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# 1 Prediction, screening and characterization of novel bioactive tetra-peptide 2 matrikines for skin rejuvenation

3 **Running head:** Novel peptide matrikines for skin rejuvenation

4  
5 Nathan Jariwala,<sup>1\*</sup> Matiss Ozols,<sup>1,2,3\*</sup> Alexander Eckersley,<sup>1,4</sup> Bezaleel Mambwe,<sup>4</sup> Rachel E.B.  
6 Watson,<sup>4,5</sup> Leo Zeef,<sup>6</sup> Andrew Gilmore,<sup>7</sup> Laurent Debelle,<sup>1,8</sup> Mike Bell,<sup>9</sup> Eleanor J. Bradley,<sup>9</sup>  
7 Yegor Doush,<sup>9</sup> Amy Keenan,<sup>9</sup> Carole Courage,<sup>9</sup> Richard Leroux,<sup>10</sup> Olivier Peschard,<sup>10</sup> Philippe  
8 Mondon,<sup>10</sup> Caroline Ringenbach,<sup>10</sup> Laure Bernard,<sup>10</sup> Aurelien Pitois<sup>10</sup> and Michael J. Sherratt<sup>1</sup>

9  
10 <sup>1</sup>Division of Cell Matrix Biology & Regenerative Medicine, School of Biological Science, Faculty  
11 of Biology, Medicine and Health, The University of Manchester, Manchester, UK

12 <sup>2</sup>Department of Human Genetics, Wellcome Sanger Institute, Genome Campus, Hinxton, UK

13 <sup>3</sup>British Heart Foundation Centre of Research Excellence, University of Cambridge, Cambridge,  
14 UK

15 <sup>4</sup>Division of Musculoskeletal & Dermatological Sciences, Faculty of Biology, Medicine and  
16 Health, The University of Manchester, Manchester, UK

17 <sup>5</sup>A\*STAR Skin Research Laboratory (A\*SRL), Agency for Science, Technology and Research  
18 (A\*STAR) and National Skin Centre, Skin Research Institute of Singapore, Republic of  
19 Singapore

20 <sup>6</sup>Bioinformatics Core Facility, Faculty of Biology, Medicine and Health, The University of  
21 Manchester, Manchester, UK

22 <sup>7</sup>Wellcome Centre for Cell Matrix Research, Division of Cancer Sciences, Faculty of Biology,  
23 Medicine and Health, University of Manchester, Manchester, UK

24 <sup>8</sup>UMR CNRS 7369 MEDyC, Université de Reims Champagne Ardenne, UFR Sciences Exactes  
25 et Naturelles, SFR CAP Santé, Moulin de la Housse, 51687 Reims cedex 2, France

26 <sup>9</sup>No7 Beauty Company, Walgreens Boots Alliance, Nottingham, UK

27 <sup>10</sup>Sederma; 29 rue du Chemin Vert, Le Perray en Yvelines, 78612, France

28 \*These authors contributed equally

29  
30 **Corresponding Author:** Michael Sherratt

1 **Email:** michael.j.sherratt@manchester.ac.uk

2  
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12 **Conflict of interests:** MB, EJB, YD and CC are employees of the No7 Beauty Company,  
13 Walgreens Boots Alliance, OP, PM, CR, LB and AP are employees of Sederma and are bound  
14 by confidentiality agreements that prevent them from disclosing their competing interests in this  
15 work. LD, AG and LZ declare no competing interests.

16 **Data availability:** Proteomics data are available via ProteomeXchange.

17 The three transcriptomics data sets (E-MTAB-12711, E-MTAB-12710 and E-MTAB-12704) can  
18 be found at ArrayExpress.

19 (<https://www.ebi.ac.uk/biostudies/arrayexpress><https://www.ebi.ac.uk/biostudies/arrayexpress>).

20 The peptide prediction code can be found at

21 [https://github.com/maxozo/Matrikine\\_Discovery](https://github.com/maxozo/Matrikine_Discovery)[https://github.com/maxozo/Matrikine\\_Discovery](https://github.com/maxozo/Matrikine_Discovery).

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

26

### 27 **What is already known about this topic?**

- 28
- 29 • Chronic skin photo-ageing induces profound remodeling of the dermal extracellular matrix
  - 30 • Small peptide fragments of extracellular matrix proteins (matrikines) can induce beneficial remodeling of the dermal matrix.
  - 31 • The discovery of new bioactive peptide matrikines has not been guided by any hypothesis.
- 32  
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## 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.

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

5  
6 Results: Using this pipeline we show that cultured fibroblasts are responsive to all applied  
7 peptides but their associated bioactivity is sequence-dependent. Based on bioactivity, toxicity  
8 and protein source we further characterised a combination of two novel peptides, GPKG and  
9 LSVD, that act *in vitro* to enhance the transcription of matrix organisation and cell proliferation  
10 genes and *in vivo*, in a short-term patch test, to promote processes associated with epithelial  
11 and dermal maintenance and remodelling. Prolonged use of a formulation containing these  
12 peptides in a split-face clinical study led to significantly improved measures of crow's feet and  
13 firmness in a mixed-ethnicity population.

14  
15 Conclusions: We conclude that this approach to peptide discovery and testing can identify new  
16 synthetic matrikines, providing insights into biological mechanisms of tissue homeostasis and  
17 repair and new pathways to clinical intervention.

## 18 19 Introduction

20  
21 Extracellular matrices (ECMs) are essential for the structure, mechanical properties, and cellular  
22 phenotype of mammalian tissues <sup>1</sup>. However, aberrant and progressive remodelling of ECM-rich  
23 tissues is a key feature in the pathology and ageing of many organs, including articular cartilage  
24 <sup>2</sup>, arteries <sup>3</sup> and skin <sup>4</sup>. Whilst some ECM proteins, are maintained through a circadian cycle <sup>5</sup>,  
25 many structural ECM components such as elastin <sup>9</sup>, aggrecan and collagen <sup>10</sup> persist in tissues  
26 for decades, making them vulnerable to oxidative damage <sup>6</sup>, pathological cross-linking <sup>7</sup> and  
27 protease-mediated degradation <sup>8</sup>. The relative susceptibility of individual ECM proteins to  
28 degradation may be determined not only by their longevity but also abundance, tissue location  
29 (for example in ultraviolet radiation [UVR]-exposed papillary dermis of photoaged skin <sup>11</sup>) and  
30 biochemistry (where amino acid composition mediates susceptibility to UVR and oxidation <sup>12</sup>).  
31 Crucially, ECM degradation may not only impair function but also release peptide fragments,  
32 known as matrikines, or reveal previously shielded active sites known as matricryptins <sup>13</sup> with  
33 cell signalling capabilities <sup>14</sup>.

