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Running head: Novel peptide matrikines for skin rejuvenation

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Data availability: Proteomics data are available via ProteomeXchange.
The three transcriptomics data sets (E-MTAB-12711, E-MTAB-12710 and E-MTAB-12704) can be found at ArrayExpress.
(https://www.ebi.ac.uk/biostudies/arrayexpresshttps://www.ebi.ac.uk/biostudies/arrayexpress).
The peptide prediction code can be found at
https://github.com/maxozo/Matrikine_Discoveryhttps://github.com/maxozo/Matrikine_Discovery.

Ethics statements: The in vitro patch-test study was conducted in accordance with the principles of the Declaration of Helsinki, with written informed consent (Manchester University Research Ethics Committee reference: 2020-7062-13677). Ethical approval for the clinical study was granted and all participants provided written informed consent prior to starting the study

What is already known about this topic?

- Chronic skin photo-ageing induces profound remodeling of the dermal extracellular matrix
- Small peptide fragments of extracellular matrix proteins (matrikines) can induce beneficial remodeling of the dermal matrix.
- The discovery of new bioactive peptide matrikines has not been guided by any hypothesis.

What does this study add?

- Our data supports the hypothesis that new matrikines can be identified by bioinformatic prediction of protease cleavage sites and hence peptide fragments.
- We describe an *in silico* to *in vitro* to *in vivo* discovery pipeline to predict, synthesize, screen and characterize peptide matrikines
- Novel matrikines exhibit sequence-specific modulation of cultured human dermal fibroblast proteomes.
- Two peptides applied to volunteer skin enhance transcription of genes which promote epithelial and dermal remodeling.

What is the translational message?

- The peptide combination characterized in this study induces repair of a key skin photo-ageing biomarker which is comparable to that achieved by the current gold standard treatment (all trans retinoic acid).
- Aberrant remodeling of extracellular matrix-rich tissues is a hallmark of many age-related diseases.
- The use of biomimicry approaches to predict the identity of naturally occurring ECM breakdown products has the potential to facilitate the development of safe, well-tolerated and targeted therapies in skin and other organs and tissues.

Abstract

Background: Extracellular matrices play a critical role in tissue structure and function and aberrant remodelling of these matrices is a hallmark of many age-related diseases. In skin, loss of dermal collagens and disorganisation elastic fibre components are key features of photo-ageing. Although application of some small matrix-derived peptides to aged skin has been shown to beneficially affect *in vitro* cell behaviour and, *in vivo,* molecular architecture and clinical appearance, the discovery of new peptides has lacked a guiding hypothesis.

Objectives: As endogenous matrix-derived peptides can act as cell-signalling molecules (matrikines), we hypothesised that protease cleavage site prediction could identify novel putative matrikines with beneficial activities for skin composition and structure.

Methods: Here, we present an *in silico* (peptide cleavage prediction) to *in vitro* (proteomic and transcriptomic activity testing in cultured human dermal fibroblasts) to *in vivo* (short term patch test and longer term split-face clinical study) discovery pipeline, which enables the identification and characterisation of peptides with differential activities.

Results: Using this pipeline we show that cultured fibroblasts are responsive to all applied peptides but their associated bioactivity is sequence-dependent. Based on bioactivity, toxicity and protein source we further characterised a combination of two novel peptides, GPKG and LSVD, that act *in vitro* to enhance the transcription of matrix organisation and cell proliferation genes and *in vivo,* in a short-term patch test, to promote processes associated with epithelial and dermal maintenance and remodelling. Prolonged use of a formulation containing these peptides in a split-face clinical study led to significantly improved measures of crow's feet and firmness in a mixed-ethnicity population.

Conclusions: We conclude that this approach to peptide discovery and testing can identify new synthetic matrikines, providing insights into biological mechanisms of tissue homeostasis and repair and new pathways to clinical intervention.

Introduction

Extracellular matrices (ECMs) are essential for the structure, mechanical properties, and cellular phenotype of mammalian tissues [1]. However, aberrant and progressive remodelling of ECM-rich tissues is a key feature in the pathology and ageing of many organs, including articular cartilage [2], arteries [3] and skin [4]. Whilst some ECM proteins, are maintained through a circadian cycle [5], many structural ECM components such as elastin [9], aggrecan and collagen [10] persist in tissues for decades, making them vulnerable to oxidative damage [6], pathological cross-linking [7] and protease-mediated degradation [8]. The relative susceptibility of individual ECM proteins to degradation may be determined not only by their longevity but also abundance, tissue location (for example in ultraviolet radiation [UVR]-exposed papillary dermis of photoaged skin [11]) and biochemistry (where amino acid composition mediates susceptibility to UVR and oxidation [12]). Crucially, ECM degradation may not only impair function but also release peptide fragments, known as matrikines, or reveal previously shielded active sites known as matricryptins [13] with cell signalling capabilities [14].

Early research into the bioactivity of ECM fragments focussed on the activity of elastin-derived peptides [15], but it is clear that matrikines can be liberated from multiple ECM proteins. For example, the collagen IV NC1 domain fragments canstatin [16] and arresten [17] are anti-angiogenic, tumour suppressive and able to regulate apoptosis. Furthermore, a smaller peptide fragment of canstatin (amino acids 78-86; known as Cans) also demonstrates biological activities [18]. The action of matrikines may not always be clinically beneficial, with the collagen-derived matrikine PGP promoting chronic inflammation and pulmonary fibrosis [19], although conversely collagen I (C-1158/59 [20]) and XVIII (endostatin [21]) matrikines have been shown to reduce cardiac fibrosis. These studies, and many others, demonstrate that endogenously generated ECM peptides can act as matrikines however; they also suggest that the application of exogenous ECM-derived peptides may beneficially affect tissue homeostasis.

The accessibility of human skin, which enables characterisation of ageing [22] and repair [23], its susceptibility to molecular damage [24] and its cellular and extracellular composition, which is similar to other connective tissue-rich organs, make it an excellent system in which to study the action of exogenous peptides in humans. Human skin may be subject to both intrinsic (passage of time) and extrinsic (action of exogenous factors, often ultraviolet radiation; UVR) ageing [25]. Whilst there are differences in the clinical manifestations, both processes affect the epidermis and dermis [26]. In our recent review of bioactive peptides used within skin anti-ageing cosmeceuticals [27], we identified 35 peptides whose sequences were found in at least one human protein. Many of these peptides were short (di- or tri-peptides), and were usually modified with a palmitoyl chain to aid penetration through the skin barrier [28] but comparison of their relative activity is difficult given the disparity of outcome measures and model systems employed in published studies and, in some cases, the inclusion of the peptide in a complex formulation. The beneficial effects of such treatments, however, are clear with, for example, enhanced collagen synthesis *in vitro* [29] and enhanced fibrillin-rich microfibril deposition and wrinkle reduction *in vivo* [23] being reported for the peptide palmitoyl-GHK. Despite the success of some peptides in inducing clinically discernible benefits in aged skin, including those developed based on protein active sites or the chemical modification of existing peptides [30], there has been no published conceptual framework to guide the prediction and characterisation of new bioactive therapeutic peptides.

In this study, we test the hypothesis that small bioactive peptides (matrikines) can be predicted by the *in silico* digestion of dermal proteins via action of prominent ECM proteases. In contrast

to enzymes such as trypsin, where cleavage sites can be predicted with a great deal of certainty [31], identifying putative cleavage sites of endogenous tissue ECM proteases (such as members of matrix metalloproteinases [MMPs] and cathepsins) requires the use of machine learning algorithms which can predict cleavage sites in protein sequences [32,33]. We have established a new discovery pipeline in which potential peptide matrikines were predicted and synthesised (following selection based on size, solubility and suitability for manufacturing; Fig. 1a) and screened for *in vitro* cell culture toxicity and biological activity (targeted immunohistochemical markers, and transcriptome and proteome discovery; Fig. 1b). A peptide combination was then progressed to an *in vivo* occluded patch test and a split face clinical study in human volunteers in order to characterise the skin rejuvenation potential of the peptides in formulation [34] (Figs. 1c and 1d). The identification of new matrikines has the potential not only to provide insights into tissue pathology, but also to translate to actives with applications in skin [35] and potentially other diverse tissues (such as brain [36], muscle [37], heart [38] and liver [39]) in which treatment of aberrant ECM-remodelling, for example fibrosis, is a pressing and unmet clinical need.

