Proteomic analysis of the psoriatic skin, and the effect of hyperosmotic stress on keratinocytes

PhD Thesis

Edit Szél, M.D.

Supervisor:

Gergely Groma, Ph.D.

University of Szeged

Faculty of Medicine

Department of Dermatology and Allergology

Doctoral School of Clinical Medicine

Szeged

2019

LIST OF PUBLICATIONS

Publications included in this thesis

I. Szél E, Bozó R, Hunyadi-Gulyás É, Manczinger M, Szabó K, Kemény L, Bata-Csörgő Zs, Groma G: Comprehensive proteomic analysis reveals intermediate stage of non-lesional psoriatic skin and points out the importance of proteins outside this trend. Sci Rep. 2019 Aug 6;9(1):11382. doi: 10.1038/s41598-019-47774-5

IF: 4.011

II. Szél E, Danis J, Sőrés E, Tóth D, Korponyai Cs, Degovics D, Prorok J, Acsai K, Dikstein S, Kemény L, Erős G: Protective effects of glycerol and xylitol in keratinocytes exposed to hyperosmotic stress. Clin Cosmet Investig Dermatol. 2019;12:323-331. doi: http://dx.doi.org/10.2147/CCID.S197946

Scientific papers not directly related to the thesis

III. Erős G, Korponyai Cs, Szabó K, Behány Z, Szél E, Kemény L: Antibacterial and skin hydrating effects of Xylinep® gel containing glycerol and xylitol. Borgyogy Venerol Sz. •2014 • 90. ÉVF. 4. 152–155. doi: 10.7188/bvsz.2014.90.4.4

IV. Szél E, Kemény L, Groma G, Szolnoky G: Pathophysiological dilemmas of lipedema. Med Hypotheses. 2014 Nov;83(5):599-606. doi: 10.1016/j.mehy.2014.08.011

IF: 1.074

V. Szél E, Polyánka H, Szabó K, Hartmann P, Degovics D, Balázs B, Németh IB, Korponyai C, Csányi E, Kaszaki J, Dikstein S, Nagy K, Kemény L, Erős G: Anti-irritant and anti-inflammatory effects of glycerol and xylitol in sodium lauryl sulphate-induced acute irritation. J Eur Acad Dermatol Venereol. 2015 Dec;29(12):2333-41. doi: 10.1111/jdv.13225

IF: 3.029

VI. Strifler G, Tuboly E, Szél E, Kaszonyi E, Cao C, Kaszaki J, Mészáros A, Boros M, Hartmann P: Inhaled methane limits the mitochondrial electron transport chain dysfunction during experimental liver ischemia-reperfusion injury. PLoS One. 2016 Jan 7;11(1):e0146363. doi: 10.1371/journal.pone.0146363

IF: 2.806

VII. Szél E, Szolnoky G, Korom I, Bata-Csörgő Zs, Adamkovich N, Annus JK, Kovács L, Krenács L, Meszes A, Modok Sz, Ondrik Z, Kemény L: What can vasculitic leg ulcers implicate? Int Wound J. 2016 Oct;13(5):1027-8. doi: 10.1111/iwj.12312

IF: 2.848

VIII. Korponyai Cs, Szél E, Behány Z, Varga E, Mohos G, Dura Á, Dikstein S, Kemény L, Erős G: The effects of locally applied glycerol and xylitol on the hydration, barrier function and morphological parameters of the skin. Acta Derm Venereol. 2017 Feb 8;97(2):182-187. doi: 10.2340/00015555-2493

IF: 3.127

IX. Manczinger M, Bodnár V, Papp BT, Bolla BS, Szabó K, Balázs B, Csányi E, Szél E, Erős G, Kemény L: Drug repurposing by simulating flow through protein-protein interaction networks. Clin Pharmacol Ther. 2018 Mar;103(3):511-520. doi: 10.1002/cpt.769. Epub 2017 Jul 29.

IF: 6.336

X. Degovics D, Hartmann P, Németh IB, Árva-Nagy N, Kaszonyi E, Szél E, Strifler G, Bende B, Krenács L, Kemény L, Erős G: A novel target for the promotion of dermal wound healing: Ryanodine receptors. Toxicol Appl Pharmacol. 2019 Mar 1;366:17-24. doi: 10.1016/j.taap.2019.01.021. Epub 2019 Jan 23.

IF: 3.585

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration
25p: 25th percentile
75p: 75th percentile
ABCA12: ATP-binding cassette sub-family A member 12
AKR1B10: aldo-keto reductase family 1 member B10
AM: acetoxymethyl ester
ANOVA: analysis of variance
AQP-3: aquaporin-3
ATP: adenosine triphosphate
ATP1B1: sodium/potassium-transporting ATPase subunit beta-1
AZU1: azurocidin
BCLAF1: Bcl-2-associated transcription factor 1
BSA: bovine serum albumin
CARD18: caspase recruitment domain family member 18
CCL-5: chemokine (C-C motif) ligand 5
CD207: C-type lectin domain family 4 member K
CHCHD6: MICOS complex subunit MIC25
CHMP5: charged multivesicular body protein 5
CNTN1: contactin-1
COLEC12: collectin-12
CRABP2: cellular retinoic acid-binding protein 2
CSE1L: exportin-2
CSPG4: chondroitin sulfate proteoglycan 4
CSTA: cystatin-A
CTSV: cathepsin L2
DAG: diacylglycerol
DAPI: 4',6-diamidino-2-phenylindole
DDX21: nucleolar RNA helicase 2
DEFA1: neutrophil defensin 1
DMEM-HG: Dulbecco's modified Eagle's medium containing 4.5 g/L glucose
DMSO: dimethyl sulfoxide
EDTA: ethylenediaminetetraacetic acid
EVPL: envoplakin
F: fluorescence intensity
FABP5: fatty acid-binding protein 5
FBS: fetal bovine serum
FDR: false detection rate
FLOT2: flotillin-2
Foxp3: forkhead box P3
GABA: gamma-aminobutyric acid
GART: trifunctional purine biosynthetic protein adenosine-3
GBP1: guanylate-binding protein 1
gly: glycerol
GnHCl: guanidine hydrochloride
GO: gene ontology
GWAS: genome-wide association study
H: healthy donor/skin sample
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGNC: HUGO Gene Nomenclature Committee
HLA-DR: human leukocyte antigen-DR
HMOX1: heme oxygenase 1
HPSE: heparanase
ICD: irritant contact dermatitis

IL-17A: interleukin-17A
IL-1α: interleukin-1α
IL-1β: interleukin-1β
IL36G: interleukin-36G
IL-6: interleukin-6
IL-8: interleukin-8
IP3: inositol 1,4,5-trisphosphate
IPA: Ingenuity Pathway Analysis
ITGA2: integrin alpha-2
ITGA7: integrin alpha-7
ITGA8: integrin alpha-8
IVL: involucrin
Kd: dissociation constant
KLK10: kallikrein-10
L: lesional psoriatic skin
LC-MS/MS: liquid chromatography-tandem mass spectrometry
LCN2: neutrophil gelatinase-associated lipocalin
LDH: lactate dehydrogenase
LEMD2: LEM domain-containing protein 2
LTF: lactotransferrin
m: mean
M: median
MHC: major histocompatibility complex
MMP-9: matrix metalloproteinase-9
MMPs: matrix metalloproteinases
MPO: myeloperoxidase
MPZ: myelin protein P0
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYBBP1A: Myb-binding protein 1A
MYH11: myosin-11
MYO18A: unconventional myosin-XVIIIa
n/a: not available
NCAM1: neural cell adhesion molecule 1
NCCRP1: F-box only protein 50
NFAT5: nuclear factor of activated T cells 5
NF-κB: nuclear factor- κB
NGS: normal goat serum
NL: non-lesional psoriatic skin
NOP56: nucleolar protein 56
OD: optical density
P: plaque-type psoriatic patient
PASI: Psoriasis Area Severity Index
PI3: elafin
PKC-α: protein kinase C-α
PKC-δ: protein kinase C- δ
PLVAP: plasmalemma vesicle-associated protein
PML: protein PML
PRINS: psoriasis susceptibility-related RNA induced by stress
PRKDC: DNA-dependent protein kinase catalytic subunit
PRX: periaxin
PSAPL1: proactivator polypeptide-like 1
PSME3: proteasome activator complex subunit 3
RPL21: 60S ribosomal protein L21
RPL23: 60S ribosomal protein L23
RPL28: 60S ribosomal protein L28
RPS11: 40S ribosomal protein S11

RPS15: 40S ribosomal protein S15
RPS27: 40S ribosomal protein S27
RRAS: Ras-related protein R-Ras
RT qRT-PCR: real-time quantitative reverse transcription polymerase chain reaction
S100A2: protein S100-A2
S100A7: protein S100-A7
S100A8: protein S100-A8
S100A9: protein S100-A9
SCCA1: squamous cell carcinoma antigen-1
SCCA2: squamous cell carcinoma antigen-2
SD: standard deviation
SDS: sodium dodecyl sulfate
SGCD: delta-sarcoglycan
SLMAP: sarcolemmal membrane-associated protein
SLS: sodium lauryl sulfate
SMARCA5: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5
snoRNP: small nucleolar ribonucleoprotein
sor: sorbitol
STAT1: signal transducer and activator of transcription 1
STAT3: signal transducer and activator of transcription 3
SYNM: synemin
TBS: Tris-buffered saline
TEWL: transepidermal water loss
TNF-α: tumour necrosis factor-alpha
TOP1: DNA topoisomerase 1
TREX2: three prime repair exonuclease 2
TRPV1b: transient receptor potential vanilloid 1b
UGDH: UDP-glucose 6-dehydrogenase
UPLC: ultra performance liquid chromatography
VSNL1: visinin-like protein 1
XP32: skin-specific protein 32
xyl: xylitol

1. INTRODUCTION

1.1 Psoriasis

Our skin connects and at the same time separates the internal and the external environment. It is constantly subjected to many different stimuli that require proper responses, through which the skin can influence the function of other organs, like the brain and the endocrine system in a mutual way[1,2].

Psoriasis is a chronic inflammatory skin disease, mainly characterised by abnormalities in the immunological response of the skin to various internal and external stimuli[3]. It affects approximately 2% of the human population world-wide with no gender differences[4]. The age of onset is typically between 20-30 years (early-onset) or 50-60 years (late-onset)[5]. The most common form (90%) is plaque-type psoriasis characterised by erythematous plaques with silvery scales, mainly on the extensor surfaces of the extremities, scalp, sacrum and umbilicus[4]. On histological sections of psoriatic plaques, elongated rete ridges, hyperkeratosis, parakeratosis and Munro's microabscesses can be found with dilated vessels in the dermal papilla[6]. The exact cause of psoriasis is unknown, however, genome-wide association studies (GWAS) have identified over 50 regions associated with the risk of the disease. The coding genes of these regions are connected to antigen presentation, the interleukin-23 (IL-23) axis, T-cell development and polarisation, innate immunity and negative regulators of immune responses[7]. Psoriasis is not only a skin disorder; it is associated with systemic inflammation[8]. Comorbid conditions are psoriatic arthritis, Crohn's disease, cancer, depression, non-alcoholic fatty liver disease, metabolic syndrome and cardiovascular disorders[9]. To date, all therapies available for psoriasis only manage symptoms. Understanding alterations behind the disease is crucially important for developing new therapies for better disease management.