34

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

12  
13 The accessibility of human skin, which enables characterisation of ageing <sup>22</sup> and repair <sup>23</sup>, its  
14 susceptibility to molecular damage <sup>24</sup> and its cellular and extracellular composition, which is  
15 similar to other connective tissue-rich organs, make it an excellent system in which to study the  
16 action of exogenous peptides in humans. Human skin may be subject to both intrinsic (passage  
17 of time) and extrinsic (action of exogenous factors, often ultraviolet radiation; UVR) ageing <sup>25</sup>.  
18 Whilst there are differences in the clinical manifestations, both processes affect the epidermis  
19 and dermis <sup>26</sup>. In our recent review of bioactive peptides used within skin anti-ageing  
20 cosmeceuticals <sup>27</sup>, we identified 35 peptides whose sequences were found in at least one  
21 human protein. Many of these peptides were short (di- or tri-peptides), and were usually  
22 modified with a palmitoyl chain to aid penetration through the skin barrier <sup>28</sup> but comparison of  
23 their relative activity is difficult given the disparity of outcome measures and model systems  
24 employed in published studies and, in some cases, the inclusion of the peptide in a complex  
25 formulation. The beneficial effects of such treatments, however, are clear with, for example,  
26 enhanced collagen synthesis *in vitro* <sup>29</sup> and enhanced fibrillin-rich microfibril deposition and  
27 wrinkle reduction *in vivo* <sup>23</sup> being reported for the peptide palmitoyl-GHK. Despite the success of  
28 some peptides in inducing clinically discernible benefits in aged skin, including those developed  
29 based on protein active sites or the chemical modification of existing peptides <sup>30</sup>, there has been  
30 no published conceptual framework to guide the prediction and characterisation of new  
31 bioactive therapeutic peptides.

32  
33 In this study, we test the hypothesis that small bioactive peptides (matrikines) can be predicted  
34 by the *in silico* digestion of dermal proteins via action of prominent ECM proteases. In contrast

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

15

## 16 **Materials and Methods**

17

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

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

11

12

## 13 Results

14

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

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

34



1  
2 *In silico prediction and in vitro selection of candidate matrikines from target proteins.*  
3 Most ECM proteins are susceptible to digestion by multiple proteases and, as a consequence,  
4 have many experimentally-validated cleavage sites<sup>50</sup>. Therefore, we developed a bespoke  
5 algorithm which utilised PROSPER<sup>32</sup> cleavage probability scores (for multiple combinations of  
6 proteases) to predict fragmentation and hence the sequence of liberated peptides (Fig. 2b).  
7 Having generated a cohort of putative peptide fragments we next selected tetra-peptides for  
8 further review, as such small molecules (~500Da or less) are more likely to penetrate the skin  
9 barrier<sup>51</sup> and may potentially be generated from 10's-100's of proteins. In contrast, penta-  
10 peptide homologues are less widely distributed in the skin proteome (Fig. 2ci), whilst di- and tri-  
11 peptides homologues are found in 1000's of human proteins and hence may lack specific  
12 matrikine activities<sup>27</sup>. Collagens IV, VI and VII are potentially rich sources of liberated  
13 matrikines, with between 25-56 unique cleaved tetra-peptide sequences predicted for the alpha  
14 chains of COL4A1, COL4A3, COL6A3, COL7A1 (Fig. 2cii). The putative matrikines generated  
15 are also predicted (collectively) to be cleaved from over 100 skin proteins, thereby increasing  
16 the probability of them being liberated *in vivo*. In addition to the collagens, elastic fibre  
17 associated proteins (e.g. EMILIN1, FBN1, LTPB4), the adhesive glycoprotein FN1, and the  
18 basement membrane component LAMB3 may also be rich sources of matrikines *in vivo* (Fig.  
19 2cii). This screening process identified 453 putative matrikine tetra-peptides.

20  
21 Ubiquitous peptides may be produced with a higher frequency in damaged tissues, resulting in  
22 the promotion of ECM synthesis and tissue repair via multiple pathways. When selecting  
23 candidates for synthesis and experimental characterisation, we chose some peptides which  
24 were predicted to be cleaved from multiple skin ECM proteins by several proteases (in order to  
25 increase the chances of observing biological activity and/or broad-spectrum activity). For  
26 example, peptide P1 was predicted to be cleaved from multiple collagen alpha chains as well as  
27 ECM glycoproteins and ECM regulators. However, we also included some peptides which were  
28 predicted to be cleaved from a smaller and alternative protein cohort (for example P8 which is  
29 found predominantly in proteoglycans; Fig. 3a and Table S2). Additional review identified eight  
30 peptides (P1: GPKG; P2: GPSG; P3: LSPG; P4: EKGD; P5: QTAV; P6: LSPD; P7: LSVD and  
31 P8: ELED) with predicted high solubility, the potential to form hydrogen bonds (which can  
32 mediate receptor/ligand interactions), and minimal issues with regards to downstream bulk-  
33 scale manufacture (Table S2). These were successfully synthesised and chemically modified  
34 with a palmitoyl chain (pal-) to increase penetration into the skin.

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

22  
23

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

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

1 term enrichment analysis highlighted the diversity of biological processes enriched by the  
2 peptides individually (e.g. P1, cellular proliferation and lipid metabolism; P7, ECM remodelling).  
3 However, there was a profound synergistic effect, with the peptides in combination (hereby  
4 referred to as P1+P7) significantly enriching the transcription of 243 processes (compared with  
5 84 for P1 and 52 for P7 alone). Of these, P1+P7 induced 32 processes involving ECM genes  
6 (as defined by the 27 ECM target genes listed in Table S1) compared with 6 for P1 and 11 for  
7 P7 (Fig. 4b and Figure S1). In particular, P1+P7 enriched processes concerned with ECM  
8 assembly and cell adhesion and with cellular proliferation. Proteomics analysis confirmed the  
9 transcriptomic clustering by HDF body site origin but identified more separation between  
10 untreated control cells and 7 days peptide treatments (Fig. 4c). Again, the peptide combination  
11 was most biologically active, upregulating the deposition of over 50 ECM proteins *in vitro* (as  
12 defined by Matrisome DB<sup>53</sup>) with a fold change  $\geq 1.2$  (Fig. 4c and d). In particular, the  
13 combination enhanced synthesis of ECM regulators and proteoglycans, including serpin  
14 peptidase inhibitor, clade E, member 1, and versican core protein.