Materials and Methods

The research objectives of this study were to test the hypothesis that protease cleavage site prediction could identify novel putative matrikines with beneficial activities. Starting with *in silico* predictions of peptide sequences, the hypothesis was tested by synthesising candidate peptides and characterizing their effects in controlled laboratory experiments (human cell culture) and small -medium scale trials in human volunteers. Detailed methods are provided in the supplementary material including samples sizes and experimental replicates, but the key steps in the discovery pipeline are summarised in Fig.1. Beginning with bioinformatic screening of the human skin proteome, the pipeline integrated existing resources (PROSPER protease cleavage prediction server [32]) with bespoke algorithms to predict cleavage sites and hence potential peptide fragments from 27 target proteins with matrikine activity (supporting information: detailed methods for target protein selection and *in silico* peptide prediction). Eight candidate matrikines were selected on the basis of predicted cleavage from multiple source proteins, reported literature involvement in skin ageing and assessments of suitability for manufacturing. These were synthesised, modified with a palmitoyl chain and characterised for biological activity in cultured HDFs (supporting information: detailed methods for synthesis and toxicity testing and initial activity screens of eight candidate peptides by ELISA, fibrillin-I immunofluorescence and LC-MS/MS proteomics). Following initial immunochemical screening with key ECM components

as outcome markers, the biological and toxicological effects of two prime candidate peptides (individually and in combination) were assessed (supporting information: detailed methods for *in vitro* characterisation of cell transcriptomes and proteomes in response to peptides 1, 7 and 1+7 in combination toxicology assessments and proteomic analysis). The peptide combination was subsequently applied in a formulation to the skin of eight human volunteers and the impact on skin histology and transcriptome characterised (supporting information: detailed methods for *in vivo* patch test of peptide combination). Finally, 38 female volunteers aged 43-74 were recruited to a 6-month split-face single-blind clinical study to assess the ability of a peptide-containing topical skincare formulation to visibly improve three key parameters associated with photoaged skin (supporting information: detail of the clinical study).

Results

Selecting key protein targets which are promising sources of bioactive matrikines in vivo.

As matrikines are most likely to be generated from abundant and/or degradation-susceptible skin proteins we first defined an initial target cohort of 69 extracellular proteins (Fig. 2a and Table S1) drawn from the human skin proteome [40]. In contrast with a protease such as trypsin, where, prediction of cleavage sites is straightforward[31], cleavage prediction for endogenous tissue proteases (such as MMPs and cathepsins) is determined by multiple factors. Therefore, to identify the initial cohort of target proteins, we used an established machine-learning algorithm, PROSPER [32] to predict the cleavage sites of skin-active enzymes (MMPs-2, -3, -7 and -9, cathepsins –G and –K, granzyme B and elastase-2 [41-45]) for all human skin ECM proteins, and selected the 20 extracellular proteins with the highest proportion of predicted cleavage sites. As photo-oxidation can also degrade ECM components in skin [46-48], we next identified the 20 extracellular human skin proteins with the highest proportion of UVR/ROS susceptible amino acid residues [12]. Finally, the cohort was supplemented with a further 33 ECM proteins with known roles in dermal function. Each protein in this cohort of 69 was further reviewed to select proteins with high relative abundance (immune staining on Human Protein Atlas [40 49]) and susceptibility to reported age-related remodelling. The final cohort of 27 target proteins included key components of the elastic fibre, system (i.e. elastin, fibrillin-1 and fibulin-1), fibrillar collagens (I and III) and the dermal-epidermal junction basement membrane (collagen IV and laminin-332) which all undergo remodelling in photoaged skin (Fig. 2a).

In silico prediction and in vitro selection of candidate matrikines from target proteins.

Most ECM proteins are susceptible to digestion by multiple proteases and, as a consequence, have many experimentally-validated cleavage sites [50]. Therefore, we developed a bespoke algorithm which utilised PROSPER [32] cleavage probability scores (for multiple combinations of proteases) to predict fragmentation and hence the sequence of liberated peptides (Fig. 2b). Having generated a cohort of putative peptide fragments we next selected tetra-peptides for further review, as such small molecules (~500Da or less) are more likely to penetrate the skin barrier [51] and may potentially be generated from 10's-100's of proteins. In contrast, penta-peptide homologues are less widely distributed in the skin proteome (Fig. 2ci), whilst di- and tri-peptides homologues are found in 1000's of human proteins and hence may lack specific matrikine activities [27]. Collagens IV, VI and VII are potentially rich sources of liberated matrikines, with between 25-56 unique cleaved tetra-peptide sequences predicted for the alpha chains of COL4A1, COL4A3, COL6A3, COL7A1 (Fig. 2cii). The putative matrikines generated are also predicted (collectively) to be cleaved from over 100 skin proteins, thereby increasing the probability of them being liberated *in vivo*. In addition to the collagens, elastic fibre associated proteins (e.g. EMILIN1, FBN1, LTPB4), the adhesive glycoprotein FN1, and the basement membrane component LAMB3 may also be rich sources of matrikines *in vivo* (Fig. 2cii). This screening process identified 453 putative matrikine tetra-peptides.

Ubiquitous peptides may be produced with a higher frequency in damaged tissues, resulting in the promotion of ECM synthesis and tissue repair via multiple pathways. When selecting candidates for synthesis and experimental characterisation, we chose some peptides which were predicted to be cleaved from multiple skin ECM proteins by several proteases (in order to increase the chances of observing biological activity and/or broad-spectrum activity). For example, peptide P1 was predicted to be cleaved from multiple collagen alpha chains as well as ECM glycoproteins and ECM regulators. However, we also included some peptides which were predicted to be cleaved from a smaller and alternative protein cohort (for example P8 which is found predominantly in proteoglycans; Fig. 3a and Table S2). Additional review identified eight peptides (P1: GPKG; P2: GPSG; P3: LSPG; P4: EKGD; P5: QTAV; P6: LSPD; P7: LSVD and P8: ELED) with predicted high solubility, the potential to form hydrogen bonds (which can mediate receptor/ligand interactions), and minimal issues with regards to downstream bulk-scale manufacture (Table S2). These were successfully synthesised and chemically modified with a palmitoyl chain (pal-) to increase penetration into the skin.

Selection of two peptides with promising in vitro activities

Initial toxicity testing (Hoechst 33258) was conducted to determine viable peptide concentrations for *in vitro* testing (Table S3). The ability of the 8 peptides to promote cellular synthesis of selected ECM components essential to dermal integrity (procollagen I, fibronectin, decorin, collagen IV, hyaluronic acid and fibrillin-1) was then assessed *in vitro* on human dermal fibroblast cells (HDFs) via enzyme-linked immunosorbent assays (ELISA) or immunofluorescence (IF) techniques. Except for hyaluronic acid, all peptides enhanced the synthesis of at least some of the ECM markers tested (Table S4). These targeted immune-assays were followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomic analysis of HDFs exposed to each peptide. Although peptides P2, P4, P6 and P8 upregulated proteins in multiple functional classes (Fig. 3b), their activity was more limited than the other peptides and peptides P3 and P5 were present in few skin proteins. Consequently, two peptides with contrasting activities (P1 and P7) were selected for characterisation of fibrillin-rich microfibrils (an early biomarker of both skin ageing and repair [34]) deposition. Both peptides induced significant elaboration of a fibrillin-rich microfibril network compared with the DMSO control (Fig. 3ci). P1 has ubiquitous distribution within skin proteins and ability to enhance pro-collagen I synthesis (by ELISA) and a broad range of proteins, including fibrillin-rich microfibrils (IF) and basement membrane components (LC-MS/MS; Fig. 3cii). P7 was also chosen as it promoted both fibrillin-1 and decorin synthesis (by IF and ELISA respectively) and showed diverse activity in the LC-MS/MS screen.