1.1.1 Biomarkers in psoriasis

Previous large-scale treatises including genomic, transcriptomic and proteomic studies have identified psoriasis-related markers playing key roles in the pathomechanism, such as AKR1B10[10], CSTA[11], FABP5[12], PI3[13], SCCA2[14], STAT1[10], STAT3[15], S100A2[16], S100A7[12–

[14], S100A8[13,17] and S100A9[13,14,17,18], among others, thereby contributing greatly to better understanding of the disease.

Several proteomic studies have led to biomarker discovery contributing to the diagnosis or the estimation of prognosis in psoriasis. According to *Williamson et al*., among proteins with altered levels in psoriatic lesional vs. non-lesional skin, profilin 1 showed a tendency to increase in the plasma of psoriatic patients[12]. Fibrinogen α-chain-derived peptide and a filaggrin-derived peptide were found to be increased in the sera of patients with psoriasis vulgaris and psoriatic arthritis compared to those of healthy individuals[19]. Patients with psoriasis could be differentiated from those with atopic or contact dermatitis by the serum level of kynureninase[20]. Desmoplakin, complement C3, polymeric immunoglobulin receptor and cytokeratin 17 plasma levels were significantly altered compared to healthy controls and correlated with the Psoriasis Area Severity Index (PASI) score[21].

1.1.2 Proteomic studies of the psoriatic skin

One of the most effective ways to study different diseases with such enormous complexity and to elucidate related mechanisms is to perform a comparative proteomic analysis of protein extracts derived from affected tissues.

A recent review summarised the novel mechanisms and hypotheses proposed by the results of proteomic studies of psoriasis[22]. In the articles reviewed, the following results are related to the alterations of the psoriatic skin: I. Proteins showing increased expression in psoriatic lesional skin were related to abnormalities in cell proliferation, the regulatory/balancing system and inflammation[23]. II. S100A8, S100A9 and complement C3 were upregulated in lesional vs. non-lesional skin[17], while kallikrein-related peptidase 6, solute carrier family 25, cystatin A and serpin B1[11] were increased in psoriatic lesions compared to healthy skin. III. *Lysvand and colleagues* found that unique epitopes on the Pso p27 complex caused by the post-translational modification of Serpin B3 (SCCA1) may be immunogenic in psoriasis[24]. IV. Some proteins with differential expression in lesional vs. non-lesional skin were targeted by IL-17A[25], while others were related to mechanisms contributing to abnormal epidermal growth[26].

Until now, none of the full-scale psoriatic proteomic studies[11,12,17,20,23,26,27], to the best of our knowledge, have compared lesional and non-lesional psoriatic skin regions (both

epidermis and dermis) with the inclusion of biopsies from healthy individuals as a reference in the comparison. The inclusion of healthy skin could provide several pieces of important additional information. I. Alterations that are similar in non-lesional and lesional skin, but differ from healthy skin, can be detected and used to identify potential novel disease markers or predisposing factors already present in the non-lesional skin. II. The comparison of non-lesional skin to healthy skin might facilitate the identification of inherent characteristics of psoriatic patients that are already present in their healthy-looking skin prior to lesion development. III. Information could be gained about the extent to which the non-lesional skin is affected with respect to lesional alterations. IV. Altered processes in the non-lesional skin that are contrary to the changes of lesional skin could be identified, some of which may contribute to the maintenance of the non-lesional state and serve as novel intervention points for disease management.

1.1.3 The psoriatic non-lesional skin

In psoriasis, the macroscopically healthy-looking non-lesional skin harbours alterations that might cause symptoms[28]. One of the most characteristic properties of the non-lesional skin is an altered response to mechanical stress or injury[29] leading to barrier disruption[30], which leads to an elevated innate immune response[31,32]. Alterations in non-lesional skin are not restricted to keratinocytes. Angiogenesis is also among those mechanisms that are already affected in the non-lesional skin, resulting in altered quantity and quality of microvessels[33]. In addition, it is becoming clear that some adaptive immune responses are also altered[34]. Abnormalities in the dermal extracellular matrix composition, such as elevated expression of the oncofetal splice variant of fibronectin[35] due to altered splicing events[36], indicate the involvement of dermal fibroblasts[28]. Several matrix metalloproteinases (MMPs), such as MMP-9, previously thought to be increased only in lesions, are now known to be elevated in non-lesional skin compared to healthy skin[37]. There is also evidence for mechanisms in non-lesional skin that contribute to the maintenance of its state. The PRINS long non-coding RNA is induced by stress and nucleic acids, and it is anticipated to have a protective function in psoriasis. Not only does PRINS decrease inflammatory responses[38] in the non-lesional skin by inhibiting IL-6 and chemokine (C-C motif) ligand 5 (CCL-5) mRNA translation, but it also influences anti-apoptotic mechanisms[39]. The elevated expression of the anti-inflammatory

regulator caspase recruitment domain family member 18 (CARD18) in non-lesional skin compared to healthy skin was found to aid the inhibition of inflammatory events[40]. These mechanisms, among many others, highlight the relevance of comparing non-lesional skin to healthy skin.

The psoriatic non-lesional skin is characterised by increased stress response to several external and internal factors, such as mechanical stress[29] and alcohol[41–44], which results in plaque formation. The central features of psoriatic plaque formation include abnormal keratinocyte proliferation and differentiation leading to the disturbance of the barrier function of the skin, among others.

1.2 Impaired barrier function of the skin and related osmotic challenge

Impaired barrier function is the main pathologic finding in irritant contact dermatitis (ICD), resulting in increased skin permeability and transepidermal water loss (TEWL)[45]. Water evaporation can lead to a higher osmotic pressure in the superficial layers of the skin, therefore local hyperosmotic challenge may contribute to the development of the disease.

Hyperosmolarity reduces cellular viability[46–50] and elevates intracellular calcium (Ca^{2+}) concentration in HaCaT keratinocytes. Although its exact molecular mechanism has not yet been revealed, it has been shown that Ca^{2+} is derived from both intra- and extracellular compartments[49,51].

Osmotic stress induces further intracellular responses. At mRNA level, the expression of tumour necrosis factor-alpha (TNF-α), IL-1β, IL-8[46], IL-6[47] and the nuclear factor of activated T cells 5 (NFAT5)[48] was found to be higher in epithelial cells under hyperosmotic condition. Elevated mRNA expression of TNF-α, IL-1β, IL-6 and IL-8 was also observed in normal human epidermal keratinocytes[52].

NFAT5 is a principal transcription factor involved in water homeostasis during normal cornification[53] and activated by hyperosmotic stress[48], however, its regulation by Ca^{2+} signals is equivocal[54–56]. Local hyperosmotic stress of the skin activates NFAT5 in macrophages[57], thereby intensifying the electrolyte clearance via lymphatic vessels[58].

Under experimental conditions, different methods are used to induce hyperosmotic stress. Sodium chloride is usually used for this aim[46–48] and non-ionic organic agents such as sorbitol[52] and sucrose[47] can also be applied on keratinocyte cultures.

1.3 Beneficial effects of polyols on the skin

Glycerol and xylitol have well-known beneficial effects on the skin[59–62]. Previously, we have shown their anti-inflammatory and anti-irritant effects[60–62]. Other *in vitro* experiments have revealed that glycerol suppresses human leukocyte antigen-DR (HLA-DR) mRNA level[63], while xylitol inhibits inflammatory cytokine expression[64] and upregulates filaggrin mRNA expression[63]. In addition, glycerol is known to compose the principal osmolyte system of several bacterial species[65]. However, the role of glycerol and xylitol as osmolytes in the skin has not yet been fully clarified.

2. AIMS

We aimed to extend previous proteomic studies, in order to get more information regarding the putative alterations in psoriasis. Therefore, a complex comparison was performed, where in addition to non-lesional and lesional skin, samples from healthy skin were also included, in a label-free, semi-quantitative proteomic analysis.

Furthermore, we aimed to investigate whether glycerol and xylitol, as known effective anti-irritant agents[60–62], provide protection against hyperosmotic stress *in vitro*. Their effects on cellular viability, cytotoxicity, intracellular Ca^{2+} concentration and expression of pro-inflammatory cytokines were studied in hyperosmotic condition.

3. MATERIALS AND METHODS

3.1 Patients and skin sample collection

Skin biopsy collection from donors, the procedure of collection and all experimental protocols were approved by the Regional and Institutional Research Ethics Committee and by the Human Investigation Review Board of the University of Szeged (SOEDAFN-002, IF-562-5/2016 and IF-15056/2015; 157/2015; 3638 and 2799, 3517), strictly following the guidelines and regulations of the Declaration of Helsinki. Prior to surgical intervention and following a detailed description of the skin biopsy donation procedure, participants provided written informed consent. No donor under the age of 18 was included in our study.

To identify alterations that are general in chronic plaque psoriasis and to keep the number of volunteers for skin biopsy collection to a minimum, for our proteomic approach, we randomly engaged individuals I.) of different age to minimise possible age-related differences; II.) with various PASI scores between 5 and 25 since the score for an individual patient varies over time and with relapse; III.) of both genders to avoid possible gender-associated differences; and IV.) with both early and late onset. A total of 9 (3x3) patients suffering from chronic plaque psoriasis and the same number of healthy donors were involved in our study. None of the psoriatic patients had received any kind of treatment for the condition for at least 6 months. 6 mm skin punch biopsies containing the epidermis and the dermis were collected from an area of the upper-middle gluteal region that is not exposed to sunlight. Both lesional and non-lesional samples were collected from patients. Non-lesional samples were taken at least 7 cm from the edge of the lesion subjected for biopsy. The presence of psoriasis was clinically verified for all patients, and clinical as well as demographic data of the donors are presented in **Table 1**.