15 Given the clear synergy between the peptides, the influence of body site on HDF phenotype,  
16 and the profound effects of TGF- $\beta$ 1 on cell phenotype, we next tested the biological activity of  
17 the peptide combination against solely buttock-derived primary HDFs (n=3) using a more  
18 clinically relevant positive control (all-*trans* retinoic acid; ATRA). The combination peptide  
19 treatment once again significantly enhanced transcription of genes relevant to ECM-rich tissues  
20 (ECM organisation, collagen biosynthesis, connective tissue development and wound healing)  
21 and lipid/steroid-related processes, and epithelial processes (Fig. 4e and f and Fig. S2). After 7  
22 days' exposure, the peptide combination induced clear clustering by the proteins induced on the  
23 PCA plots, which was distinct from both the control and those treated with ATRA (Fig. 4g). Over  
24 20 ECM proteins in each donor dataset were found to be upregulated by the peptide  
25 combination treatment, with ECM glycoproteins and regulators being in the majority.

26  
27 *The peptide combination acts in vivo to promote significant epithelial and dermal remodelling*

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

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

3  
4 Chronic UVR exposure results in epidermal thinning and significant dermal remodelling,  
5 including loss of fibrillin-rich microfibrils from the superficial papillary dermis and accumulation of  
6 dystrophic elastin (solar elastosis) in both the papillary and reticular dermis <sup>26</sup>. To determine the  
7 efficacy of peptides P1 and P7 in mitigating epidermal and dermal remodelling, the peptide  
8 formulation was applied to the photoaged extensor forearm skin of otherwise healthy volunteers,  
9 using a validated, occluded patch test assay <sup>34</sup> alongside an occluded but untreated area  
10 (occluded control), a vehicle control and ATRA (as positive control).. In the bulk RNA-Seq  
11 analysis, for all donors, the peptide formulation modulated the transcriptome compared with  
12 both the occluded and vehicle controls (Fig. 5a), demonstrating that the peptides exert a  
13 significant influence on cell physiology additional to the vehicle. Specifically, GO-term analysis  
14 of RNA-Seq data for the top 20 (by q-value) biological processes identified multiple differentially  
15 enriched processes against the vehicle control: at 10ppm these included peptide hormone  
16 responses and retinol metabolism. At 30ppm, these included expression of key skin  
17 homeostasis and repair processes, such as keratinization and cornification (Fig. 5b). Compared  
18 with the occluded control transcriptome, the peptide formulation enhanced transcription of  
19 multiple ROS- and ECM-related processes (Fig, 5c).

20  
21 In addition to modulating expression of collagens, laminins and proteoglycans, the peptide  
22 formulation enriched transcription of elastic fibre components, including elastin, fibulins and  
23 microfibril associated protein 4 (MFAP4). Immunohistochemical analysis demonstrated that  
24 compared with the occluded control, the peptide formulation significantly enhanced deposition of  
25 fibrillin-rich microfibrils in the papillary dermis (Fig. 6a and b).

26  
27 *A peptide-containing formulation improves measures of wrinkle and firmness in a split-face*  
28 *clinical study*

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

1 1-month ( $P < 0.002$ ) and all time points beyond. By 6-months, 81.6% of participants showed  
2 improvement vs the non-intervention side of the face, with an average NET grade change of -  
3 0.63. For crow's feet wrinkles, a statistically significance difference between the product treated  
4 and non-intervention side was reached by 2-months ( $P < 0.002$ ) and all time points beyond. By 6  
5 months, 71.1% of participants showed improvement vs the non-intervention side, with an  
6 average NET grade change of -0.55. For firmness, a statistically significant difference between  
7 the product treated and non-intervention side was reached by 1-month ( $P < 0.002$ ) and all time  
8 points beyond. By 6 months, 97.4% of participants showed improvement vs the non-intervention  
9 side, with an average NET grade change of -0.57.

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

## 15 Discussion

16  
17 In this study we test the hypothesis that small bioactive peptides (matrikines) can be predicted  
18 by the *in silico* digestion of dermal ECM proteins by proteases. Our discovery pipeline: i)  
19 provides *in vitro* evidence for diverse, sequence-dependent, biological activities induced in  
20 cultured HDFs exposed to exogenous tetra-peptides and; ii) reveals *in vivo* that the combination  
21 of two peptides modulated key measures and visible signs of skin photo-damage; highlighting  
22 the therapeutic potential of matrikines. Although there is evidence that some ECM fragments  
23 (such as collagen XVIII-derived endostatin<sup>21</sup>) can reduce fibrosis, the role played by  
24 endogenous matrikines in mediating self-repair in ageing and/or diseased tissues remains  
25 unclear. The complex nature of ageing and chronic diseases, where a diverse array of  
26 proteases may act on hundreds of proteins to produce thousands of peptides at low  
27 concentrations, could explain the lack of evidence for endogenous matrikines in preventing  
28 tissue degeneration together with the challenges of identifying these small peptides in the  
29 complex skin proteome. Even with a small cohort of ECM derived peptides, our data  
30 demonstrates that each sequence (peptides P1-P8) can mediate the expression of numerous  
31 and disparate proteins and pathways. By applying relatively high concentrations of a peptide,  
32 the cells receive a strong and consistent signal, in contrast to the potential low-level signals of  
33 many endogenously derived peptides.

34

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

23  
24 Whilst this study has demonstrated the ability of these peptides to influence cell behaviour and,  
25 in a formulation, to induce beneficial skin remodelling, there are clear limitations. Currently the *in*  
26 *vivo* influence of the peptides alone (distinct from the formulation) is not defined and will require  
27 further studies with increased cohort sizes. In order to determine the mechanism of action for  
28 clinical improvements, it will be necessary to assess clinical outcomes alongside spatial  
29 measures of cellular transcriptomes. We have previously shown that skin composition  
30 (particularly dermal) and structure differs between white Caucasian and African/Caribbean  
31 populations<sup>61</sup>. Therefore, although the ability of the peptide formulation to induce beneficial  
32 remodelling in a multi-ethnicity study is a key observation, there remains the possibility it may be  
33 possible to tailor matrikine peptide treatments to diverse populations and skin disorders.