Peptides pal-GPKG (P1) and pal-LSVD (P7) act synergistically in vitro

Primary HDFs from five age- and sex- matched donors (derived from breast skin (n=1), labia (n=2) and buttock(n=2)) were treated with P1, P7, P1+P7 in combination and TGF-β1 (which acting as a positive control profoundly affected both the cellular transcriptome and proteome; Figure S1ai). Total RNA was extracted after 12 hrs (in order to characterise the initial response to peptides) and extracellular proteins after seven days of treatment (to characterise protein deposition). RNA-Seq transcriptome analysis identified over 6,000 genes with peptide-treatment associated fold changes of ≥1.2 [52] in each of the peptide treatments. Crucially, whilst principal component analysis (PCA) demonstrated strong clustering of samples by body site (Fig. 4ai), PCAs for each individual donor (Fig. 4aii and Figures S1ai-vi) showed that in all cases, peptide treatments modulated the transcriptome compared with untreated controls. Gene Ontology (GO)

term enrichment analysis highlighted the diversity of biological processes enriched by the peptides individually (e.g. P1, cellular proliferation and lipid metabolism; P7, ECM remodelling). However, there was a profound synergistic effect, with the peptides in combination (hereby referred to as P1+P7) significantly enriching the transcription of 243 processes (compared with 84 for P1 and 52 for P7 alone). Of these, P1+P7 induced 32 processes involving ECM genes (as defined by the 27 ECM target genes listed in Table S1) compared with 6 for P1 and 11 for P7 (Fig. 4b and Figure S1). In particular, P1+P7 enriched processes concerned with ECM assembly and cell adhesion and with cellular proliferation. Proteomics analysis confirmed the transcriptomic clustering by HDF body site origin but identified more separation between untreated control cells and 7 days peptide treatments (Fig. 4c). Again, the peptide combination was most biologically active, upregulating the deposition of over 50 ECM proteins *in vitro* (as defined by Matrisome DB [53]) with a fold change >=1.2 (Fig. 4c and d). In particular, the combination enhanced synthesis of ECM regulators and proteoglycans, including serpin peptidase inhibitor, clade E, member 1, and versican core protein.

Given the clear synergy between the peptides, the influence of body site on HDF phenotype, and the profound effects of TGF-β1 on cell phenotype, we next tested the biological activity of the peptide combination against solely buttock-derived primary HDFs (n=3) using a more clinically relevant positive control (all-*trans* retinoic acid; ATRA). The combination peptide treatment once again significantly enhanced transcription of genes relevant to ECM-rich tissues (ECM organisation, collagen biosynthesis, connective tissue development and wound healing) and lipid/steroid-related processes, and epithelial processes (Fig. 4e and f and Fig. S2). After 7 days' exposure, the peptide combination induced clear clustering by the proteins induced on the PCA plots, which was distinct from both the control and those treated with ATRA (Fig. 4g). Over 20 ECM proteins in each donor dataset were found to be upregulated by the peptide combination treatment, with ECM glycoproteins and regulators being in the majority.

The peptide combination acts in vivo to promote significant epithelial and dermal remodelling

Before the peptide combination could be progressed to *in vivo* efficacy testing on human skin, the peptides (500 ppm) were solubilised into a suitable and stable excipient for use in topical formulations and subjected to a range of *in vitro* and *ex vivo* toxicology assessments following Organisation for Economic Co-operation and Development (OECD) guidelines [54]. QSAR and Xenosite analysis, SkinEthic, Epiocular, Uvs spectra, DPRA and keratinosens tests were performed on two peptides (P1 and P7). The peptide formulation (containing 50:50 ratio of P1

and P7) was deemed to be neither a skin sensitizer nor an eye irritant and could, therefore, be safely progressed to *in vivo* efficacy assessment.

Chronic UVR exposure results in epidermal thinning and significant dermal remodelling, including loss of fibrillin-rich microfibrils from the superficial papillary dermis and accumulation of dystrophic elastin (solar elastosis) in both the papillary and reticular dermis [26]. To determine the efficacy of peptides P1 and P7 in mitigating epidermal and dermal remodelling, the peptide formulation was applied to the photoaged extensor forearm skin of otherwise healthy volunteers, using a validated, occluded patch test assay [34] alongside an occluded but untreated area (occluded control), a vehicle control and ATRA (as positive control).. In the bulk RNA-Seq analysis, for all donors, the peptide formulation modulated the transcriptome compared with both the occluded and vehicle controls (Fig. 5a), demonstrating that the peptides exert a significant influence on cell physiology additional to the vehicle. Specifically, GO-term analysis of RNA-Seq data for the top 20 (by q-value) biological processes identified multiple differentially enriched processes against the vehicle control: at 10ppm these included peptide hormone responses and retinol metabolism. At 30ppm, these included expression of key skin homeostasis and repair processes, such as keratinization and cornification (Fig. 5b). Compared with the occluded control transcriptome, the peptide formulation enhanced transcription of multiple ROS- and ECM-related processes (Fig, 5c).

In addition to modulating expression of collagens, laminins and proteoglycans, the peptide formulation enriched transcription of elastic fibre components, including elastin, fibulins and microfibril associated protein 4 (MFAP4). Immunohistochemical analysis demonstrated that compared with the occluded control, the peptide formulation significantly enhanced deposition of fibrillin-rich microfibrils in the papillary dermis (Fig. 6a and b).

A peptide-containing formulation improves measures of wrinkle and firmness in a split-face clinical study

Validation measures undertaken at baseline and 2-months showed the high level of accuracy and reproducibility of the gradings conducted during the study (Table S5). Healthy but photoaged female participants (n=38, aged 43-74) completed the 6-month clinical study. 53% of these participants were white Caucasian, 47% had skin of colour (either Asian, Hispanic or Black African American) (Fig. 7 and Tables 1 and 2). For under eye wrinkles, a statistically significant difference between the product treated and the non-intervention side was reached by

1-month (P<0.002) and all time points beyond. By 6-months, 81.6% of participants showed improvement vs the non-intervention side of the face, with an average NET grade change of -0.63. For crow's feet wrinkles, a statistically significance difference between the product treated and non-intervention side was reached by 2-months (P<0.002) and all time points beyond. By 6 months, 71.1% of participants showed improvement vs the non-intervention side, with an average NET grade change of -0.55. For firmness, a statistically significant difference between the product treated and non-intervention side was reached by 1-month (P<0.002) and all time points beyond. By 6 months, 97.4% of participants showed improvement vs the non-intervention side, with an average NET grade change of -0.57.

Given the broad, inclusive recruitment criteria in terms of age range and ethnicity, further stratified analysis at the final 6-month time point showed significant improvement on the product treated vs non-intervention side in participants who were under 60 (n=18) and over 60 (n=20); and in participants with skin of colour (n=18) as well as white Caucasian participants (n=20).

Discussion

In this study we test the hypothesis that small bioactive peptides (matrikines) can be predicted by the *in silico* digestion of dermal ECM proteins by proteases. Our discovery pipeline: i) provides *in vitro* evidence for diverse, sequence-dependent, biological activities induced in cultured HDFs exposed to exogenous tetra-peptides and; ii) reveals *in vivo* that the combination of two peptides modulated key measures and visible signs of skin photo-damage; highlighting the therapeutic potential of matrikines. Although there is evidence that some ECM fragments (such as collagen XVIII-derived endostatin [21]) can reduce fibrosis, the role played by endogenous matrikines in mediating self-repair in ageing and/or diseased tissues remains unclear. The complex nature of ageing and chronic diseases, where a diverse array of proteases may act on hundreds of proteins to produce thousands of peptides at low concentrations, could explain the lack of evidence for endogenous matrikines in preventing tissue degeneration together with the challenges of identifying these small peptides in the complex skin proteome. Even with a small cohort of ECM derived peptides, our data demonstrates that each sequence (peptides P1-P8) can mediate the expression of numerous and disparate proteins and pathways. By applying relatively high concentrations of a peptide, the cells receive a strong and consistent signal, in contrast to the potential low-level signals of many endogenously derived peptides.

