marker for the endothelial vascular network and Collagen IV as a marker for the ECM. Oil Red O staining specific for lipid droplets was used to visualize adipocytes. Picro-sirius Red staining was used as a specific staining for collagen I and collagen III [26].

Qualitative and quantitative analysis of the immunofluorescence and histological staining techniques revealed that the abdominal and chin hypodermis exhibit a comparable vascular network, adipocyte content and collagen IV organization (***Fig.1a,b***). A notable difference was observed on the Picro-sirius Red stained samples (***Fig.1a,b***), with an increased staining intensity in abdominal tissue as compared to chin tissue, indicating a different composition and structure of collagen I and III fibers (***Fig.1c***).

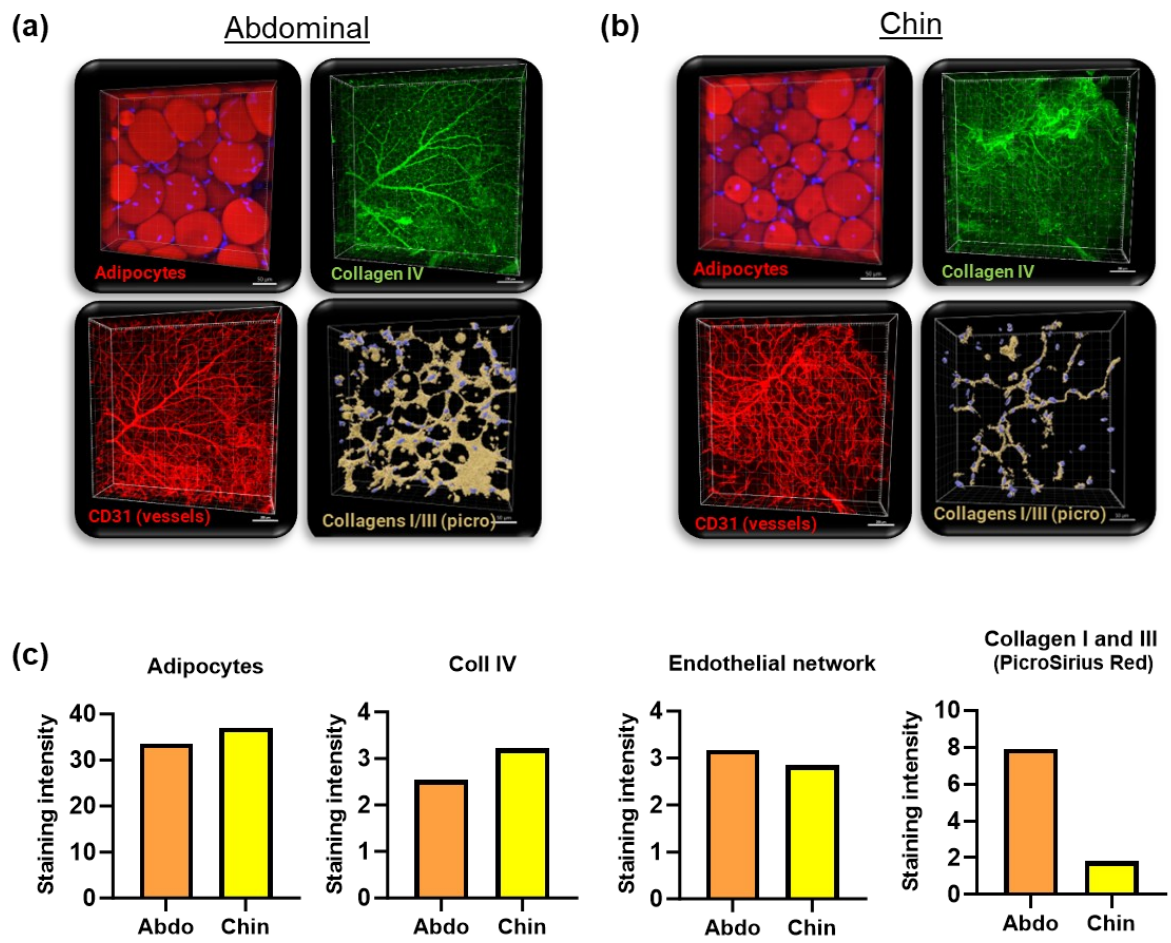

Figure 1. Characterization of hypodermis (subcutaneous tissue) derived from different donor-derived body depots. (a,b) Immunofluorescence for CD31 and collagen IV, ORO and Picro-sirius Red statining on hypodermis tissue material collected from aesthetic surgery procedures on donor #1 from the abdominal (a) and chin (b) area. (c) Quantification of staining signal intensity by confocal microscopy for ORO staining (adipocytes), collagen IV, CD31 staining (endothelial network) and Picro-sirius Red staining (collagen I and III).