34

1 In summary, by applying an *in silico* to *in vivo* discovery pipeline we have identified and  
2 characterised the ability of novel peptides (GPKG and LSVD) to enhance the transcription of  
3 ECM organisation and cell proliferation genes and to promote epithelial and dermal remodelling  
4 (Fig. 6c). The use of such biomimicry approaches to predict the identity of naturally occurring  
5 ECM breakdown products which promote cell signalling could facilitate the development of safe  
6 and well-tolerated technologies and therapies. The development of improved techniques for  
7 predicting and detecting the cleavage of small peptides and for the localisation of peptide action  
8 within organs and target cells will be critical to enabling better understanding of the mechanisms  
9 of matrikine-induced tissue repair with both patient and consumer benefits. Our data  
10 underscores the importance of endogenous matrikines in mediating self-repair in ageing and/or  
11 diseased tissues.  
12  
13  
14

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## 27 28 29 **Figure legends**

30  
31 **Figure 1. *In silico* to *in vivo* discovery pipeline.** (a) Bioinformatic prediction of putative  
32 matrikines. The entire human proteome was filtered to identify 205 human skin ECM proteins.  
33 An initial protein cohort (key structural ECM and/or higher predicted susceptibility to proteases  
34 and UVR/ROS) was further filtered based on reported age-related remodelling to a target cohort

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

16  
17 **Figure 2. Bioinformatic prediction and selection of candidate peptides.** (a) Defining protein  
18 targets for *in silico* protease cleavage. An initial cohort of 69 proteins (unique protease and  
19 UVR/ROS-susceptible and key structural ECM components) was filtered to identify 27 abundant  
20 and/or skin ageing-susceptible target proteins classified into seven categories. (b) *In silico*  
21 prediction of protease cleavage sites and liberated small peptides. Each of the 27 proteins was  
22 screened using a bespoke algorithm parsing the PROSPER protease cleavage server results  
23 combining all possible cleavage site combinations with a cleavage score probability of  $\geq 0.7$ . In  
24 this hypothetical example a single tetra peptide (red dotted box) would be liberated. (c)  
25 Comparable numbers of penta- (429) and tetra- (482) peptides were predicted to be cleaved  
26 from the target cohort but penta-peptide homologues (orange) were less widely distributed in  
27 skin proteins. (cii) For each protein, the number of unique cleaved tetra-peptides is plotted  
28 against the number of proteins which share predicted cleaved peptides with that protein. For  
29 example, COL7A1 harbours 49 predicted cleaved matrikines, which, collectively, are predicted  
30 to be cleaved from 198 skin proteins. In contrast, ELN contains 10 unique cleaved peptides,  
31 which are shared with 37 other proteins. In general, structural collagens, followed by some  
32 elastic fibre associated proteins and fibronectin, were predicted to be the most likely source of  
33 tetra-peptides. Collectively this screening process identified 453 unique putative matrikines.

34

1 **Figure 3. Characterisation of eight potential matrikine peptides.** (a) Potential skin protein  
2 sources of peptide 1-8. Peptides P1, P2, P3, P4, P5, P6, P7 and P8 were originally identified as  
3 putative cleavage products of collagen I, emilin-1, collagen XVII, fibronectin, collagen VI, fibulin-  
4 1 and biglycan respectively. However, these peptides also have 100% homology to sequences  
5 found in many other skin proteins. Pie charts are sized relative to the number of proteins from  
6 which each peptide is predicted to be cleaved (i.e. 22 for P1 and 2 for P5). (b) Influence of  
7 peptides on the cultured HDF proteome. Cells were incubated for seven days either without any  
8 active added (untreated control), or with each peptide (n= 3 experimental replicates) for analysis  
9 of the combined secretome and cell matrix by LC-MS/MS. (bi) All peptides exhibited some  
10 ability to influence the cell with P1, P3, P5 and P7 showing the broadest range of activity. P1  
11 significantly enhanced protein synthesis in wide range of protein families (bi and bii volcano plot  
12 red points). Whilst P7 induced both significant up- and down-regulation of proteins (bi and bii  
13 volcano plot). The identity of proteins up- and down-regulated by P1 and P7 is reported in the  
14 supplementary data. (c) Induction of fibrillin-1 synthesis in cultured HDFs (at optimum  
15 concentration for each peptide) following 5 days treatment (n = 3 experimental replicates). Both  
16 P1 and P7 peptides enhanced deposition of a fibrillin-rich microfibrils network (green stained  
17 filaments in **ci**) compared with the DMSO-treated control Scale bar = 100µm. Fibrillin rich-  
18 microfibril deposition was significantly higher for P1 (adjusted p = 0.0069) and P7 (adjusted p =  
19 0.0117) by one-way ANOVA using Dunnett correction for multiple comparisons (cii).

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

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

8  
 9 **Figure 5. In vivo transcriptomic characterisation of peptide treated skin.** (a) PCA of RNA-  
 10 Seq data for three individual donors: occluded control, vehicle (control), ATRA (positive control)  
 11 and P1+P7 at concentrations of 10ppm and 30ppm. For all three donors ATRA treated  
 12 transcriptomes segregated according to PC1. Peptide treated transcriptomes segregated from  
 13 the occluded control and (except for donor 2) the vehicle, by PC2. (b) GO-term analysis of RNA-  
 14 Seq data for the top 20 (q-value) enriched biological processes for peptides at both  
 15 concentrations' vs occluded and vehicle controls. Typically applied peptides preferentially  
 16 enriched immune cell proliferation (occluded control: yellow arrows) and skin remodelling  
 17 (vehicle: red arrows) processes. (c) GO-term enrichment analysis for ECM genes of interest  
 18 (COL1A1, COL1A2, COL3A1, COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6,  
 19 COL6A1, COL6A2, COL6A3, COL7A1, LAMB1, LAMC2, LAMB3, LAMB2, LAMA2, LAMA3,  
 20 LAMA4, LAMA5, ELN, EMILIN1, FBLN5). At both concentrations, the peptide combination  
 21 enriched multiple ECM remodelling processes (green arrows). Whilst ROS- and UV-related  
 22 processes were predominantly enriched by 30 ppm P1+P7.

23  
 24 **Figure 6. Peptide-treated skin: immunohistological characterisation and impact on**  
 25 **biological processes.** (a) Immunofluorescence staining of fibrillin-rich microfibrils, following 12-  
 26 day patch test with occluded control, vehicle, ATRA (4-day occluded patch test as a positive  
 27 control) and 10ppm and 30ppm peptide blends. A (n = 8 biological replicates per treatment). (b)  
 28 Analysis of immunofluorescence of fibrillin-rich microfibrils. Sub-epidermal microfibril coverage  
 29 was measured as a percentage of area covered with microfibrils within an area 30µm  
 30 immediately below the epidermis. Repeated Measures one-way ANOVA was used for statistical  
 31 analysis with where significance \*p < 0.05 \*\*p< 0.005. Error bars represent mean ± SEM (n=8).  
 32 (c) The *in vivo* patch-test transcriptome analysis demonstrates that exposure to the peptide  
 33 formulation enhances biological processes key to skin health including the epidermal maturation  
 34 of keratinocytes and development of a skin barrier and the expression of dermal ECM

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

3  
4 **Figure 7. Efficacy of peptide formulation in a medium-term split face clinical study of**  
5 **female volunteers. (a-c)** Average grade changes (n=38, healthy females aged 43-74) in (a)  
6 visible under eye wrinkles (b) crow's feet wrinkles and (c) visible and tactile firmness on cheek  
7 at monthly intervals for the non-intervention vs matrikine-containing product treated side of the  
8 face. On graphs, \* = p<0.05, \*\* = p<0.01 and \*\*\* = p<0.002 (2-Sided Wilcoxon matched-pairs  
9 signed-ranks test) and error bars show standard error of the mean.