Predicting and selecting peptides with specific activities remains challenging. Our data indicates that some peptides (i.e. P3) may act as relatively specific alarm signals prompting cells to primarily synthesise enzyme inhibitors (Fig. 3c) but other peptides (particularly P1, P3, P5 and P7) appear to be non-specific alarm signals, inducing fibroblasts to synthesise a varied proteome (Fig.3c). The disparate nature of outcome measures reported for commercially-available peptides makes it difficult to make comparisons with the transcriptomic and proteomic measures used in this study, but our data confirms previous observations [27] that ECM-derived peptides promote ECM synthesis. It is possible that longer (and hence protein source-specific) peptide sequences may have more targeted effects but, for use in topical skin treatments, penetration through the *stratum corneum* may be challenging [27] and smaller peptides (Cans [18]) can exhibit similar activities to the parent molecule (canstatin [16]). Relative peptide activity will also be subject to inter-individual variation (Figs. 4-6). In the case of skin, even the gold-standard topical treatment ATRA varies in its effectiveness between individuals [34,55,56]. The biological effects of exogenous peptides will also be target-cell dependent and therefore single cell transcriptomic and proteomic profiling followed by expression of quantitative trait loci (eQTL) analysis on well-characterised cell lines such as HipSci fibroblast lines may provide insight towards personalised treatment approaches [57,58]. It is clear that dermal fibroblasts are responsive to the exogenous peptides, but our *in vivo* data also highlights the enrichment of key epithelial processes including skin barrier formation, keratinization and cornification [59,60] indicating that the effects of this peptide combination are not confined to the dermis. The clinical study demonstrates that a peptide containing formulation can improve objective and visible measures of skin age, including facial wrinkles and firmness in a mixed-ethnicity population.

Whilst this study has demonstrated the ability of these peptides to influence cell behaviour and, in a formulation, to induce beneficial skin remodelling, there are clear limitations. Currently the *in vivo* influence of the peptides alone (distinct from the formulation) is not defined and will require further studies with increased cohort sizes. In order to determine the mechanism of action for clinical improvements, it will be necessary to assess clinical outcomes alongside spatial measures of cellular transcriptomes. We have previously shown that skin composition (particularly dermal) and structure differs between white Caucasian and African/Caribbean populations [61]. Therefore, although the ability of the peptide formulation to induce beneficial remodelling in a multi-ethnicity study is a key observation, there remains the possibility it may be possible to tailor matrikine peptide treatments to diverse populations and skin disorders.

In summary, by applying an *in silico* to *in vivo* discovery pipeline we have identified and characterised the ability of novel peptides (GPKG and LSVD) to enhance the transcription of ECM organisation and cell proliferation genes and to promote epithelial and dermal remodelling (Fig. 6c). The use of such biomimicry approaches to predict the identity of naturally occurring ECM breakdown products which promote cell signalling could facilitate the development of safe and well-tolerated technologies and therapies. The development of improved techniques for predicting and detecting the cleavage of small peptides and for the localisation of peptide action within organs and target cells will be critical to enabling better understanding of the mechanisms of matrikine-induced tissue repair with both patient and consumer benefits. Our data underscores the importance of endogenous matrikines in mediating self-repair in ageing and/or diseased tissues.

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Figure legends

Figure 1. *In silico* to *in vivo* discovery pipeline. (a) Bioinformatic prediction of putative matrikines. The entire human proteome was filtered to identify 205 human skin ECM proteins. An initial protein cohort (key structural ECM and/or higher predicted susceptibility to proteases and UVR/ROS) was further filtered based on reported age-related remodelling to a target cohort

of 27 proteins. This cohort was subjected to in silico digestion and peptide prediction. The 453 tetra-peptides were then assessed for potential issues with manufacture yielding 8 peptides for synthesis and characterisation. (b) *In vitro* characterisation. The activity of these 8 peptides was assessed by immunological and proteomic screens and 2 peptides were selected for further characterisation. Two rounds of proteomic and transcriptomic assays established that these peptides induced differential and synergistic effects on cell physiology. Toxicological assessment established the safety of the peptide combination in a formulation for topical in vivo testing in human skin. (c) Human volunteers were treated with a positive control (ATRA), the vehicle and the peptide formulation at two concentrations (10ppm and 30ppm) for 12 days. Histological, immunohistochemical and transcriptomic analysis established that the peptide combination exerted beneficial effects on key biomarkers of skin photoageing and upregulated expression pathways. (d) The efficacy of a modified peptide-containing formulation was further tested in a 6-month split face single-blinded randomised clinical study against clinical grading for visual wrinkles and visual and tactile firmness. Experimental data relating to each step in the discovery pipeline is indicated by colour-coded regions for each figure.

Figure 2. Bioinformatic prediction and selection of candidate peptides. (a) Defining protein targets for *in silico* protease cleavage. An initial cohort of 69 proteins (unique protease and UVR/ROS-susceptible and key structural ECM components) was filtered to identify 27 abundant and/or skin ageing-susceptible target proteins classified into seven categories. (b) In silico prediction of protease cleavage sites and liberated small peptides. Each of the 27 proteins was screened using a bespoke algorithm parsing the PROSPER protease cleavage server results combining all possible cleavage site combinations with a cleavage score probability of >=0.7. In this hypothetical example a single tetra peptide (red dotted box) would be liberated. (ci) Comparable numbers of penta- (429) and tetra- (482) peptides were predicted to be cleaved from the target cohort but penta-peptide homologues (orange) were less widely distributed in skin proteins. (cii) For each protein, the number of unique cleaved tetra-peptides is plotted against the number of proteins which share predicted cleaved peptides with that protein. For example, COL7A1 harbours 49 predicted cleaved matrikines, which, collectively, are predicted to be cleaved from 198 skin proteins. In contrast, ELN contains 10 unique cleaved peptides, which are shared with 37 other proteins. In general, structural collagens, followed by some elastic fibre associated proteins and fibronectin, were predicted to be the most likely source of tetra-peptides. Collectively this screening process identified 453 unique putative matrikines.

Figure 3. Characterisation of eight potential matrikine peptides. (**a**) Potential skin protein sources of peptide 1-8. Peptides P1, P2, P3, P4, P5, P6, P7 and P8 were originally identified as putative cleavage products of collagen I, emilin-1, collagen XVII, fibronectin, collagen VI, fibulin-1 and biglycan respectively. However, these peptides also have 100% homology to sequences found in many other skin proteins. Pie charts are sized relative to the number of proteins from which each peptide is predicted to be cleaved (i.e. 22 for P1 and 2 for P5). (b) Influence of peptides on the cultured HDF proteome. Cells were incubated for seven days either without any active added (untreated control), or with each peptide (n= 3 experimental replicates) for analysis of the combined secretome and cell matrix by LC-MS/MS. (bi) All peptides exhibited some ability to influence the cell with P1, P3, P5 and P7 showing the broadest range of activity. P1 significantly enhanced protein synthesis in wide range of protein families (bi and bii volcano plot red points). Whilst P7 induced both significant up- and down-regulation of proteins (bi and bii volcano plot). The identity of proteins up- and down-regulated by P1 and P7 is reported in the supplementary data. (**c**) Induction of fibrillin-1 synthesis in cultured HDFs (at optimum concentration for each peptide) following 5 days treatment (n = 3 experimental replicates). Both P1 and P7 peptides enhanced deposition of a fibrillin-rich microfibrils network (green stained filaments in **ci**) compared with the DMSO-treated control Scale bar = 100µm. Fibrillin rich-microfibril deposition was significantly higher for P1 (adjusted p = 0.0069) and P7 (adjusted p = 0.0117) by one-way ANOVA using Dunnett correction for multiple comparisons (cii).