Proteomic experiment	Group of donors	Donors	Age	Gender	PASI score
No. 1	Healthy	H I.	46	Male	n/a
		H II.	59		
		H III.	51		
	Plaque-type psoriasis	P I.	65		17.1
		P II.	63		9.9
		P III.	50		5.5
No. 2	Healthy	H IV.	23	Female	n/a
		H V.	48		
		H VI.	51		
	Plaque-type psoriasis	P IV.	25		9.2
		P V.	62		21.5
		P VI.	70		17.5
No. 3	Healthy	H VII.	37	Male	n/a
		H VIII.	39		
		H IX.	61		
	Plaque-type psoriasis	P VII.	49		22.4
		P VIII.	55		12.1
		P IX.	61		12

Table 1. Basic demographic and clinical characteristics of donors involved in the proteomic analysis (H: healthy donor, P: plaque-type psoriatic patient, PASI: Psoriasis Area Severity Index, n/a: not available)

3.2 Comparative proteomic workflow of healthy, non-lesional and lesional skin

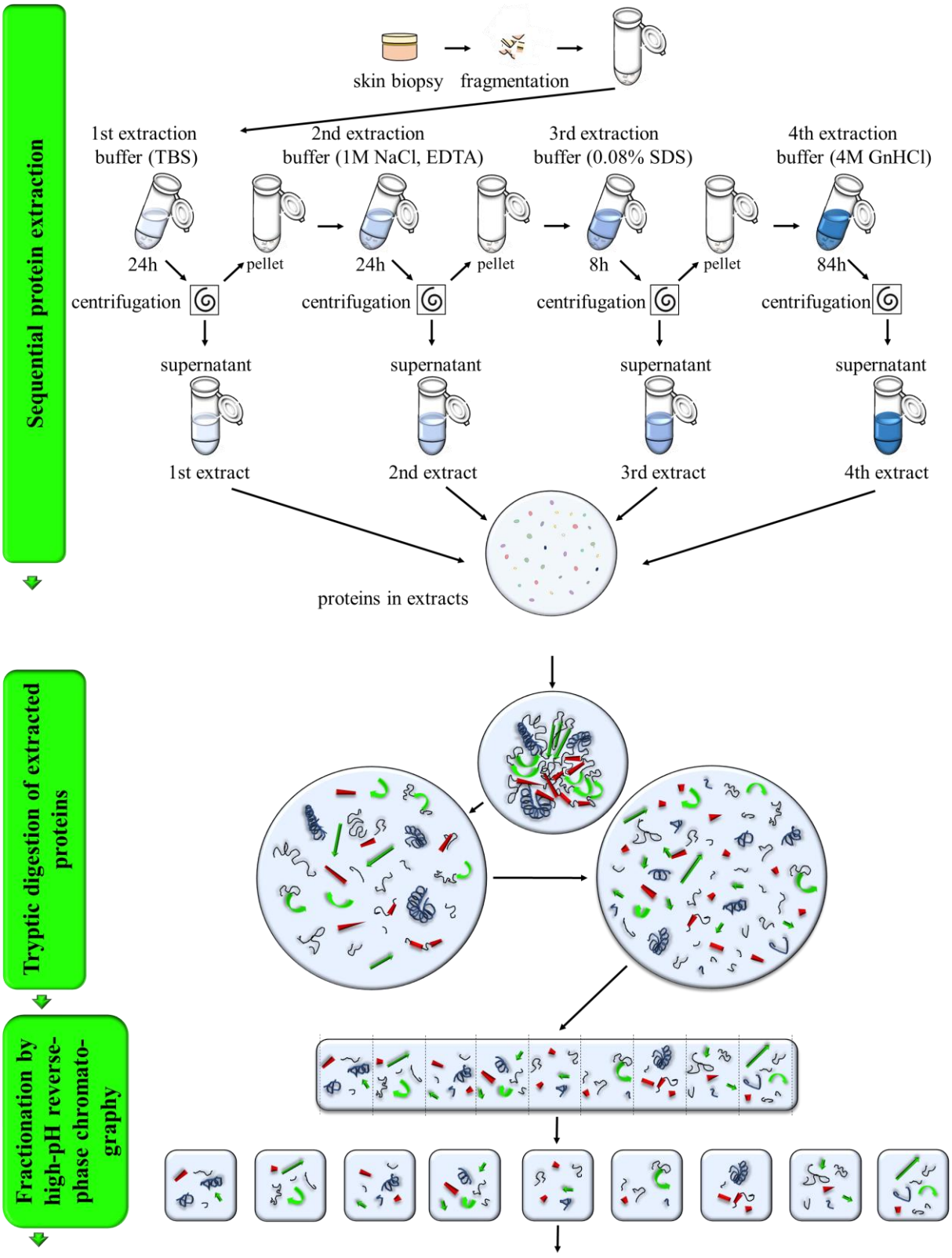
3.2.1 Four-step sequential protein extraction

Samples were cut with a razor blade. Skin proteins were extracted sequentially in four consecutive, solubility-based extraction steps. Extraction buffers were used in increasing order of their solubilising properties for a better separation of proteins. Samples were initially incubated in extraction buffer I (0.15 M NaCl, 50 mM Tris-HCl, pH=7.4) for 24 h at 4◦C in the presence of protease inhibitors. Protein extracts were then clarified by centrifugation and separated from the pellet. This step was repeated by resuspending the pellet in extraction buffer II, which contained 1 M NaCl, 25 mM EDTA, 50 mM Tris-HCl, pH=7.4. Following extraction with 250mM SDS-containing extraction buffer III (8 h at room temperature), guanidine hydrochloride-containing extraction buffer IV (4 M GnHCl, 10 mM EDTA, 50 mM Tris-HCl, pH=7.4) was applied for 84 h at 4◦C. The same protein extracts of three donors

were pooled in each investigated group (healthy, non-lesional, lesional). The extraction procedure was carried out three times and each contained extracted proteins of three donors following the pooling of the samples, which were then subjected to downstream proteomic analysis.

3.2.2 Two-dimensional LC-MS/MS analysis

A total of 35 µg protein from each sample was applied for mass spectrometry analysis. A modified filter-aided sample preparation method was used for tryptic digestion of the protein extracts[66]. High-pH reversed-phase chromatography was performed on a C18 column (Kinetex 5 µm EVO C18 100 A, 2.1 x 100 mm; cat. no. 00D-4622-AN, flow rate: 150 µl/min, Phenomenex, California, USA). 48 fractions were collected from 1 to 25 min. (half minute/fraction) and 4-4 fractions were combined (1,13,25,37; 2,14,26,38 and so on) to get 12 final fractions. Each fraction was subjected to nano LC-MS/MS analysis on an Orbitrap Elite Hybrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled with a nanoAcquity UPLC system (Waters, Milford, Massachusetts, USA), using a gradient elution after trapping the samples onto the trap column. Data-dependent analyses were applied; the 20 most intense peaks were selected for ion-trap collision-induced dissociation after each survey scan had been measured in the Orbitrap. Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to generate MS/MS peak-list files and our in-cloud ProteinProspector (version 5.16.0, http://prospector.ucsf.edu © Copyright (1996-2019) The Regents of the University of California) database search engine was used for protein identification against the human sequences from the UniProtKB.2015.12.14.random.concat (149781/55820795 entries searched) database (European Bioinformatics Institute (EMBL-EBI), Hinxton Cambridge, UK). The schematic overview of the applied proteomic strategy is summarised in **Figure 1.**


Sequential protein extraction
skin biopsy
fragmentation
1st extraction buffer (TBS)
2nd extraction buffer (1M NaCl, EDTA)
3rd extraction buffer (0.08% SDS)
4th extraction buffer (4M GnHCl)
24h
pellet
24h
pellet
8h
pellet
84h
centrifugation
centrifugation
centrifugation
centrifugation
supernatant
supernatant
supernatant
supernatant
1st extract
2nd extract
3rd extract
4th extract
proteins in extracts
Tryptic digestion of extracted proteins
Fractionation by high-pH reverse-phase chromato-graphy

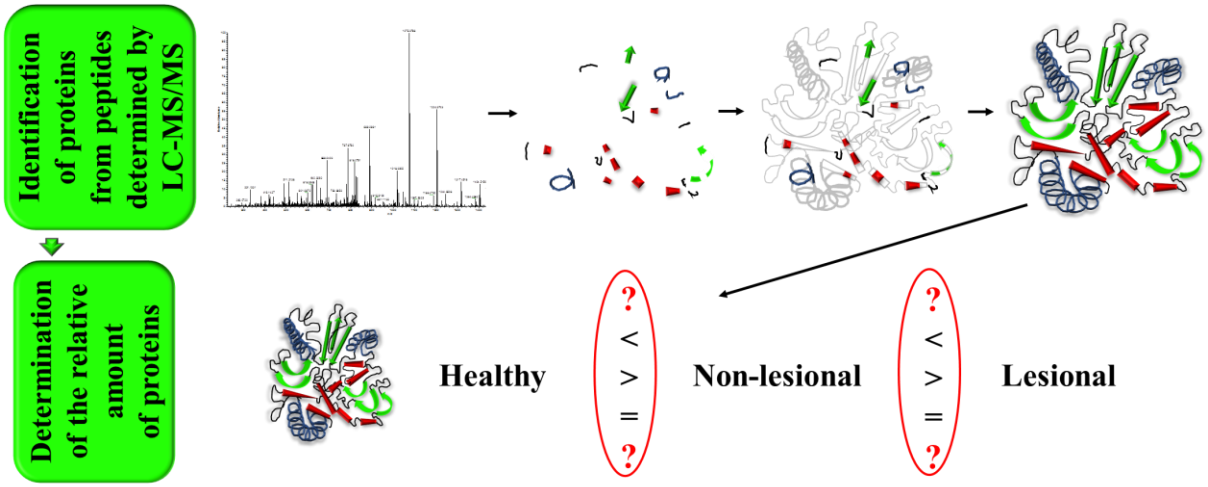


Figure 1. Schematic illustration of the applied proteomic workflow (TBS: Tris-buffered saline, EDTA: ethylenediaminetetraacetic acid, SDS: sodium dodecyl sulfate, GnHCl: guanidine hydrochloride, LC-MS/MS: liquid chromatography – tandem mass spectrometry)

Detailed protocols and applied counting for semiquantitative analysis is described as supplementary information: https://www.nature.com/articles/s41598-019-47774-5#Sec18.

3.3 Immunofluorescent staining for UDP-glucose 6-dehydrogenase (UGDH)

For immunofluorescence analysis, 5 µm sections of frozen embedded skin biopsies from psoriatic patients (non-lesional and lesional skin) and healthy individuals were used. After fixation and permeabilisation (Foxp3 staining buffer set, fixation/permeabilisation kit - Miltenyi Biotec, Bergisch Gladbach, Germany - used according to the description of the manufacturer), the sections were blocked in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 1% normal goat serum (NGS) for 1 h at room temperature. Samples were incubated overnight at 4°C in TBS with 1% NGS and primary antibody against UGDH (rabbit polyclonal antibody, ab155005, Abcam, Cambridge, UK), diluted to 1:100. Following washing in TBS, AF546 secondary antibody (Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546, A-11035, Invitrogen, Carlsbad, California, USA), diluted in TBS containing 1% NGS to 1:500, was applied for 1 h at room temperature.

3.4 Bioinformatic analysis

To identify proteins not yet linked with the pathomechanism of psoriasis, literature mining was carried out using protein names or the encoding gene's symbol(s), applying the following

strategy: each protein or gene name was searched together with "psoriasis" as a keyword using the RISmed R package.

For the identification of cellular mechanisms that may be associated with the proteins that were detected in altered amounts in the proteomic approach, we used the Ingenuity Pathway Analysis software (IPA, Qiagen, Redwood City, California, USA).

3.5 Cell culture and preparation of the cell-treating media

HaCaT cells kindly provided by Dr. N. E. Fusenig (Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 1.8 mM Ca^{2+} (DMEM-HG) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and grown at 37°C in a humidified atmosphere containing 5% CO_2. Three to four days after plating, cells were collected from semi-confluent cultures by 5-10 min. trypsinisation (0.25% trypsin EDTA solution). The proteolic digestion was stopped by FBS and the cells were sedimented (10 min., 4°C, 1500 g) and resuspended in the medium for further use. Cellular viability was determined by the trypan blue exclusion test. The experiments were performed on cultures, where trypan blue was excluded from 95% of the cells.

For osmolarity measurement, Model 5600 Vapro® Vapor Pressure Osmometer (Dieren, The Netherlands) was used. The basal osmolarity of the serum-free DMEM-HG medium was 338 mOsm. The media contained 3 or 30 mM polyols, respectively, which was equivalent to 0.027 or 0.27 w/w% glycerol and 0.045 or 0.45 w/w% xylitol[63]. The osmolarity of these solutions was also measured. The final osmolarity of 450, 500 and 600 mOsm was reached by the addition of the appropriate amount of 1.83 M sorbitol stock solution. Serum-free DMEM-HG media containing only glycerol, xylitol or sorbitol in concentrations mentioned above, respectively, were also applied.