10  
11 **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

13  
14 NET grade change between non-intervention and peptide-containing product treated side of the  
15 face; and % positive NET responders at 6 months (n=38).

16  
17 **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 (%)
<b>Under Eye Wrinkles</b>	-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
<b>Crow's Feet Wrinkles</b>	-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
<b>Firmness</b>	-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

18  
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20 NET grade and % positive responders between non-intervention and peptide-containing product  
21 treated side of the face stratified according to age and ethnicity.

Figure 1. Jariwala and Ozols et al.

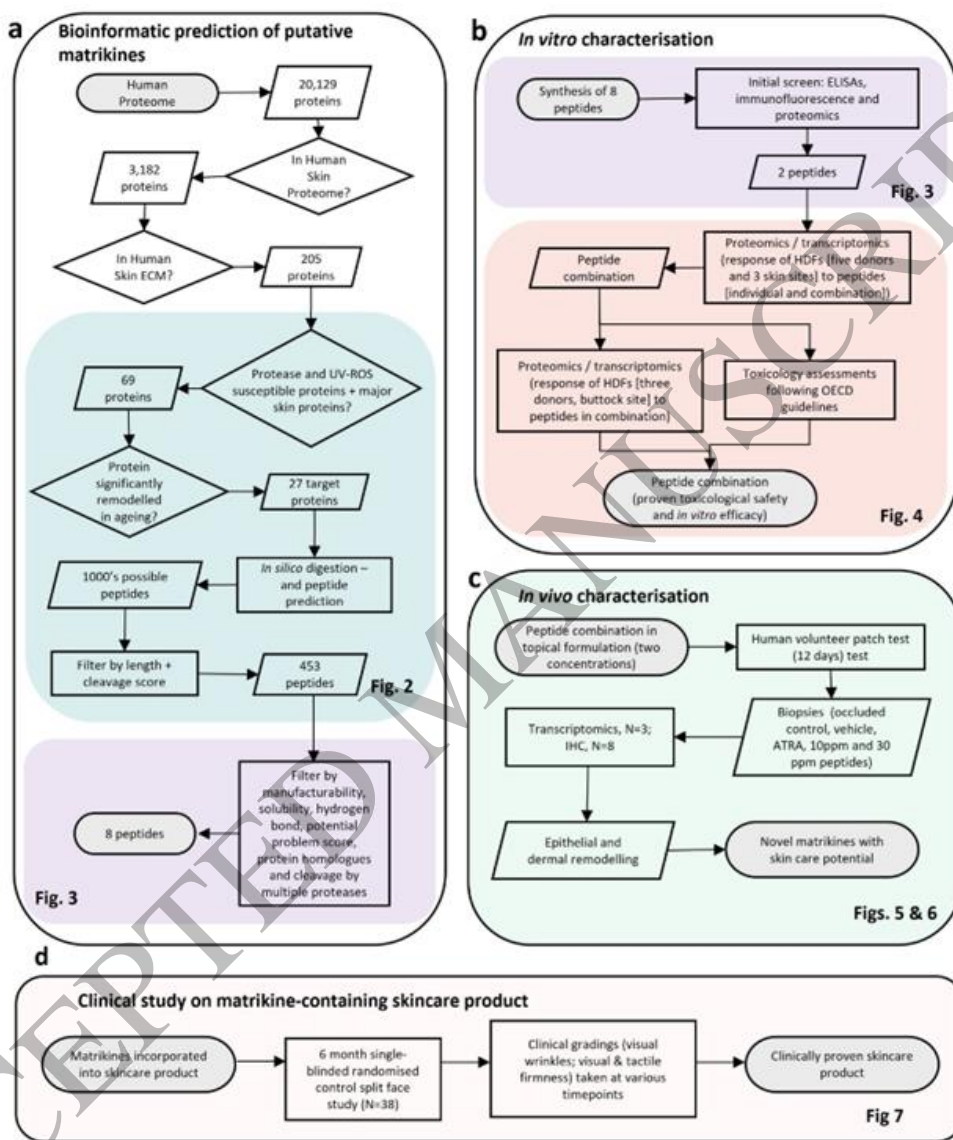
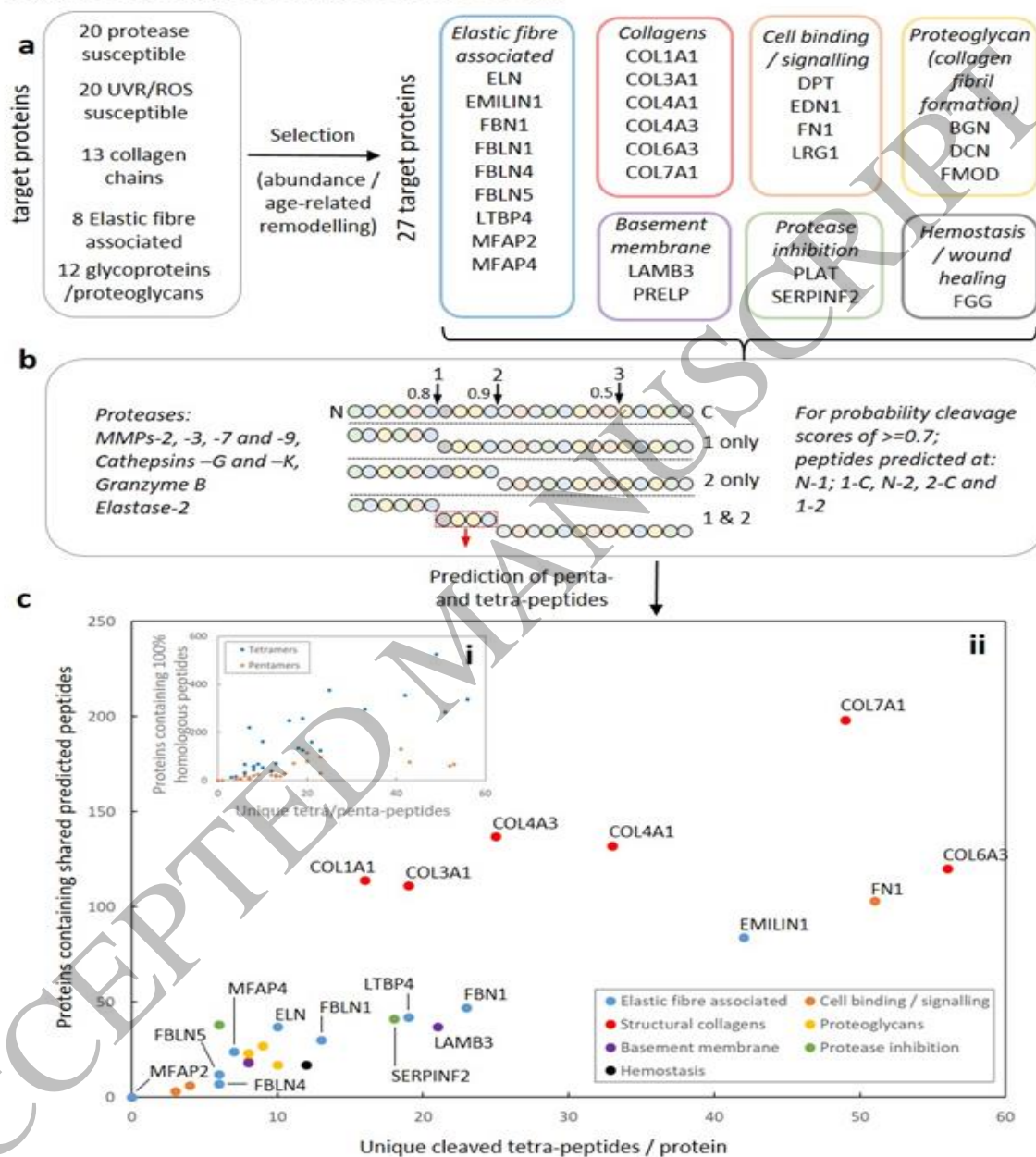