Figure 4. *In vitro* characterisation of the biological activities of peptides 1, 7 and the combination. (**a**-**d**) *In vitro* 'omics characterisation of HDFs derived from multiple skin sites exposed to individual peptides and peptides in combination (n= 5 biological replicates per treatment). (**ai**) Principal component analysis (PCA) of RNA-Seq data for peptide exposed HDFs. Gene expression clustered by cell donor and by cell donor site. However, for each individual donor (**aii**: example PCA for buttock 1) there was clear separation between treatments. (**b**) GO-term enrichment analysis of RNA-Seq data for the top 20 (q-value) enriched biological processes. Both P1 and P7 treatments enriched multiple yet distinct processes but the peptides in combination acted synergistically to upregulate a wider range of processes with lower q values and higher gene ratios. (**c**) Proteomic analysis: PCA of LC-MS/MS data. After 7 days exposure to treatments, whilst protein synthesis remained clustered by skin site, the influence of the peptides was more readily discernible. (**d**) Upregulated ECM proteins with a fold change >=1.2. Of the 700 proteins upregulated by the peptide combination, over 50 were ECM components. (**e**-**g**) Characterisation of buttock-derived HDFs exposed to the peptide

combination with ATRA as a positive control (n= 3 biological replicates with 3 experimental replicates per treatment). (**e**) PCAs of individual donor RNA-Seq data. Both ATRA and peptide treatments induced clear and distinct clustering compared to the control. (**f**) In the top 20 (q-value) enriched biological processes, sterol biosynthesis (yellow arrows) was enhanced in all three donors and ECM-related tissue development/repair processes in 2/3 donors. (**g**) PCAs of proteomic data. Peptide treatment induced clear clustering in all three donors, which was distinct from the control and ATRA treated cells.

Figure 5. In vivo transcriptomic characterisation of peptide treated skin. (**a**) PCA of RNA-Seq data for three individual donors: occluded control, vehicle (control), ATRA (positive control) and P1+P7 at concentrations of 10ppm and 30ppm. For all three donors ATRA treated transcriptomes segregated according to PC1. Peptide treated transcriptomes segregated from the occluded control and (except for donor 2) the vehicle, by PC2. (**b**) GO-term analysis of RNA-Seq data for the top 20 (q-value) enriched biological processes for peptides at both concentrations' vs occluded and vehicle controls. Topically applied peptides preferentially enriched immune cell proliferation (occluded control: yellow arrows) and skin remodelling (vehicle: red arrows) processes. (**c**) GO-term enrichment analysis for ECM genes of interest (COL1A1, COL1A2, COL3A1, COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6, COL6A1, COL6A2, COL6A3, COL7A1, LAMB1, LAMC2, LAMB3, LAMB2, LAMA2, LAMA3, LAMA4, LAMA5, ELN, EMILIN1, FBLN5). At both concentrations, the peptide combination enriched multiple ECM remodelling processes (green arrows). Whilst ROS- and UV-related processes were predominantly enriched by 30 ppm P1+P7.

Figure 6. Peptide-treated skin: immunohistological characterisation and impact on biological processes. (**a**) Immunofluorescence staining of fibrillin-rich microfibrils, following 12-day patch test with occluded control, vehicle, ATRA (4–day occluded patch test as a positive control) and 10ppm and 30ppm peptide blends. A (n = 8 biological replicates per treatment). (**b**) Analysis of immunofluorescence of fibrillin-rich microfibrils. Sub-epidermal microfibril coverage was measured as a percentage of area covered with microfibrils within an area 30µm immediately below the epidermis. Repeated Measures one-way ANOVA was used for statistical analysis with where significance *$p < 0.05$ **$p< 0.005$. Error bars represent mean ± SEM (n=8). (**c**) The *in vivo* patch-test transcriptome analysis demonstrates that exposure to the peptide formulation enhances biological processes key to skin health including the epidermal maturation of keratinocytes and development of a skin barrier and the expression of dermal ECM

components involved in elastic fibre deposition and modulation of tissue biomechanical properties. Scale bars = 50µm.

Figure 7. Efficacy of peptide formulation in a medium-term split face clinical study of female volunteers. (**a-c**) Average grade changes (n=38, healthy females aged 43-74) in (a) visible under eye wrinkles (b) crow's feet wrinkles and (c) visible and tactile firmness on cheek at monthly intervals for the non-intervention vs matrikine-containing product treated side of the face. On graphs, * = p<0.05, ** = p<0.01 and *** = p<0.002 (2-Sided Wilcoxon matched-pairs signed-ranks test) and error bars show standard error of the mean.

Table 1. Medium-term split face clinical study – cohort-wide effects.

Parameter	NET Change	Responder (%)
Under Eye Wrinkles	-0.63 (p<0.002)	81.
Crow's Feet Wrinkles	-0.55 (p<0.002)	71.7
Firmness	-0.57 (p<0.002)	97.4

NET grade change between non-intervention and peptide-containing product treated side of the face; and % positive NET responders at 6 months (n=38).

Table 2. Medium-term split face clinical study – stratified effects.

Param-eter	Under 60 (n=18)		Over 60 (n=20)		Caucasian (n=20)		Skin of Colour (n=18)	
	NET Change	Respond-ers (%)	NET Change	Respond-ers (%)	NET Change	Respond-ers (%)	NET Change	Respond-ers (%)
Under Eye Wrinkles	-0.64 (p<0.01)	77.8	-0.63 (p<0.002)	85	-0.53 (p<0.01)	75	-0.75 (p<0.002)	88.9
Crow's Feet Wrinkles	-0.39 (p<0.02)	61.7	-0.70 (p<0.002)	80	-0.43 (p<0.01)	70	-0.69 (p<0.002)	72.2
Firmness	-0.56 (p<0.002)	94.4	-0.58 (p<0.002)	100	-0.53 (p<0.002)	95	-0.61 (p<0.002)	100

NET grade and % positive responders between non-intervention and peptide-containing product treated side of the face stratified according to age and ethnicity.

Figure 1. Jariwala and Ozols et al.