3.6 Measurement of cellular viability and cytotoxicity

Cells were seeded into 96-well culture plates at a density of 10^4 cells/well in DMEM-HG supplemented with the appropriate agents detailed above, and serum-starved 24 h before any treatments. For 60 min., cells were incubated with or without 0.27% glycerol or 0.45% xylitol in serum-free DMEM-HG, followed by incubation with 450, 500 or 600 mOsm, sorbitol-

containing culture medium with or without 0.27% glycerol or 0.45% xylitol for 24 h. Experiments were carried out in triplicates, data are presented as the means of three experiments.

For the cell viability assay, 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to the cells at 37^{o}C for 4 h, then the produced formazan crystals were solubilised and the optical density (OD) was measured at 540 nm by a Multiskan EX spectrophotometer (Thermo Labsystems, Beverly, Massachusetts, USA) and Ascent Software (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The absorbance rate was compared to untreated samples and represented as the percentage rate of living cells.

Cytotoxicity was assessed by using the Cytotoxicity Detection Kit PLUS (Roche Diagnostics, Risch, Switzerland) according to the manufacturer's instructions. Briefly, released lactate dehydrogenase (LDH) was quantified from the supernatant by a colorimetric method and OD was measured at 492 nm. Measured values were corrected with the background values of the cell-free culture medium. Results are presented in percentage of cytotoxicity, where lysed cells correspond to 100% cytotoxicity.

3.7 Determination of changes in intracellular Ca^{2+} concentration

HaCaT keratinocytes were plated onto 13 mm diameter uncoated sterile coverslips (VWR, Radnor, Pennsylvania, USA) and were left to attach and proliferate for 24 h. Coverslips with the attached cells were transferred and incubated in Tyrode's solution (144 mM NaCl, 0.4 mM NaH_2PO_4, 4 mM KCl, 0.53 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5.5 mM glucose and 5 mM HEPES, pH=7.4). HaCaT cells were loaded by incubation for 20 min. with the acetoxymethyl ester (AM) form of a single wavelength Ca^{2+}-sensitive fluorescent dye (Fluo-4, Molecular Probes Inc., Eugene, Oregon, USA, 5μM from a stock of 1mM in DMSO + 20% pluronic acid Pluronic F-127 Sigma-Aldrich, Saint Louis, Missouri, USA and 6.25 nM/mL Probenecid, Molecular Probes Inc., Eugene, Oregon, USA) at room temperature in dark. After the incubation period, the cells were washed in indicator-free Tyrode's solution to remove any dye. The technique for intracellular Ca^{2+} detection was based on established procedures described earlier[67]. Subsequently, cells were incubated for 30 min. with 0.027 or 0.27% glycerol and 0.045 or 0.45% xylitol[63] in Tyrode's solution, respectively.

Optical measurements were performed using a Zeiss Axiovert 100 microscope (Zeiss, Oberkochen, Germany) equipped with a xenon lamp and used in epifluorescent mode at 100x magnitude. The coverslips were placed into a low-volume imaging chamber (Warner Instruments, Hamden, Connecticut, USA) (37°C) at the microscope stage and cells were superfused with Tyrode alone for at least 10 min. (control period). Hyperosmotic stimulus was added to the cells in rapid perfusion (2-3 μL/sec) of 450 mOsm, sorbitol-containing solution followed by the addition of 30 nmol/mL A23187 ionophor (Sigma-Aldrich, Saint Louis, Missouri, USA) in Tyrode. Cells in the 75x75 μm frame were illuminated at 485 nm and the emitted light was recorded at 535 nm. Images of relative fluorescence intensity of a cell group (3-5 cell/group) were collected in 8-10 independent experiments per treatment group with the Mintron 7266pd Color CCD Camera (New Taipei City, Taiwan). Raw data were analysed with WinWCP V4.5.0 (Glasgow, Scotland) and Clampfit 10.6 (San Jose, California, USA) softwares.

Calibration was performed by the $[Ca^{2+}]_i$ = Kd (F-Fmin)/(Fmax-F) method[68] where $[Ca^{2+}]_i$ is the intracellular Ca^{2+} concentration, Kd is the dissociation constant (345 nM), F is the fluorescence intensity evoked with the hyperosmotic stimulus, Fmin values were developed from corrected fluorescence intensity before osmotic stress and Fmax values were derived from the maximal fluorescence response after the addition of the ionophor solution. Background correction was calculated with the autofluorescence of unloaded cells and the decrease of fluorescence intensity in untreated cells caused by bleaching or dye efflux. For representative figures, $\Delta Ca^{2+} = \Delta F/F = (F - Frest)/Frest)$ was calculated[68].

3.8 RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT qRT-PCR)

Cells were seeded into 6-well plates at a density of $2x10^5$ cells/mL and serum-starved overnight. Cells were pre-incubated for 60 min. with 0.27% glycerol or 0.45% xylitol, followed by incubation with 450 mOsm, sorbitol-containing medium for 2 and 6 h[46–48]. Untreated or only sorbitol-treated cells served as negative or positive controls, respectively. Total RNA was isolated using TRIzol reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. To avoid DNA contamination, DNAse treatment was performed and intron-spanning assays were used. cDNA was synthesised from 1 μg total

RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA). RT qRT-PCR experiments were carried out with the Universal Probe Library system (Roche Diagnostics, Risch, Switzerland) using TaqMan probe-based assays and a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The expression of each gene was normalised to the 18S rRNA gene and relative mRNA levels were calculated by the ΔΔCt method, compared to untreated, time-matched control samples.

3.9 Statistical analysis

3.9.1 Statistical analysis of the proteomic study

To compare protein abundance from healthy, lesional and non-lesional skin extracts, significant differences were determined based on relative peptide ion chromatograms and spectrum counting, and evaluated using two different approaches: 1) modified t-test (limma) and 2) rank product test (as described by *Schwämmle et al*.[69] following a t-test. We considered a protein amount to be different between two samples if at least one test was significant ($p<0.05$) and the absolute fold change was at least two or higher.

3.9.2 Statistical analysis of the *in vitro* experiments

Data analysis was performed with SigmaStat for Windows 11.0 software (Jandel Scientific, San Rafael, California, USA). Differences among groups were analysed with one-way ANOVA and Holm-Sidak post-hoc test and data are presented as mean (m) values with standard deviation (SD). When the normality test failed, Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison was performed. In such cases, median values (M) with 25th and 75th percentiles (25p and 75p, respectively) are given. $P<0.05$ was considered statistically significant.

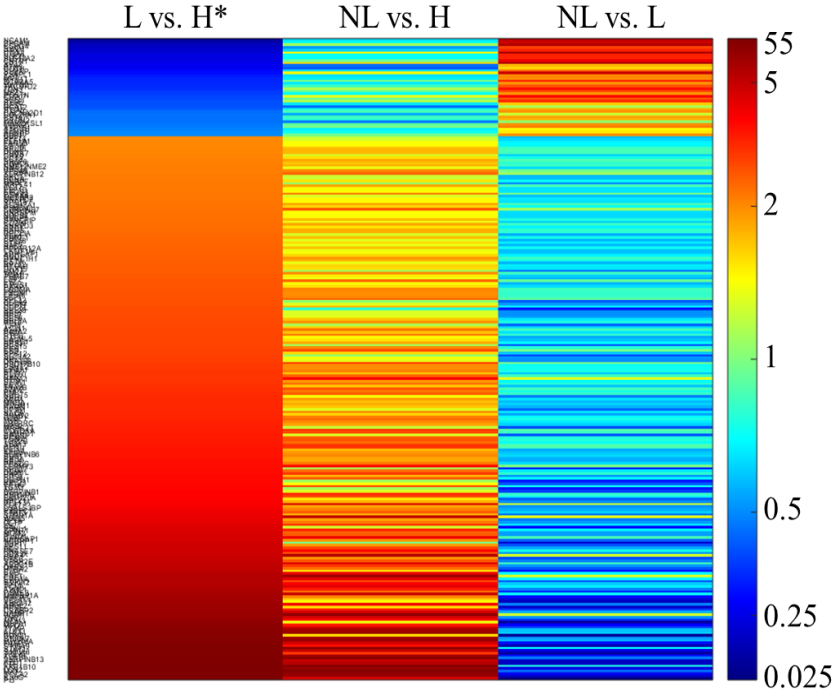
4. RESULTS

4.1 Proteomic results

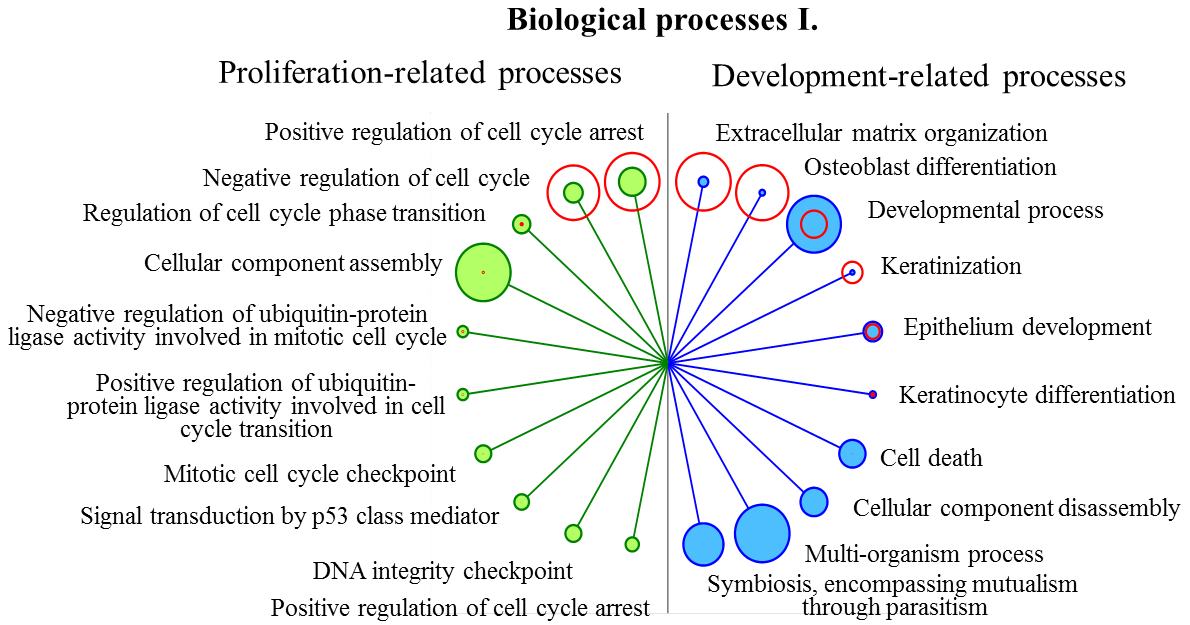
4.1.1 Comparison of healthy and lesional skin, and the associated biological processes