Figure 1  
190x275 mm (x DPI)

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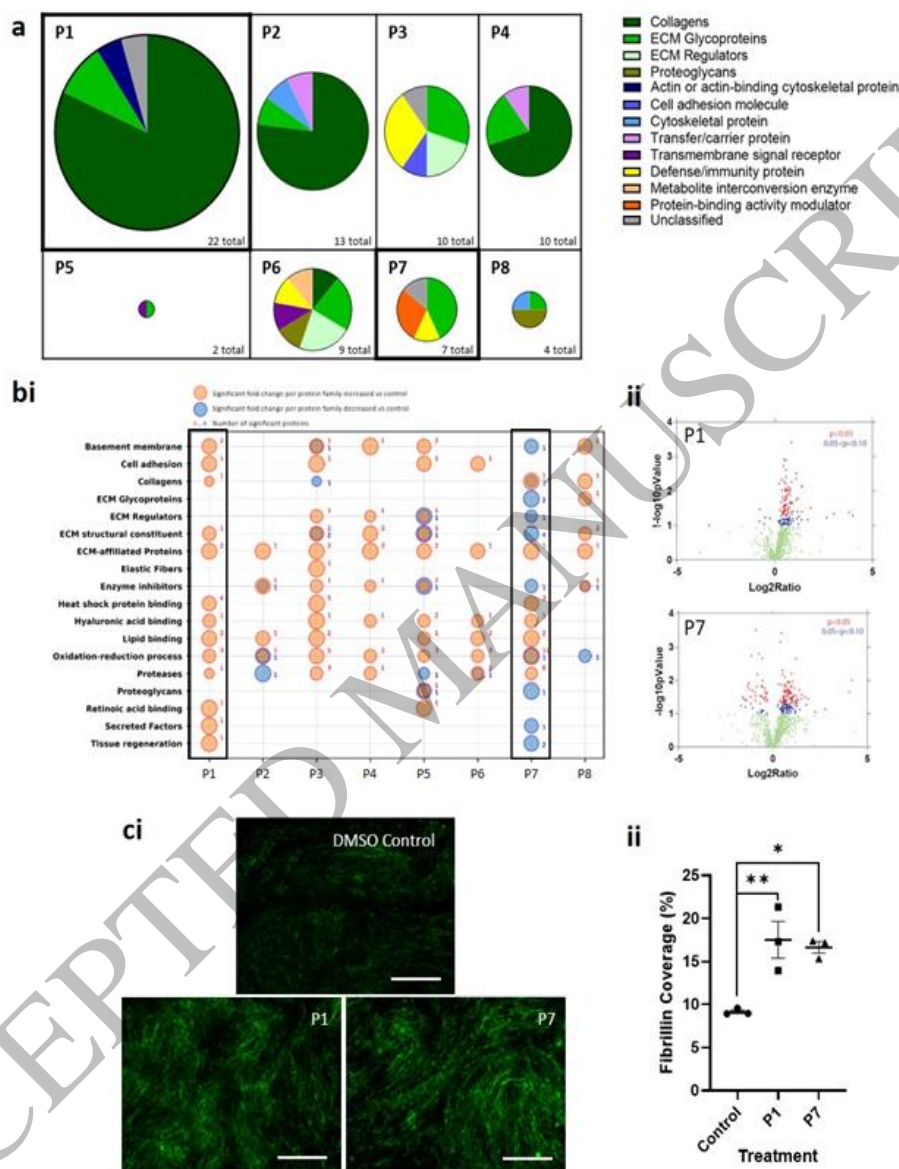
Figure 2. Jariwala and Ozols et al.



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Figure 2  
190x275 mm (x DPI)

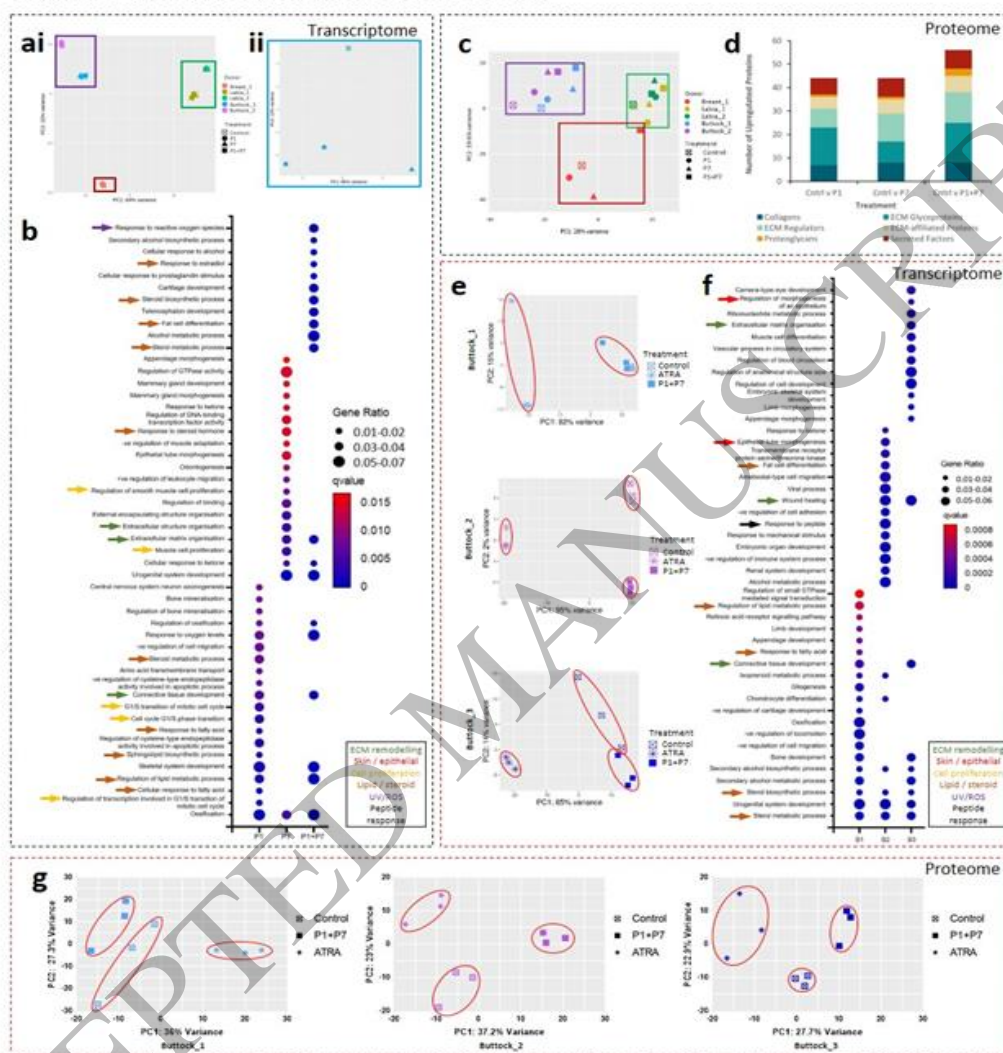
Figure 3. Jariwala and Ozols et al.