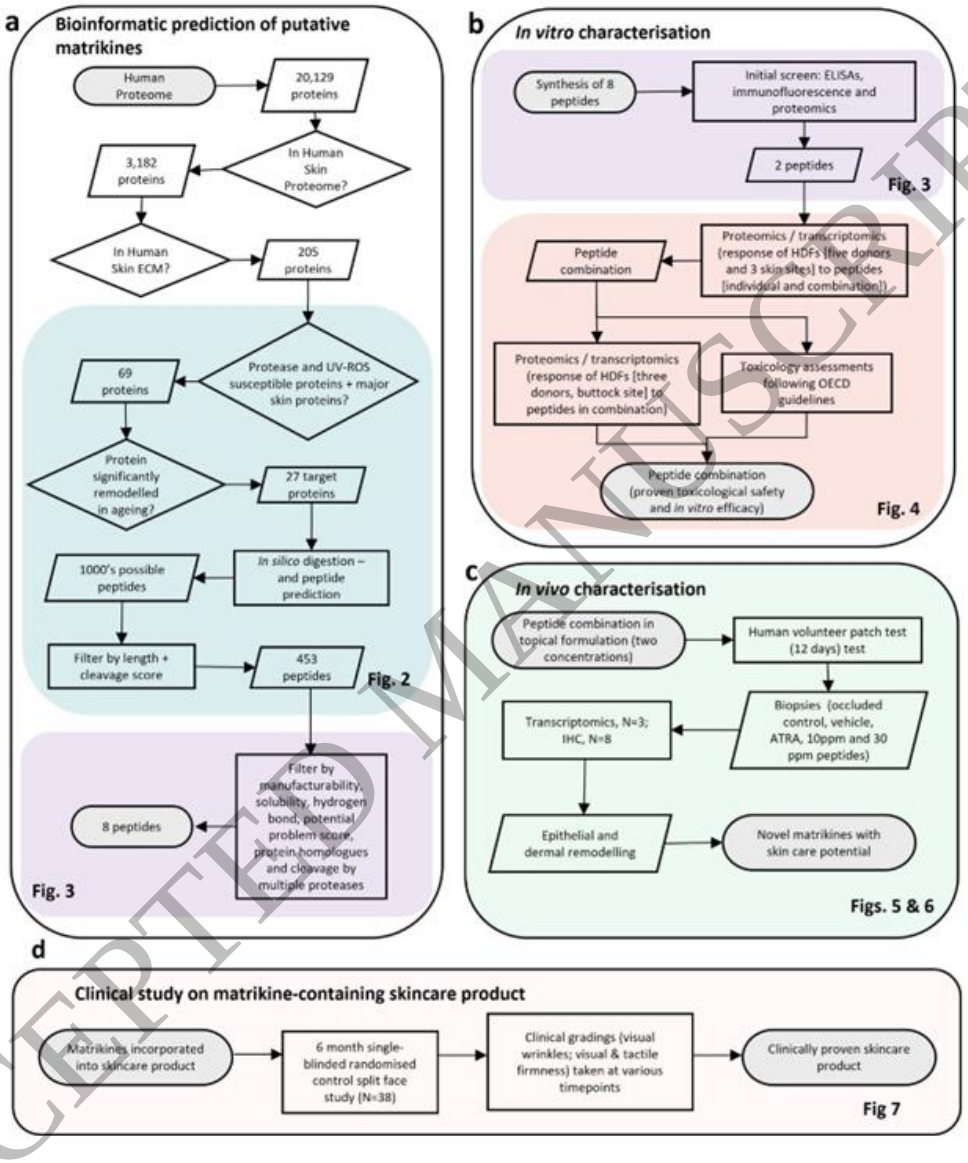

Figure 1
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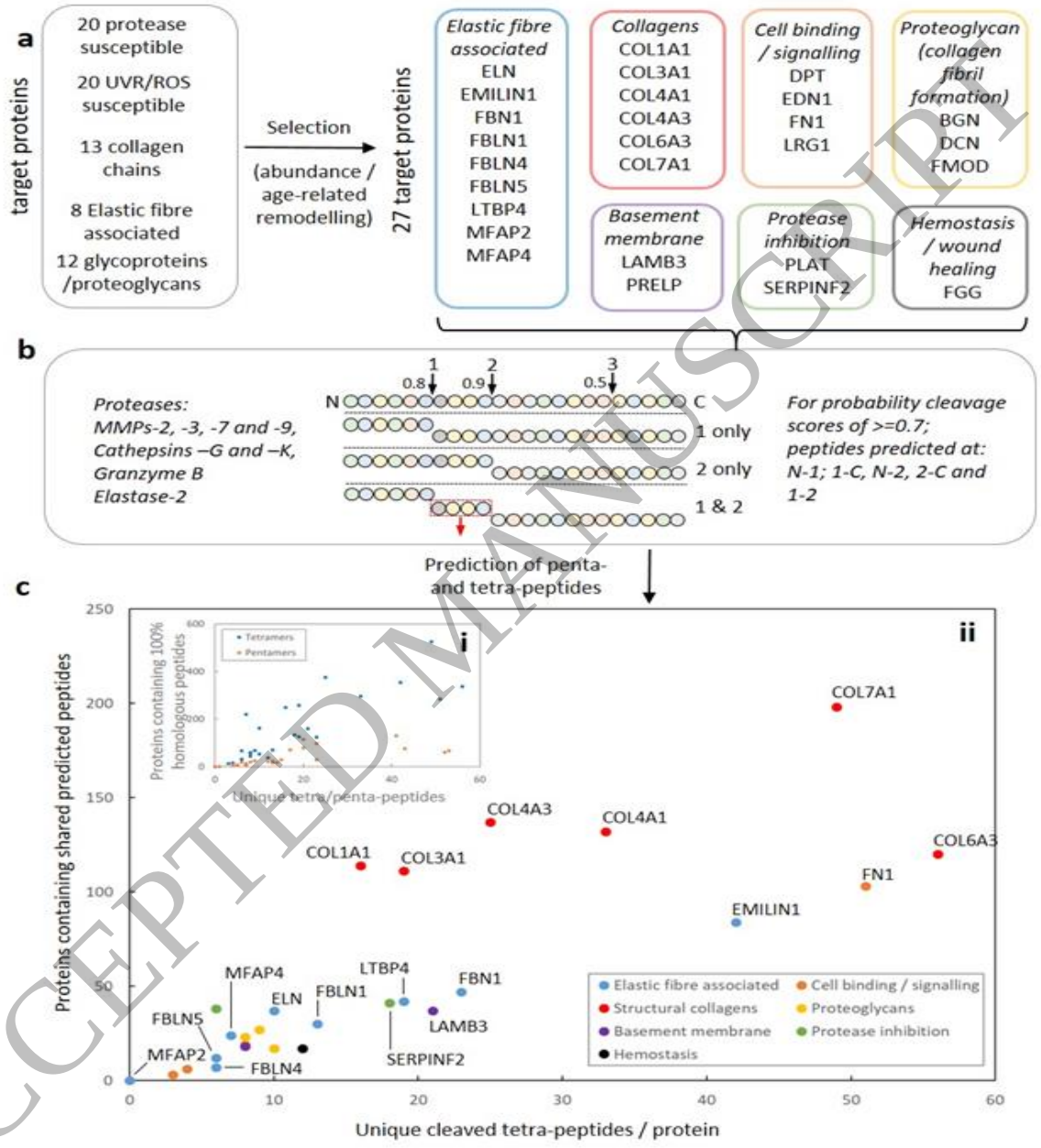
Figure 2. Jariwala and Ozols et al.



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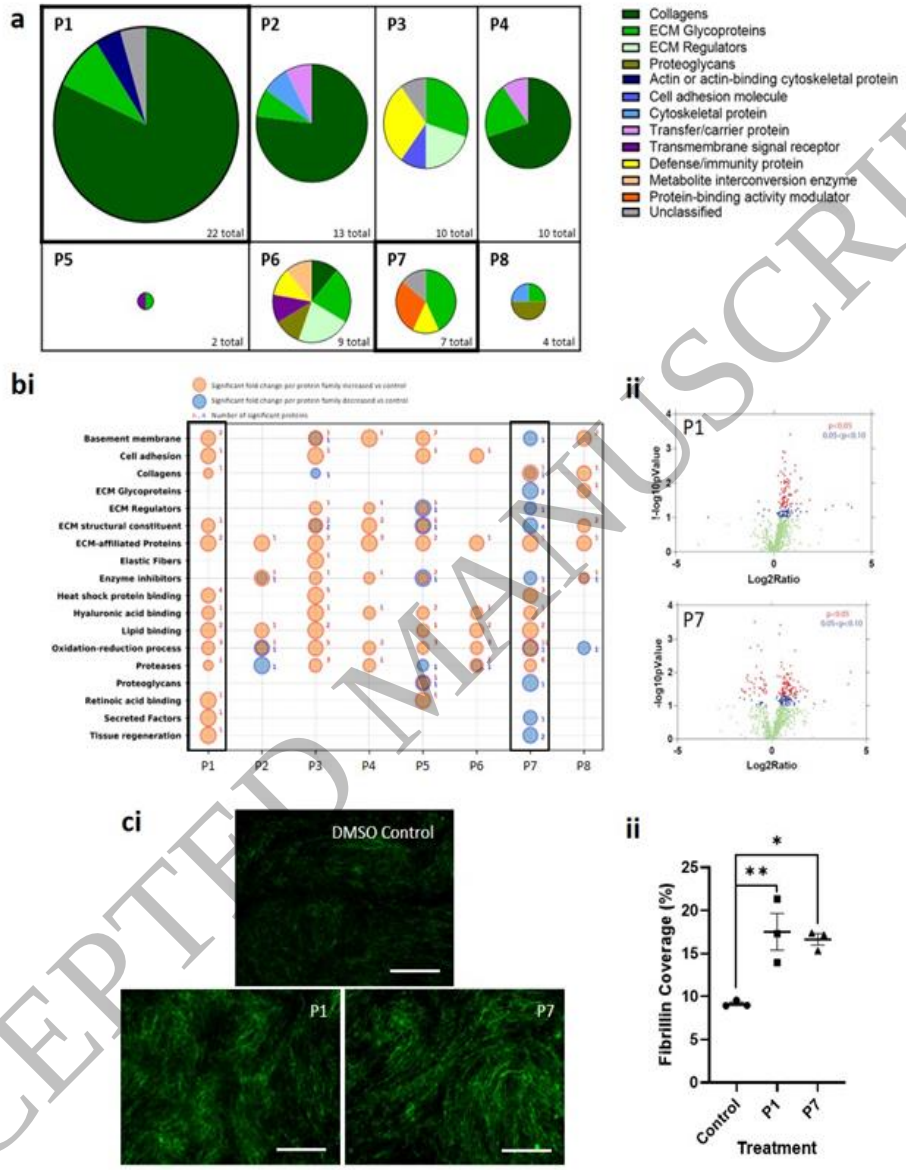