As an initial step, the proteomic results of lesional and healthy skin samples were compared and the relative abundance of 249 proteins was found to be different (**Figure 2a**). A protein–protein interaction-based enrichment analysis was performed with these proteins. We screened for interaction networks and biological processes related to the observed differences in expression using Gene Ontology (GO) analysis of the STRING database (version 10.5). Based on the GO nomenclature and protein composition, the identified biological processes could be classified into the following categories: development, proliferation, regulation of expression and response to stimulus. The ten most significantly different biological processes of each category are listed in **Figures 2b** and **2c**.

a)



b)



c)

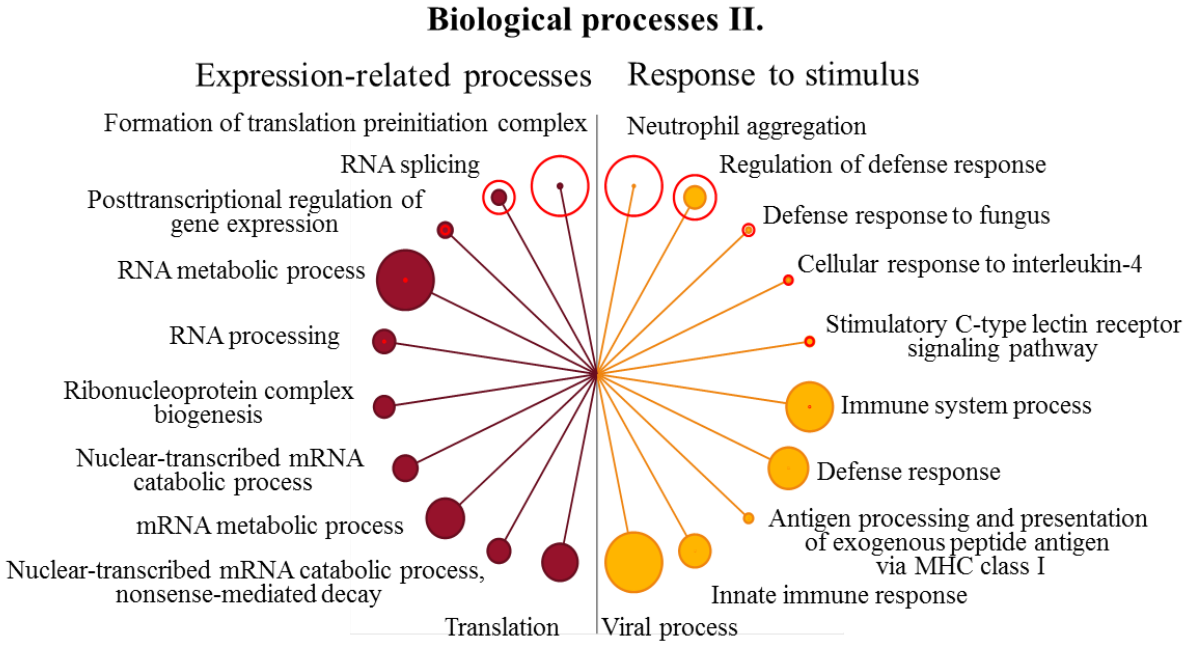
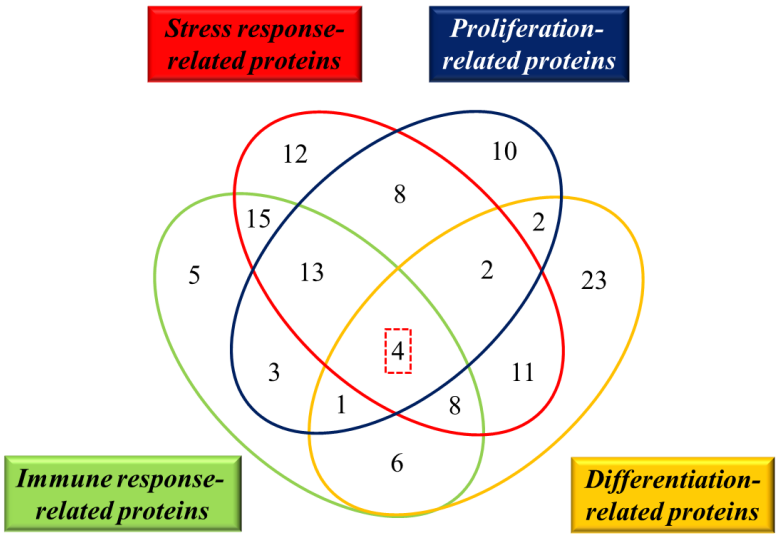


Figure 2. Characterisation of altered protein expression of lesional (L) skin compared to healthy (H) skin. (a) Heatmap of relative expression of proteins differentially expressed in L and H skin (left column), **and their expression in non-lesional (NL) and H skin** (middle column) **and NL and L skin** (right column). (*Significant difference in relative protein expression at least by two-fold in L and H comparison.) **(b), (c) Biological processes for which proteins were differentially expressed in L and H**. The top ten processes are depicted for proliferation (b, left, green circles) and

development (b, right, blue circles), expression (c, left, filled red circles) and response to stimulus (c, right, orange circles). False detection rate (FDR) values are indicated with unfilled red circles around the filled circles for the various biological processes. The size of each circle is proportional to FDR values (unfilled circles) or to the number of proteins (filled circles).

Since the major characteristics of psoriatic alterations include altered stress and immune responses, as well as dysregulation of proliferation and differentiation, we screened among proteins expressed differentially in lesion compared to healthy skin for central regulators participating in all four of these mechanisms (**Figure 3a**). As a result, four central proteins — MYBBP1A, PML, PRKDC and STAT1 — were identified, however, only STAT1 had previously been associated with psoriasis (PML had been related to psoriasis as ”progressive multifocal leukoencephalopathy” not as “promyelocytic leukemia protein”) (**Figure 3b**).

a)



b)

Protein name	Gene ID of protein	Association with psoriasis
Myb-Binding Protein 1A	MYBBP1A	novel
Protein PML	PML	novel
DNA-Dependent Protein Kinase Catalytic Subunit	PRKDC	novel
Signal Transducer And Activator Of Transcription 1	STAT1	known

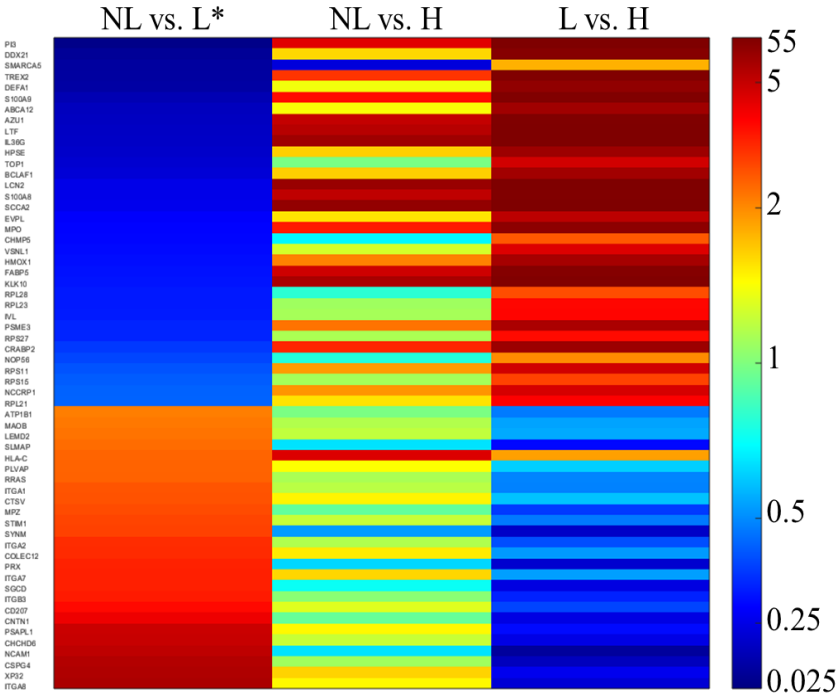
Figure 3. (a) Four proteins differentially expressed in healthy and lesional skin are believed to participate in all four mechanisms of stress, immune response, proliferation and differentiation, (b) however, only STAT1 has already been associated with psoriasis.

To determine which lesional alterations and to what extent are manifest in non-lesional skin, we selected the 249 proteins that exhibited differential expression in healthy and lesional skin, and their expression level was compared to those in non-lesional skin. In non-lesional skin, the expression of 79.9% (199) of the 249 proteins differed from the expression in healthy and lesional skin by less than two-fold. Therefore, this category was termed as intermediate, as they may represent a discrete step in the healthy-to-lesional transition.

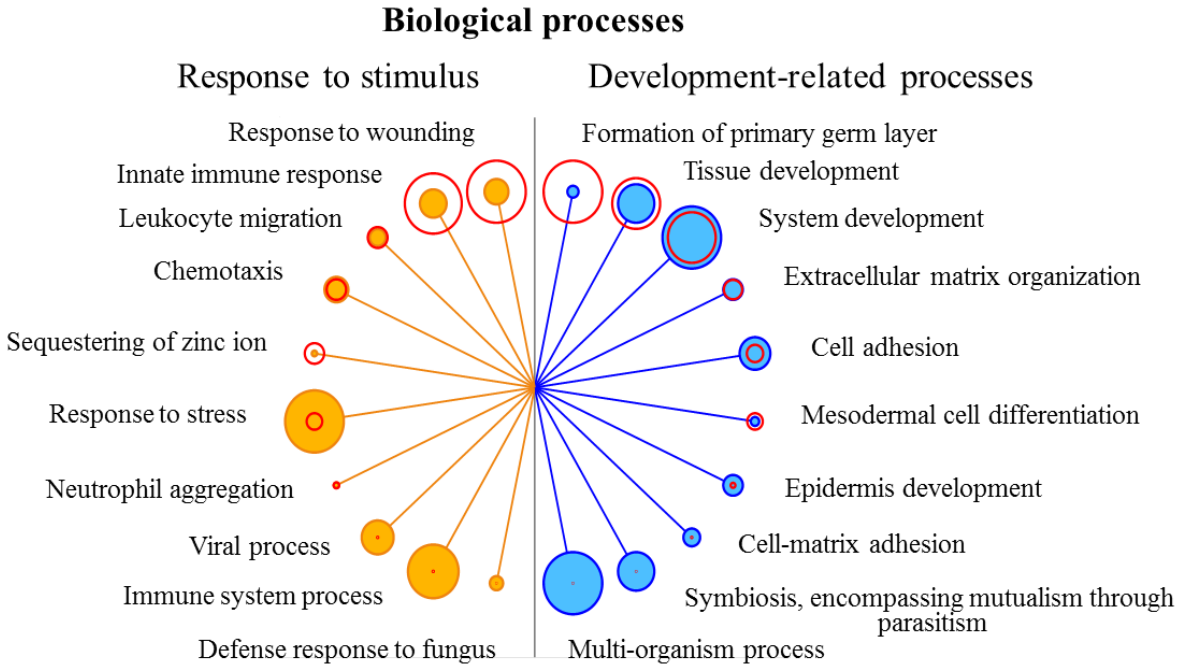
4.1.2 Differential protein expression in non-lesional and lesional skin, and the biological processes associated with these proteins

Comparison of non-lesional and lesional skin proteomes led to the identification of 60 proteins exhibiting at least two-fold differences in relative abundances. Of these proteins, 34 exhibited lower protein abundance in non-lesional skin compared to lesions, whereas 26 exhibited higher abundance (**Figure 4a**). Functional enrichment analysis of these 60 proteins revealed several biological processes identified in psoriasis pathomechanism, including development and response to stimulus (**Figure 4b**). We also found a subset of proteins to be differentially expressed in non-lesional and lesional skin, that were not differentially expressed in healthy skin and lesions (**Figure 4c**).