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Figure 3  
190x275 mm (x DPI)

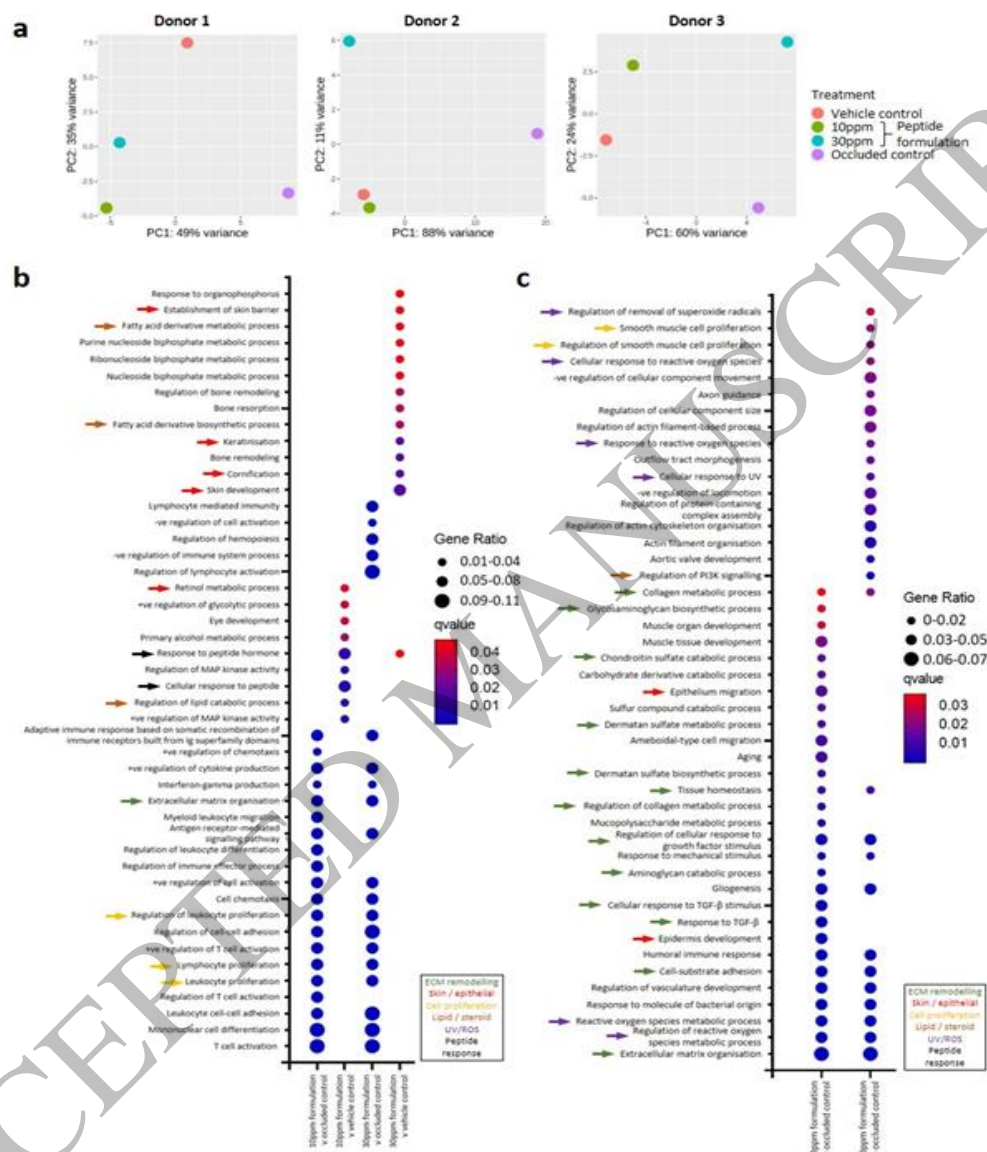
Figure 4. Jariwala and Ozols et al.



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Figure 4  
190x275 mm (x DPI)