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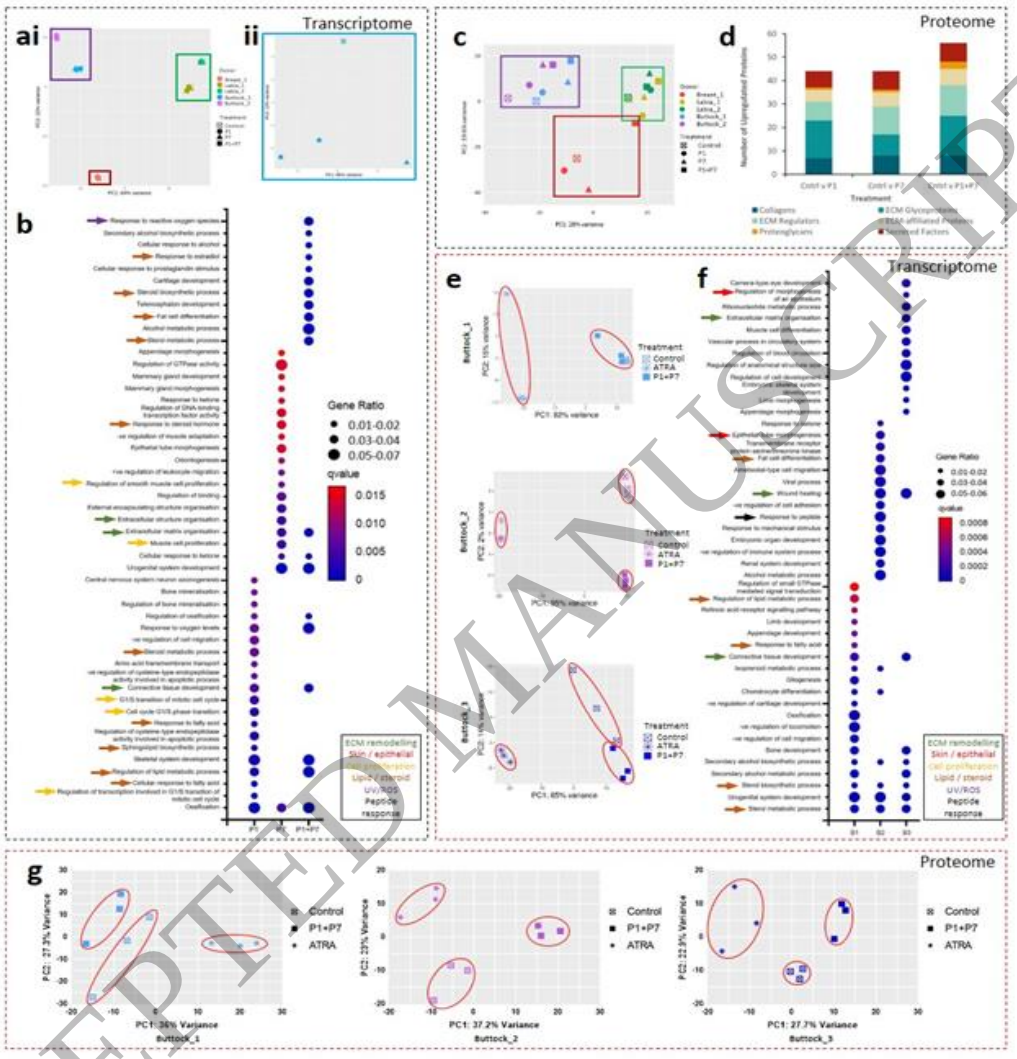

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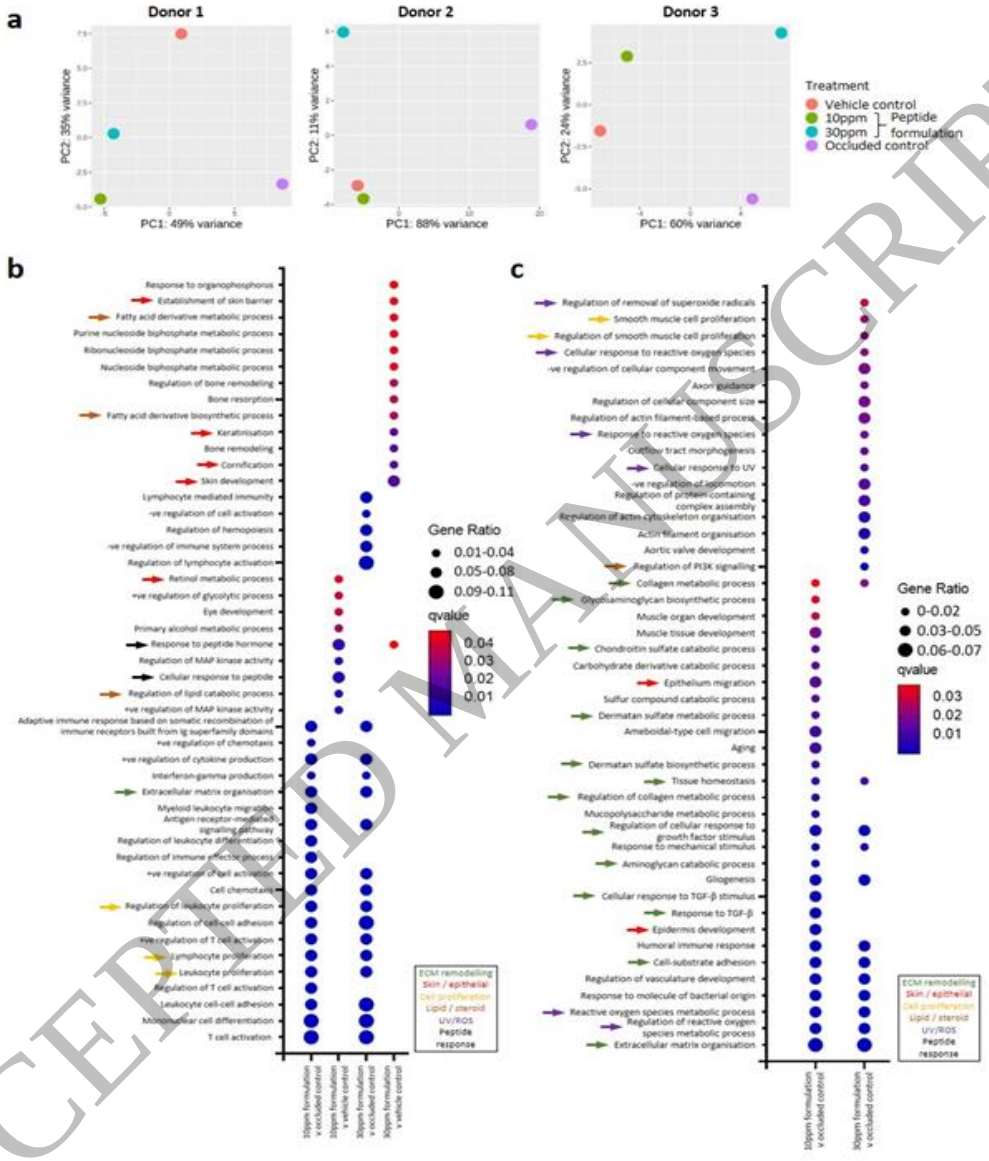