3.2. Signature of donor-derived hypodermis from facial and lower body depots

The molecular signature of hypodermis tissue from the abdominal and chin area was analysed using RT-qPCR to assess the expression of key genes involved in adipocyte function, adipose progenitor cells and adipogenesis, ECM composition, and vascularization (***Fig.2***).

Data revealed that the main adipose tissue effector *Adiponectin*, adipocyte progenitor marker *MSCA1* and glucose transporter *Glut4* were all overexpressed in the abdominal hypodermis compared to the chin region for the donor tissues analysed in this study *(**Fig.2***).

Inter-donor variability was observed for vascular *CD31/PECAM1* and lymphatic *LYVE-1* markers, as well as for fibro-adipogenic progenitor cell marker *PDGFRα* and mature adipocyte marker *PLN1*, thus we could not observe any major trend in these markers expression in the donor tissues analysed in this study (***Fig.2***).

Regarding the ECM composition, we found a strong overexpression of Collagen I (*Col1A1*) in chin hypodermis compared to abdominal hypodermis for all donors analysed (***Fig.2***),

confirming the observed trend for Picro-sirius Red staining (***Fig.1c***). In addition, we found that *Laminin* (*Laminin A4*) was overexpressed in chin hypodermis compared to abdominal hypodermis for 3 out of 4 donor tissues analysed (***Fig.2***).