a)


NL vs. L*
NL vs. H
L vs. H
PI3
DDX21
SMARCA5
TREX2
DEFA1
S100A9
ABCA12
AZU1
LTF
IL36G
HPSE
TOP1
BCLAF1
LCN2
S100A8
SCCA2
EVPL
MPO
CHMP5
VSNL1
HMOX1
FABP5
KLK10
RPL28
RPL23
IVL
PSME3
RPS27
CRABP2
NOP56
RPS11
RPS15
NCCRP1
RPL21
ATP1B1
MAOB
LEMD2
SLMAP
HLA-C
PLVAP
RRAS
ITGA1
CTSV
MPZ
STIM1
SYNM
ITGA2
COLEC12
PRX
ITGA7
SGCD
ITGB3
CD207
CNTN1
PSAPL1
CHCHD6
NCAM1
CSPG4
XP32
ITGA8
55
5
2
1
0.5
0.25
0.025

b)



c)

Protein name	Gene ID of protein	NL vs. L*	L vs. H	NL vs. H	Limma (NL vs. L)	Rankprod (NL vs. L)	Protein function and functional complexes
MICOS complex subunit MIC25	CHCHD6	4.887*	0.248	1.213	0.281	0.014	MICOS complex
Charged multivesicular body protein 5	CHMP5	0.293*	2.376	0.695	0.194	0.044	ESCRT-III complex
Collectin-12	COLEC12	2.842*	0.524	1.489	0.240	0.018	Scavenger receptor
Flotillin-2	FLOT2	2.043*	0.607	1.240	0.394	0.037	CSRM and CIE complex
Integrin alpha-7	ITGA7	2.983*	0.532	1.586	0.329	0.022	Cell surface receptor
LEM domain-containing protein 2	LEMD2	2.163*	0.552	1.194	0.099	0.039	Nuclear lamina and nuclear pore complexes
Nucleolar protein 56	NOP56	0.390*	1.994	0.778	0.281	0.045	snoRNP complex
Plasmalemma vesicle-associated protein	PLVAP	2.285*	0.615	1.406	0.319	0.021	Endothelial diaphragm
Ras-related protein R-Ras	RRAS	2.293*	0.488	1.120	0.258	0.037	Lysosomal degradation
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMARCA5	0.131*	1.767	0.232*	0.050	0.006	Nucleosome remodeling factor complex

Figure 4. Differential protein expression in lesional (L) and non-lesional (NL) skin, and affected biological processes. (a) Heatmap of relative expression for proteins differentially expressed in L and NL skin (left column) and the relative expression of these proteins in NL and healthy (H) skin (middle column) and L and H skin (right column). (*Significant difference in relative protein expression at least by two-fold in L and NL comparison.) **(b) Biological processes for which proteins were differentially expressed in L and NL are listed.** The top ten processes depicted to be affected

in response to stimulus (left, filled orange circles) and development (right, filled blue circles). False detection rate (FDR) values are indicated with unfilled red circles around the filled circles for the various biological processes. The size of each circle is proportional to FDR values (unfilled red circles) or to the number of proteins (filled circles). **(c) Proteins differentially expressed in L and NL but not in H and L skin are listed.** (*Significant difference in relative protein expression at least by two-fold in L and NL comparison.)

The levels of 8 proteins were greater in non-lesional skin and lower in lesional skin compared to the levels in healthy skin (non-lesional>healthy>lesional), and 1 protein exhibited the opposite trend (non-lesional<healthy<lesional). Although the non-lesional and lesional differences in the abundance of these proteins were mostly not statistically significant when compared to healthy skin, the difference in abundance between non-lesional and lesional samples differed significantly by more than two-fold (**Table 2**).

Protein name	Gene ID of protein	NL vs. L*	L vs. H	NL vs. H	Limma (NL vs. L)	Rankprod (NL vs. L)
Integrin alpha-7	ITGA7	2.983*	0.532	1.586	0.329	0.022
Plasmalemma vesicle-associated protein	PLVAP	2.284*	0.615	1.406	0.319	0.021
Cathepsin L2	CTSV¥	2.431*	0.596	1.450	0.213	0.020
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMARCA5	0.131*	1.768	0.232*	0.050	0.006
Collectin-12	COLEC12	2.842*	0.524	1.489	0.240	0.018
Skin-specific protein 32	XP32¥	6.118*	0.262	1.601	0.010	0.005
Proactivator polypeptide-like 1	PSAPL1¥	4.776*	0.300*	1.432	0.012	0.005
Integrin alpha-8	ITGA8	6.435*	0.222*	1.43	0.035	0.026
C-type lectin domain family 4 member K	CD207¥	3.335*	0.387*	1.292	0.051	0.039

Table 2. Proteins for which the changes in non-lesional (NL) and lesional (L) skin compared to healthy (H) skin are in different directions (increased vs. decreased and vice versa) (*Significant difference in relative protein expression at least by two-fold in L and NL comparison. ¥: indicates proteins with known association with psoriasis.)

4.1.3 Comparison of protein expression in non-lesional and healthy skin

Proteins that were differentially expressed in non-lesional skin compared to healthy were also identified. 7 proteins exhibited higher expression levels in non-lesional skin compared to healthy skin and 1 showed lower expression (**Table 3**). Out of the 8 proteins GBP1, KLK10 and S100A7 have already been associated with psoriasis pathogenesis, while the other 5 are

potential novel, early markers of the disease. The relative amount of 4 proteins (GART, CSE1L, GBP1 and UGDH) was similar in the non-lesional and lesional skin samples.

Protein name	Gene ID of protein	NL vs. H*	L vs. H	NL vs. L	Limma (NL vs. H)	Rankprod (NL vs. H)
Trifunctional purine biosynthetic protein adenosine-3	GART	4.451*	4.325	1.029	0.389	0.047
Guanylate-binding protein 1	GBP1¥	5.925*	4.745*	1.249	0.161	0.046
Kallikrein-10	KLK10¥	6.549*	20.793*	0.315	0.060	0.047
Exportin-2	CSE1L	7.204*	5.799	1.242	0.253	0.024
Protein S100-A7	S100A7¥	7.519*	14.74*	0.510	0.027	0.024
Unconventional myosin-XVIIIa	MYO18A	8.191*	14.777*	0.554	0.027	0.024
UDP-glucose 6-dehydrogenase	UGDH	10.175*	8.269*	1.231	0.027	0.024
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMARCA5	0.232*	1.767	0.131*	0.050	0.006

Table 3. Differentially expressed proteins in non-lesional (NL) and healthy (H) skin. Proteins with expression that differs at least by two-fold in NL skin and H skin are listed. (*: indicates statistical significance, ¥: indicates proteins with known association with psoriasis, L: lesional skin.)

4.1.3.1 Verification of the proteomic results: UGDH expression in healthy, non-lesional and lesional skin

To verify our proteomic results, immunofluorescent staining was performed to gain additional information regarding protein localisation, deposition and distribution. UGDH had the largest expression differences in non-lesional and healthy skin. As UGDH had not been linked to psoriasis previously, this protein was chosen for further analysis. UGDH staining showed similar epidermal distribution in all three sample types, with the highest protein levels detected in basal keratinocytes (n=10 different individuals in each group). Despite the similarities in the UGDH localisation pattern, clear differences in staining intensities were observed. The non-lesional and lesional psoriatic samples displayed more robust intensities compared to that of healthy samples, confirming our proteomic results (**Figure 6**).

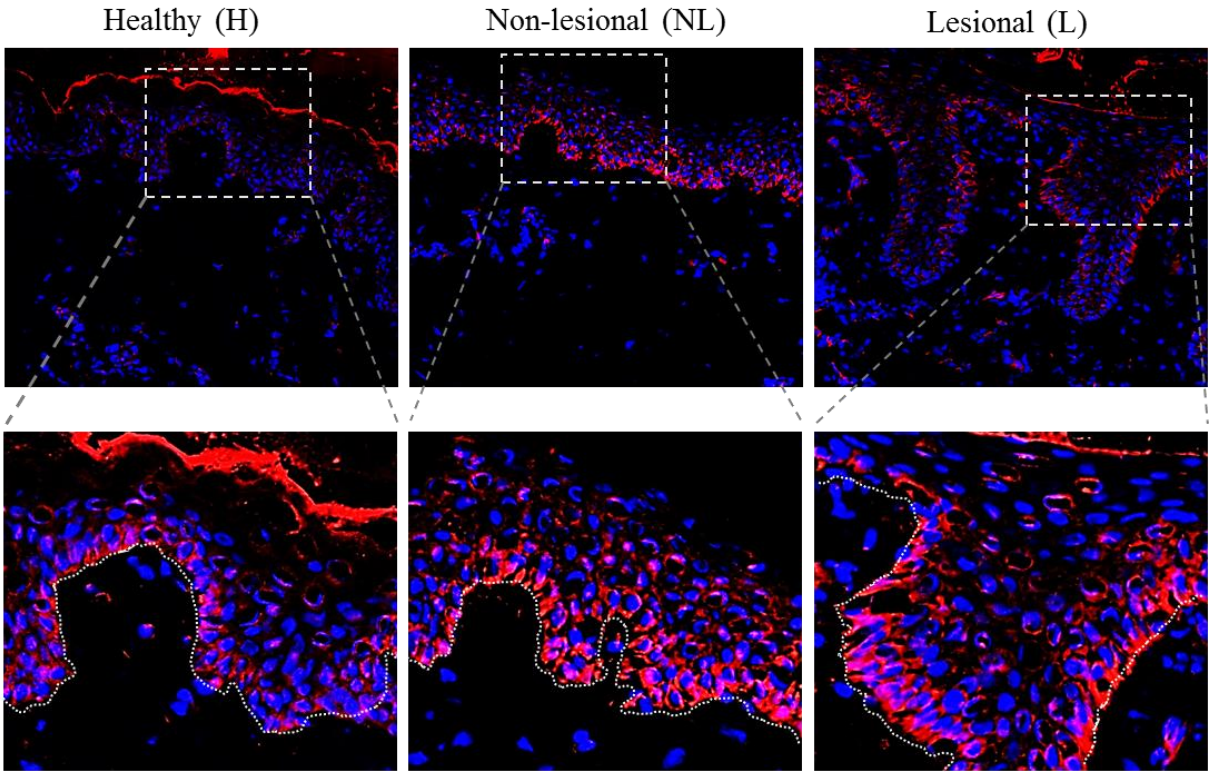


Figure 6. **UGDH protein expression is similarly increased in non-lesional (NL) and lesional (L) psoriatic skin compared to healthy (H) controls**. The highest difference in expression for NL and H was seen with immunohistochemical characterisation of UGDH (n=10), which indicated similar patterns of distribution in the three sample types. The strongest staining was observed in basal keratinocytes, and weaker staining was observed in the upper parts of the epidermis. Higher intensity staining of UGDH was observed in NL and L skin compared to H skin A higher magnification of the epidermis is also provided (DAPI nuclear staining and UGDH are shown in blue and red, respectively.)