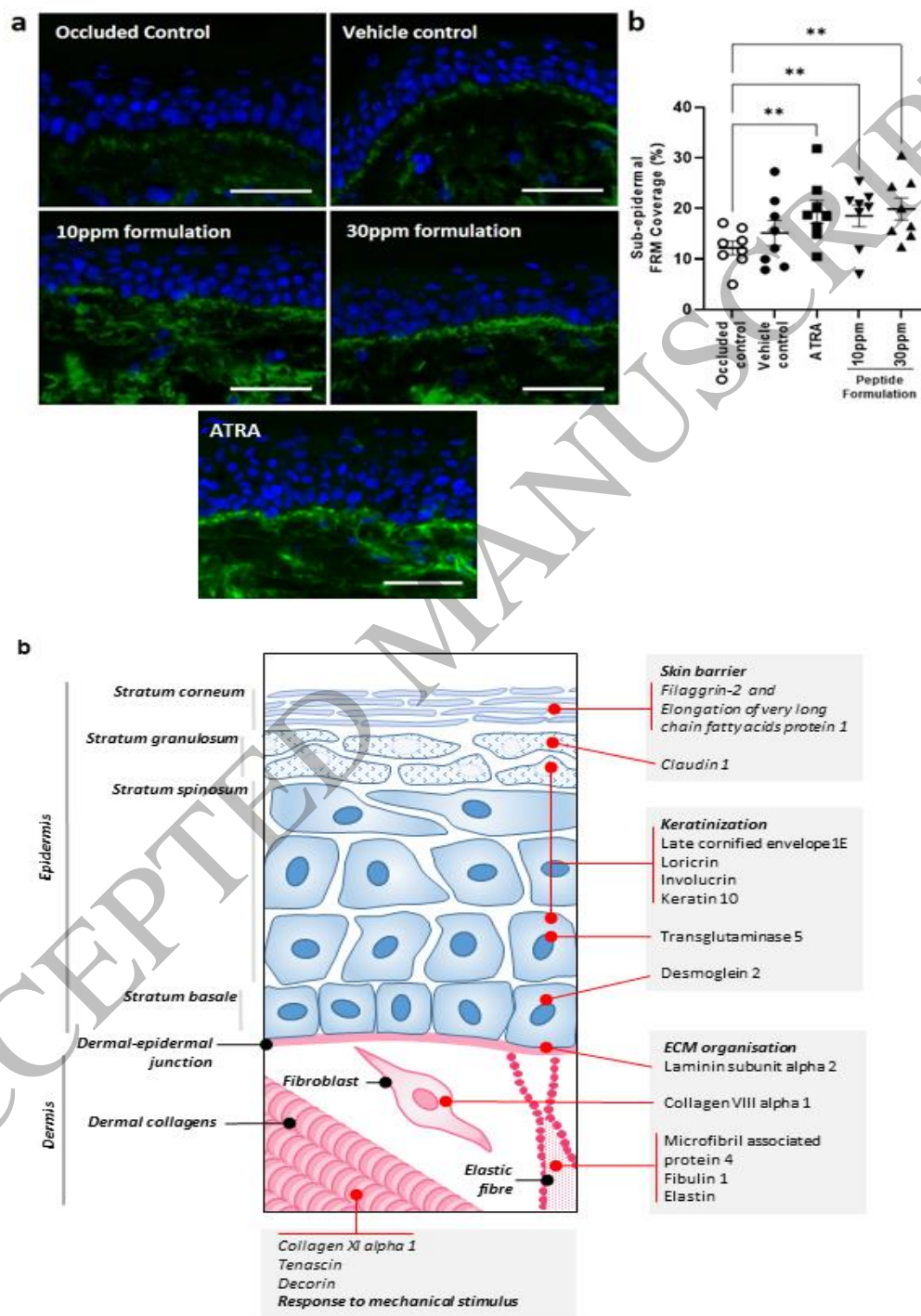
Figure 5. Jariwala and Ozols et al.



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Figure 5  
190x275 mm (x DPI)

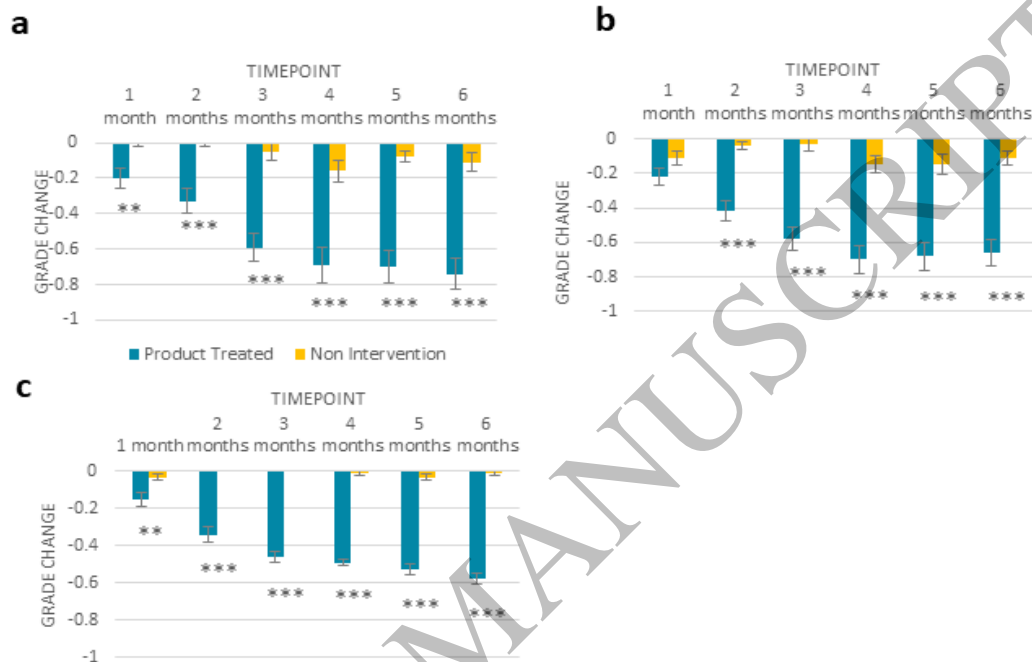
Figure 6. Jariwala and Ozols et al.



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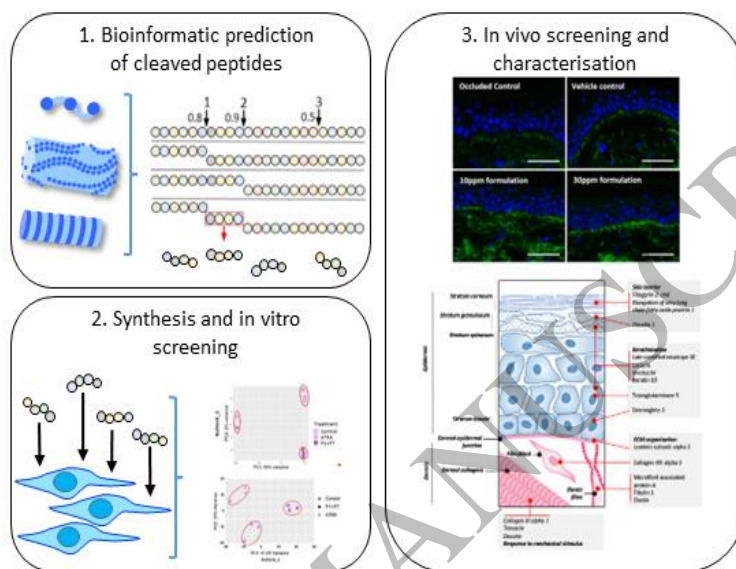
Figure 6  
190x275 mm (x DPI)

Figure 7. Jariwala and Ozols et al.



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Figure 7  
190x275 mm (x DPI)



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Graphical Abstract  
190x275 mm (x DPI)