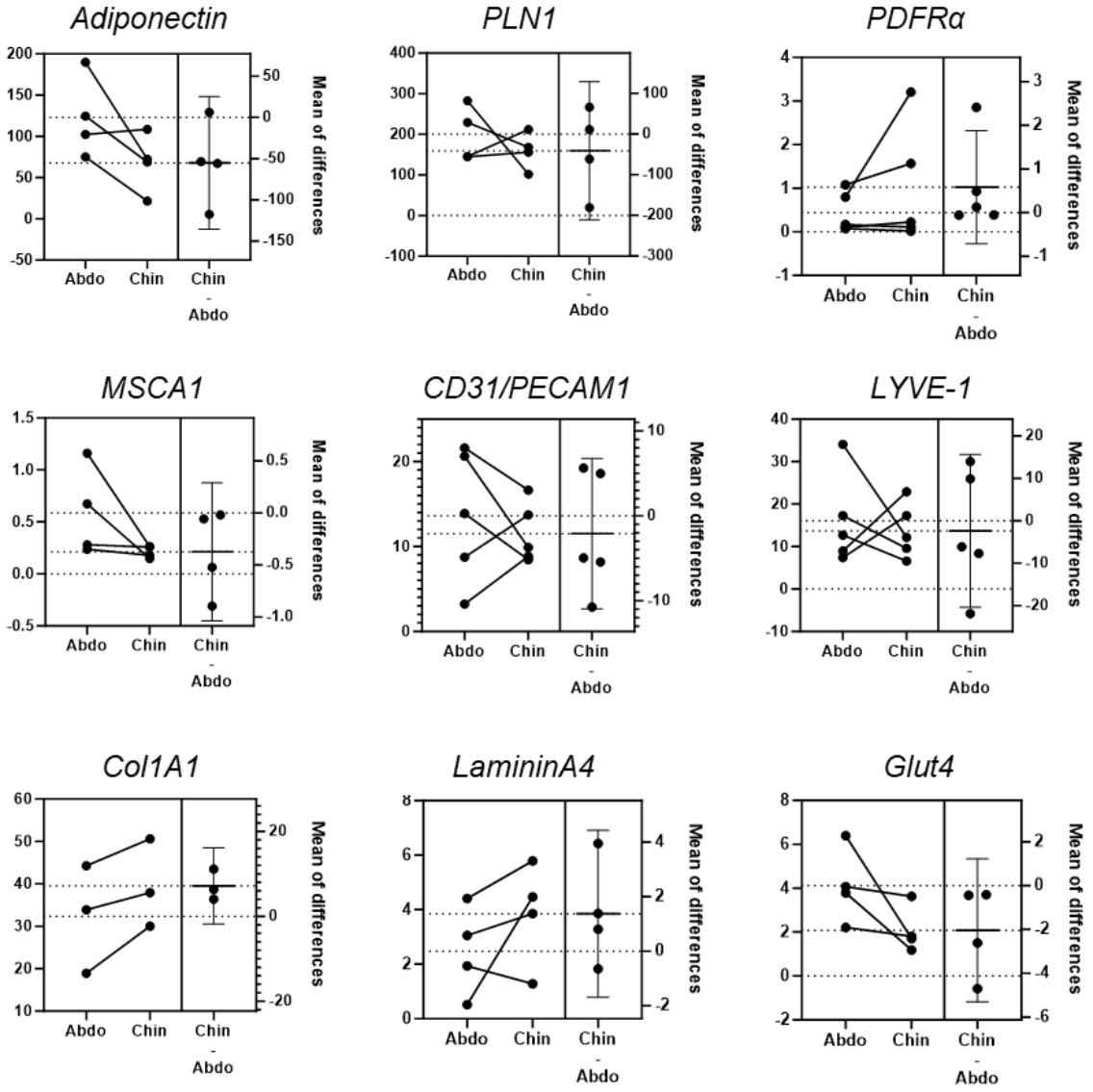

Figure 2. Transcriptomic characterization of hypodermis (subcutaneous tissue) derived from different body depots from the same donor. RT-qPCR analysis on hypodermis tissue material collected from aesthetic surgery procedures from donors from the abdominal and chin area. Each dot corresponds to a donor.

We also analysed the levels of secreted Adiponectin and Procollagen Iα in culture from donor-derived hypodermis tissue.

An increased secretion of Adiponectin was observed in the abdominal hypodermis compared to the chin region (***Fig.3a***), confirming the results obtained by RT-qPCR (***Fig.2***).

Procollagen Iα secretion was lower in the chin hypodermis compared to the chin region (***Fig. 3b***), in contrast to the observed molecular data on *Col1A1* gene (***Fig.2***), suggesting post-transcriptional and/or post-translational regulation of collagen genes.

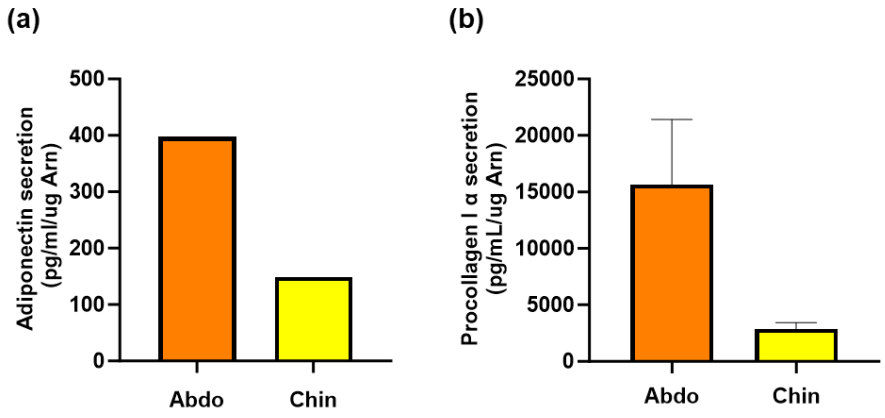

Figure 3. Secretome characterization of hypodermis (subcutaneous tissue) derived from different body depots. (a,b) ELISA analysis on hypodermis tissue material collected from aesthetic surgery procedures from donors from the abdominal and chin area for Adiponectin (a) and Procollagen Iα (b). For adiponectin secretion (a), tissues from n=1 donor were analysed. For Procollagen Iα (b), tissues from n=4 donors were analysed for the abdominal area and from the chin area.

We then generated 3D-cultured human donor hypodermis-derived models from both abdominal and chin hypodermis according to the method previously described (ExAdEx method) [24] and we cultured them for 2 weeks. Metabolic activity and lipolysis were assessed.