4.1.4 Known and novel psoriasis-associated trigger proteins

We also identified 44 proteins that had altered expression only in the comparison of lesional skin to either non-lesional or healthy skin. It is anticipated that these proteins play a role in the manifestation and/or maintenance of lesions. The results of a computer-aided, keyword-based literature search suggests that, of these 44 proteins, 23 are already associated with the disease (**Figure 5a**), whereas 21 have not yet been associated with psoriasis pathogenesis (**Figure 5b**).

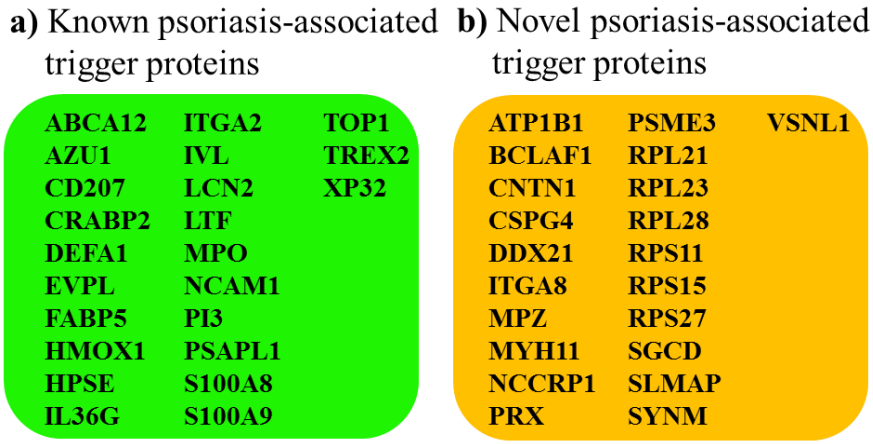


Figure 5. Proteins that exhibited altered expression only in lesions (potentially trigger proteins) with known (a) and novel (b) association with psoriasis

Proteins without previous association to psoriasis that exhibited decreased expression in lesions compared to expression in both non-lesional and healthy skin include modulators of apoptosis, signaling, endothelial cell proliferation, neurite outgrowth, migration, resistance to mechanical stress, cell–cell and extracellular matrix interactions, myelination of peripheral nerves, osmotic and membrane potential regulation. In contrast, proteins with increased expression in lesions compared to both non-lesional and healthy skin are involved in cell death, cell proliferation, transcription and translation, Ca^{2+} sensing (neuronal) and processing of class I MHC peptides.

4.1.5 Psoriatic biomarkers, biological functions, canonical pathways and annotation of diseases associated with the detected alterations in protein amounts

To examine the validity of our experimental approach, we further screened our proteomic dataset (lesional vs. healthy) for known, major biomarkers characteristic of psoriasis. These were identified previously in large-scale genomic, transcriptomic and/or proteomic studies. Out of these biomarkers AKR1B10, CSTA, FABP5, PI3, SCCA2, STAT1, STAT3 and members of the S100 family, including S100A2 and S100A7-9 were also found in our study. These molecules exhibited elevated expression levels in psoriatic lesions, compared to healthy control skin (**Table 4**).

Gene ID of protein	L vs. H	NL vs. H	NL vs. L
AKR1B10	32.769*	25.318	0.773
CSTA	2.335*	1.752	0.75
FABP5	15.076*	4.678	0.31*
PI3	52.616*	4.105	0.078
S100A2	5.878*	2.527	0.43
S100A7	14.74*	7.519	0.51
S100A8	20.639*	5.234	0.254*
S100A9	19.679*	3.306	0.168*
SCCA2	35.468*	9.221	0.26*
STAT1	20.504*	14.478	0.706
STAT3	3.766*	2.309	0.613

Table 4. Detected expressional differences of classic protein biomarkers for psoriasis (*: indicates statistical significance, L: lesional, NL: non-lesional psoriatic skin, H: healthy skin).

Further analysis was performed to identify the cellular mechanisms that may be associated with the proteins that were detected in altered amounts in the proteomic approach, using the IPA software. Diseases annotation revealed "psoriasis" as the first hit when lesional and healthy, or lesional and non-lesional differences were compared. Annotation of biological functions by IPA highlighted "initiation of protein translation" and "killing of *Staphylococcus aureus*" as the main functions likely to be affected, respectively. Ingenuity canonical pathway screening identified the "role of IL-17A in psoriasis" among the top ten most significant canonical pathways, when either lesional or non-lesional protein expression was compared to healthy samples. In addition, several cancer, neurological, neuromuscular canonical pathways were also highlighted.

Detailed proteomic results are found in the main article (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6684579/) and in the supplementary material (https://www.nature.com/articles/s41598-019-47774-5#Sec18).

4.2 *In vitro* results

4.2.1 Effects of glycerol and xylitol on the viability of HaCaT cells exposed to hyperosmotic stress

During a period of 24 h, osmotic stresses of 450 and 500 mOsm did not influence cellular viability, and the average cytotoxicity values were negligible. However, 600 mOsm, sorbitol-containing solution significantly reduced viability. The reduction was also considerable in the

additional polyol-treated groups, but glycerol treatment resulted in significantly higher viability as compared to the positive control (DMEM-HG + sorbitol (600 mOsm)) group. 0.45% xylitol failed to improve the survival of the cells. The average cytotoxicity value of the 0.27% glycerol + sorbitol (600 mOsm)-treated group was somewhat lower than that of its control group (DMEM-HG + sorbitol (600 mOsm)), but the difference was not significant. Viability and cytotoxicity in groups exposed to 0.27% glycerol and 0.45% xylitol alone did not differ significantly from that of their matched DMEM-HG control group (**Figure 7**).

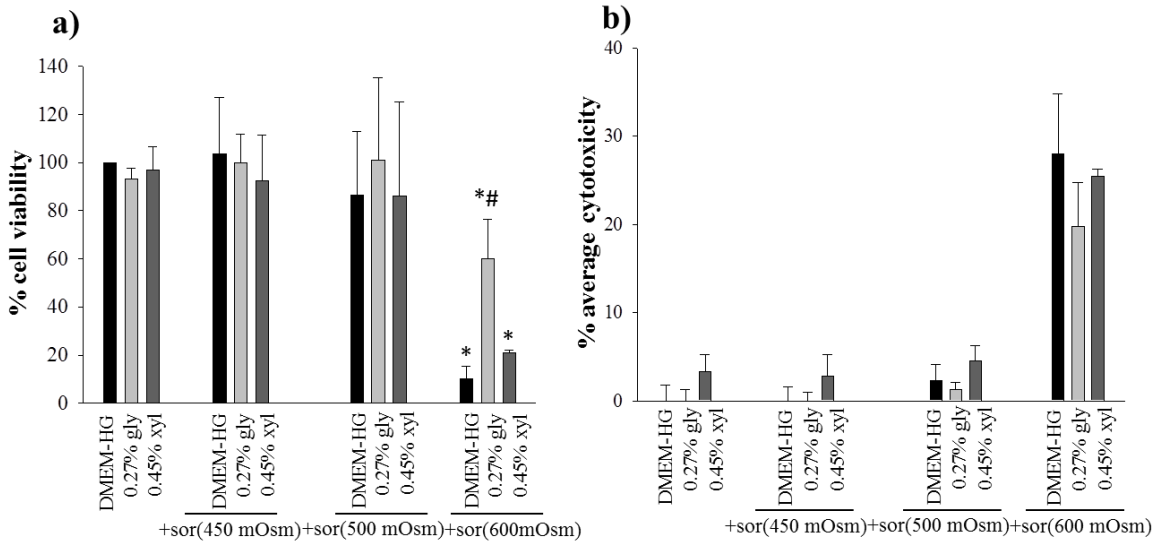


Figure 7. (a) The effects of glycerol and xylitol on cellular viability in hyperosmotic stress. (b) The effects of polyols in osmotic stress-induced cytotoxicity. (DMEM-HG: Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, sor: sorbitol, gly: glycerol, xyl: xylitol.) Statistical analysis was performed with one-way ANOVA and Holm-Sidak post-hoc test (mean+SD), *$p<0.05$ vs. DMEM-HG., #$p<0.05$ vs. DMEM-HG + sor (600 mOsm), n=3.

4.2.2 0.45% xylitol protected against the hyperosmotic stimulus-induced increase in intracellular Ca^{2+} concentration

Hyperosmotic stress induced by 450 mOsm, sorbitol-containing solution was accompanied by a short elevation of intracellular Ca^{2+} concentration. This elevation (M=328.6 nM, 25p=232.0, 75p=602.4) was prevented neither by glycerol nor the lower concentration of xylitol, but it was suppressed by the higher concentration (0.45%) of xylitol (M=78.76 nM, 25p=45.92, 75p=140.72) (**Figure 8a**). **Figure 8** also shows two representative curves of this response from the sorbitol-treated (**Figure 8b**) and the sorbitol and 0.45% xylitol-treated groups (**Figure 8c**).

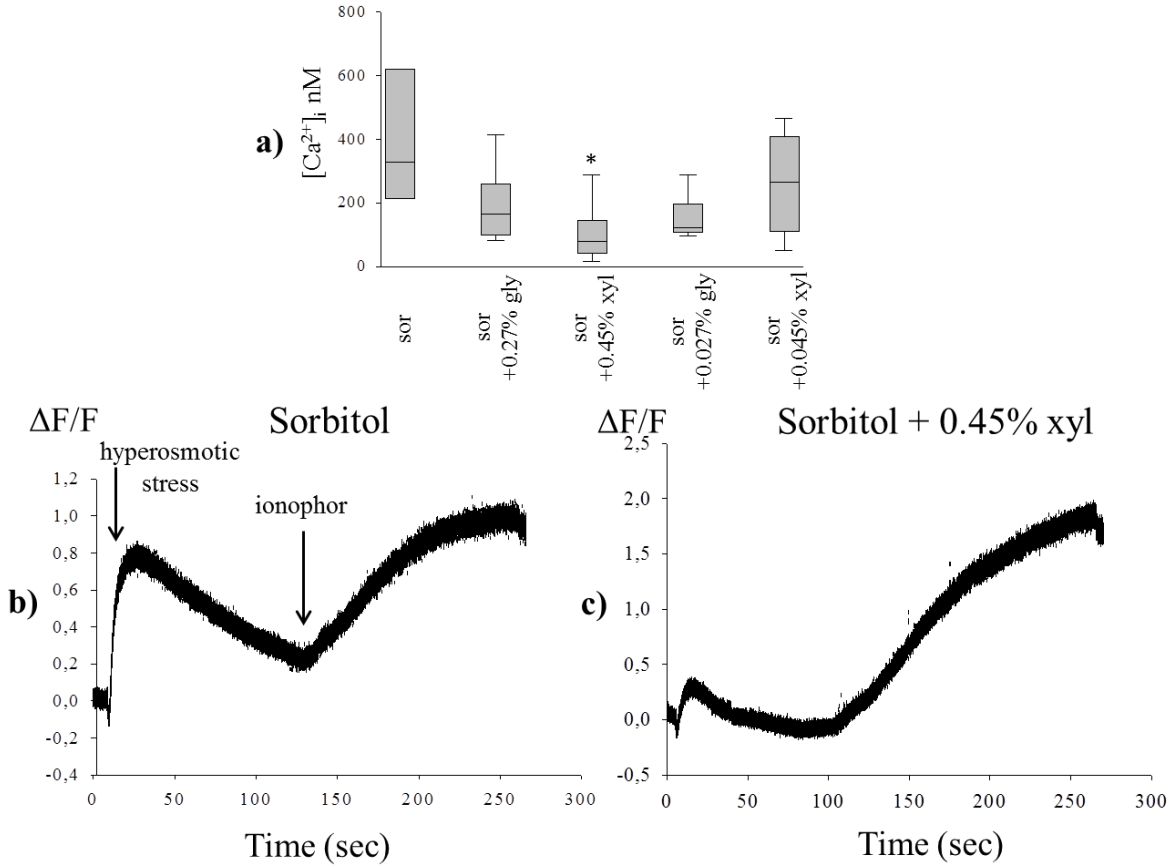


Figure 8. (a) Xylitol protected against the hyperosmotic stimulus-induced increase in intracellular Ca^{2+} concentration. (Sor: sorbitol, gly: glycerol, xyl: xylitol.) Statistical analysis was performed with Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method (median, 25th and 75th percentile), *p<0.05 vs. sor, n=8-10. **(b-c) A representative curve of the relative fluorescence on sorbitol (450 mOsm) (b) and additional xylitol-treated group (c)**