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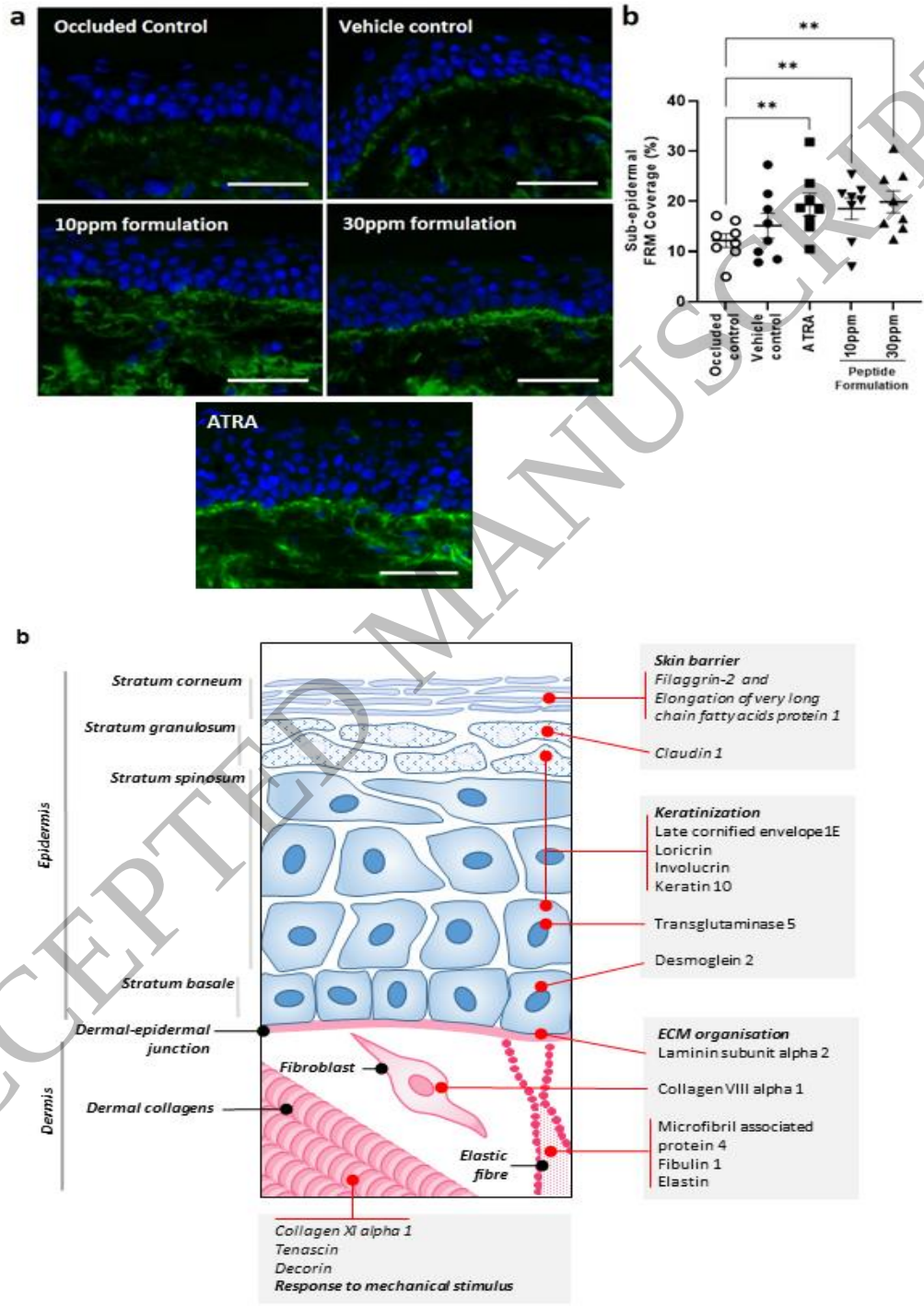

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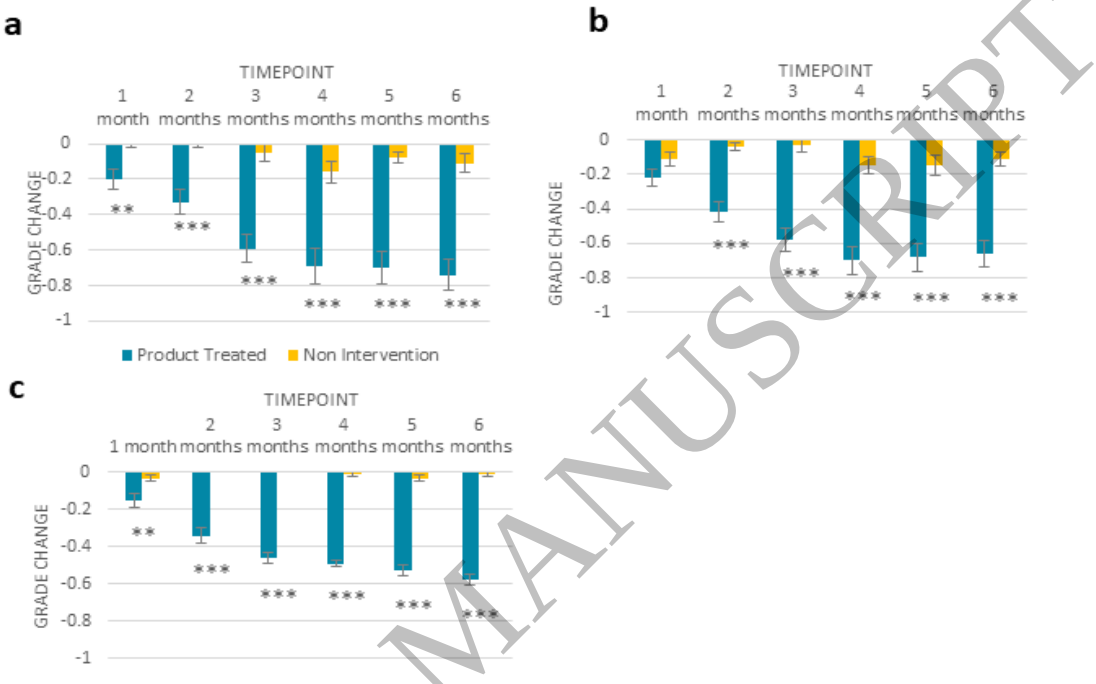

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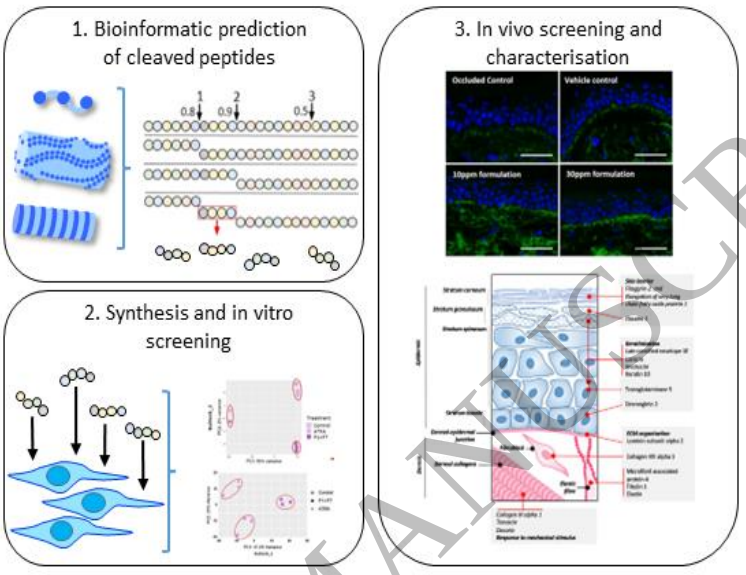

Graphical Abstract
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