3D ExAdEx models derived from abdominal tissue exhibited a higher metabolic activity (***Fig.4a***) and lipolytic response following stimulation with isoproterenol (***Fig.4b***), compared to the chin-derived model, confirming previous studies generated on 2D cell models [27].

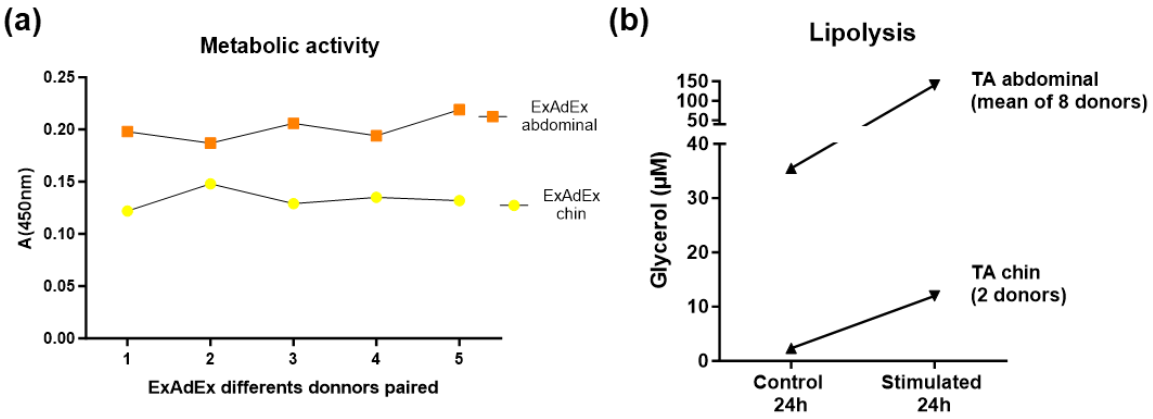

Figure 4. Different hypodermis body depots show different metabolic activity. Analyses performed on ExAdEx models derived from abdominal and chin area of different donors and cultured *ex vivo* for 2 weeks. (a) Measurement of metabolic activity through XTT assay. (b) Measurement of lipolysis through glycerol release assay in cell medium.

These findings suggest that the hypodermis from different body sites exhibits distinct transcriptomic and secretory profiles, which may influence their functional properties and responses to external stimuli. These results highlight distinct gene expression profiles of hypodermis tissue from different body sites, which may have implications for targeted skincare product development.

3.3. Generation of a 3D-cultured human donor hypodermis-derived model mimicking facial hypodermis

To overcome limitations in surgical leftover material of facial tissues, precluding studies of multiple parameters in a long-term basis from the same donors, we later wanted to mimic a chin-like signature in ExAdEx models derived from abdominal hypodermis, as this is abundantly available as surgical waste material from aesthetic surgeries. In order to mimic a chin-like phenotype, we derived and cultured 3D ExAdEx models from abdominal hypodermis according to the method previously described [24] and used Dexamethasone (DEX) in culture medium as a known agent able to reduce procollagen and collagen expression [28,29].

We found a decrease in Adiponectin (***Fig.5a***) and procollagen Iα (***Fig.5b***) secretion in DEX-treated ExAdEx models as compared to control, mimicking the differences observed in donor hypodermis profiles previously analysed (Fig.3). More interestingly, when incubated with TGFβ1, an effector known to induce an increase in collagen production [30], we found that these chin-like models showed a dramatic increase in procollagen Iα secretion (***Fig.5b***), counteracting the effect of DEX.

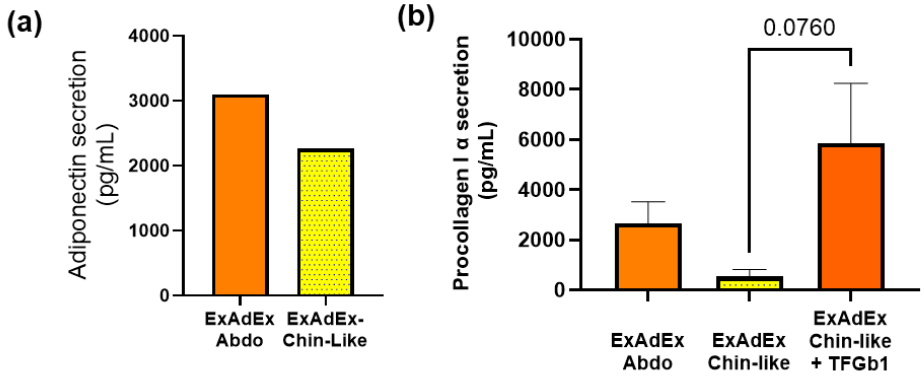

Figure 5. 3D models derived from human donor abdominal hypodermis can replicate procollagen secretion of donor tissues from the chin area. Analyses performed on ExAdEx models derived from from abdominal donor tissue (ExAdEx Abdo) incubated with DEX to mimic chin tissue signature (ExAdEx Chin-like) or with DEX+TFGβ1 (ExAdEx Chin-like + TFGβ1). Measurement of adiponectin (a) and procollagen Iα (b) secretion through ELISA.

These results demonstrate that the ExAdEx model can replicate the distinct functional properties of hypodermis tissue from different body sites, providing a valuable tool for targeted skincare product development.

4. Discussion

The results of this study provide valuable insights into the distinct morphological, molecular, and functional profiles of hypodermis tissue from different body sites.

The abdominal hypodermis exhibited an increased expression of markers associated to metabolism homeostasis (such as *Adiponectin, Glut4*) and adipogenesis (*MSCA1*). In addition, an increased metabolic activity and lipolytic response were observed in 3D cultured models derived from this tissue. Together, these data are consistent with the role of this subcutaneous

adipose tissue in regulating body metabolism and previous literature [24]. No major differences were observed with regards to vascular and lymphatic network in the study conditions.

In contrast, chin hypodermis showed a different ECM compared to abdominal hypodermis, with a reduced expression of Collagen I and III as visualized through histological staining and a reduced procollagen Iα secretion. Interestingly, gene expression for *collagen I* and *laminin* were found higher in chin hypodermis compared to abdominal hypodermis, suggesting more complex regulation of ECM proteins and structure through transcriptional, translational and post-translational modifications. Further studies will be needed to elucidate these phenomena.

These findings align with previous research indicating regional variations in adipose tissue characteristics and functions [13-19]. The distinct molecular signatures and secretory profiles of hypodermis tissue from different body sites may have significant implications for targeted skincare product development. For instance, the lower procollagen Iα secretion in the chin hypodermis supports the observations that facial tissues are more prone to aging than other tissues and that this region may be more responsive to skincare products designed to promote collagen synthesis. This is particularly relevant given that collagen synthesis decreases with age, and promoting its production is a viable strategy to delay skin aging [12].

In addition, the ExAdEx model's ability to replicate and mimic the distinct characteristics of abdominal and chin hypodermis tissue highlights its potential for developing targeted skincare products that address the specific needs of different body regions. Understanding these differences is crucial especially for the tissues from the facial area, as aging affects the distribution and structure of facial fat pads and their interactions with the dermis, contributing to wrinkles, laxity, and overall skin thinning.

5. Conclusion

In conclusion, this study demonstrates the distinct morphological, molecular, and functional profiles of hypodermis tissue from different body sites. The abdominal hypodermis exhibits a profile that is mostly related to its function as a master regulator of metabolic homeostasis, in contrast to the chin hypodermis which exhibits specific features in the structure and composition of the ECM, supporting its role in maintaining mechanical stability of the skin and relevance in the development of aging symptoms such as wrinkles [31]. These findings have important implications for targeted skincare product development, as they highlight the need for region-specific approaches to address the unique characteristics of hypodermis tissue from different body sites.

The availability of tissue models for research that can more precisely mimic phenotypic signature of facial tissues will ultimately allow to tailor the development of dermocosmetic active ingredients depending on consumers' needs and real-life use and will open perspectives as to personalized and targeted skincare approaches. In this context, the ExAdEx facial model provides a valuable tool for further investigating the functional properties of hypodermis tissue and developing targeted skincare products that have an impact on the overall aging processes of the skin. Future research should focus on exploring the mechanisms underlying the distinct profiles of hypodermis tissue from different body sites and their responses to various skincare ingredients. This will enable the development of more effective and personalized skincare products that address the specific needs of different body regions, particularly in the context of aging and collagen production claims.

By leveraging these insights, the skincare industry can develop products that not only address visible signs of aging but also support the underlying biological processes that contribute to skin health and appearance.

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