4.2.3 Preventive effects of polyols on the elevation in the mRNA expression of inflammatory cytokines and NFAT5 induced by osmotic stress

The mRNA expression of inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6 and IL-8) and the osmosis-related NFAT5 was measured after 2 and 6 h hyperosmotic stress. After 6 h, no significant changes were detected (data not presented). Following 2 h treatment, the cytokine levels of glycerol- or xylitol-treated groups were similar to that of the untreated control group. 450 mOsm, sorbitol-containing medium induced considerable increase in IL-1α, IL-1β, IL-8 and NFAT5 expression. Both 0.27% glycerol and 0.45% xylitol prevented the elevation in the expression of IL-1α (**Figure 9a**). Furthermore, both 0.27% glycerol and 0.45% xylitol led to considerably lower expression of IL-8 in osmotic stress; the expression levels did not differ significantly from those of the untreated control group (**Figure 9d**). As

concerns IL-1β and NFAT5, only 0.27% glycerol diminished considerably their expression (**Figure 9b, c**). No significant difference was found among the six study groups in TNF-α and IL-6 expression (data not shown).

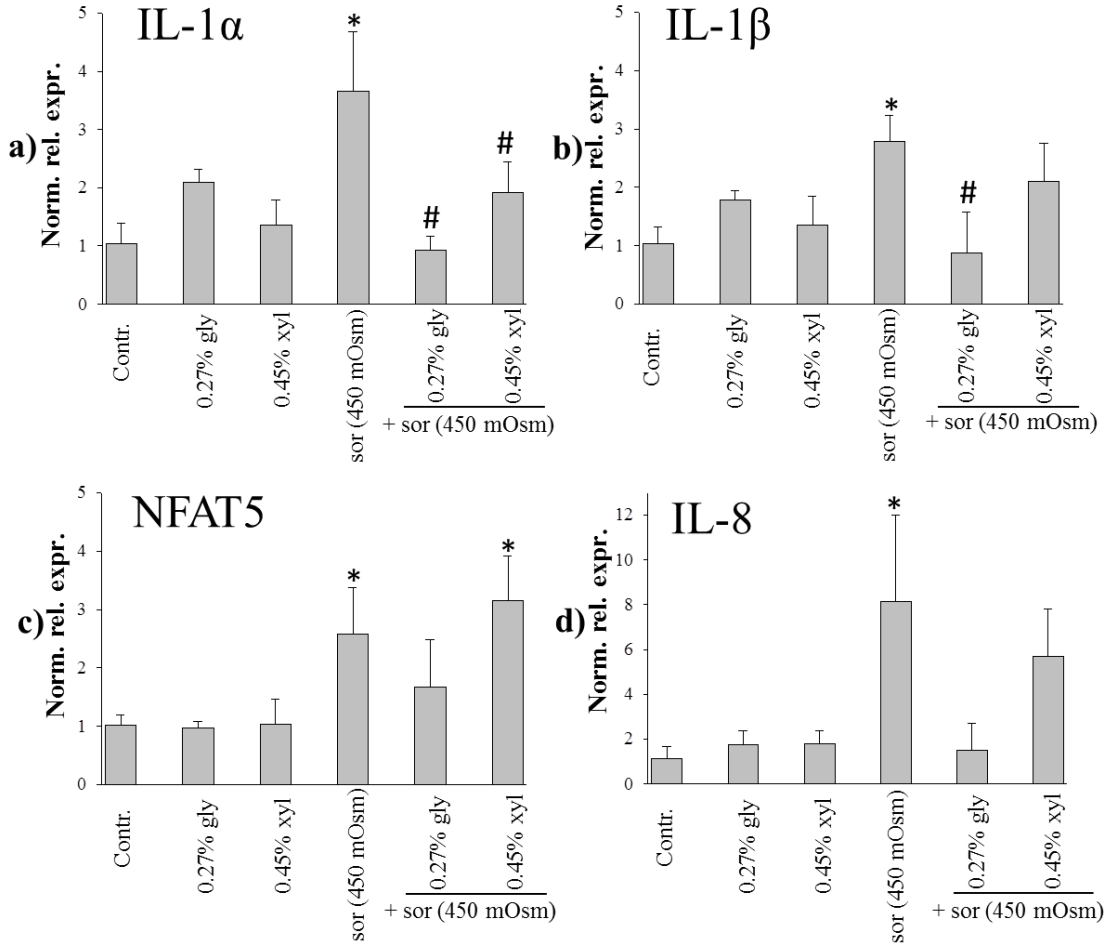


Figure 9. ***In vitro*** **effects of polyols on (a) IL-1α, (b) IL-1β, (c) NFAT5 and (d) IL-8 expression** (Sor: sorbitol, gly: glycerol, xyl: xylitol.) The expression of each gene was normalised to the 18S rRNA gene and relative mRNA levels were calculated by the ΔΔCt method, compared to the untreated, time-matched control samples. Statistical analysis was performed with one-way ANOVA and Holm-Sidak post-hoc test (mean+SD), *p<0.05 vs. Contr., #p<0.05 vs. sor (450 mOsm), n=3 (a-c) and Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method (median, 25th and 75th percentile), *p<0.05 vs. Contr., n=3 (d). Despite the difference in the statistical tests, mean+SD values are shown in all subfigures for uniform presentation.

5. DISCUSSION

To expand knowledge about the pathomechanism of psoriasis, many extensive, large-scale comparative proteomic approaches have been performed[11,20,26]. However, the comparison of healthy, non-lesional and lesional skin at the proteomic level has been missing from these studies. To fill this gap, our proteomic analysis included healthy skin as well as non-lesional and lesional psoriatic samples.

In order to check the validity of our proteomic approach, we compared major known psoriatic biomarkers published in previous genetic (genome-wide association studies)[15], transcriptomic[13,15,18] and proteomic studies[11,12,14,17] with our proteomic dataset. Known psoriatic lesional biomarkers also found in our study include AKR1B10[10], CSTA[11], FABP5[12], PI3[13], SCCA2[14], STAT1[10], STAT3[15], S100A2[16], S100A7[12–14], S100A8[13,17] and S100A9[13,14,17,18]. Moreover, annotation of diseases resulted in the identification of psoriasis with the strongest correlation based on differentially expressed proteins in either lesional vs. healthy or in lesional vs. non-lesional comparison. Canonical pathway analysis of either lesional or non-lesional differences compared to healthy skin resulted in the identification of the "role of IL-17A in psoriasis". However, these annotations also highlighted cancer, neurological, neuromuscular or muscular disease-related mechanisms, suggesting their potential involvement in disease pathomechanism, or some similarities between these diseases.

Since our proteomic and *in silico* analysis cannot distinguish between cell types and provide information whether mechanistically linked alterations take place within the same or different cell types, further experiments are required to clarify the exact relevance of these predicted connections to psoriasis pathomechanism.

We performed a literature search for known functions of proteins found to be altered in amounts in our study to suggest mechanisms through which they may potentially participate in the pathomechanism of the disease. The detected differences in the expression of proteins in healthy and lesional skin highlighted the involvement of cell proliferation[70], development[71], response to stimulus[72] and expression[73]-related processes in psoriasis. Comparing non-lesional and lesional skin, we identified 60 proteins with differential expression, which represent only 24.1% of the number of proteins, which showed altered expression in the comparison of healthy and lesional skin (60 vs. 249). This highlights the importance of

studying healthy skin in comparisons using patient samples for pinpointing disease-associated alterations. Qualitative literature-based analysis of these 60 proteins led to the identification of several mechanisms, the association of which with psoriasis has already been described, including processes related to development[71], response to stimulus[26] and expression[73].

We searched for potential central proteins in disease pathogenesis participating in key mechanisms of psoriasis including regulation of stress and immune response, proliferation and differentiation. Out of the central proteins, STAT1 had already been linked to psoriasis[74], however, we also identified 3 proteins, MYBBP1A, PML and PRKDC, which had not previously been highlighted in context with the disease. The suggested altered expression by our results of the transcription factor MYBBP1A may also be among the potentially important proteins implicated in the pathogenesis of psoriasis since it functions as a co-repressor of NF-κB that may regulate responses to stress and cytokines[75]. An isoform of PML (PML-4) is known to regulate apoptosis and growth suppression, while the nuclear isoforms are involved in gene expression regulation at the MHC-I locus[76]. The PRKDC may play a role in the detection and repair of breaks in double-stranded DNA[77] and mediates the phosphorylation of c-Myc[78] and p53[79] suggesting a potentially important role in psoriasis.

Proteins whose expression was affected only in lesions are often considered “trigger” proteins, as changes in the expression of these proteins are linked to the shift of the disease state. The proteins that had not previously been associated with psoriasis were categorised into two groups. The first group of proteins might contribute to the mechanosensitivity of the tissue (SGCD[80], SYNM[81], MYH11[82], ATP1B1[83]). The second group functions within the nervous system (MPZ, PRX[84], CSPG4[85], CNTN1 and ITGA8[86], ATP1B1[87]), which could suggest the involvement of the peripheral nervous system in psoriasis[88].

By comparing non-lesional and healthy skin, differential expression was observed for 8 proteins (CSE1L, GART, GBP1, KLK10, MYO18A, S1007A, SMARCA5 and UGDH). 4 of these proteins (CSE1L, GART, GBP1 and UGDH) might be predisposing factors, as their expression was similar in non-lesional and lesional skin, and their significance would have been missed in comparisons in which healthy samples were not included. Of these 4 proteins, UGDH was detected with the highest relative difference. UGDH had not been highlighted previously in association with psoriasis. We therefore decided to analyze it further. An immunohistochemical analysis confirmed our proteomic results: higher UGDH levels were

found in non-lesional and lesional skin compared to healthy skin, that was mainly associated with keratinocytes. Elevated UGDH levels may increase chondrocyte proliferation indirectly, probably through increased hyaluronan production that binds different cytokines[89]. However, *in vitro* downregulation of UGDH and consequently decreased hyaluronan amounts did not influence keratinocyte proliferation[90]. These results are in line with our observation, suggesting that elevated UGDH levels observed in non-lesional keratinocytes are not sufficient to modify their proliferation.

A further analysis focused on gaining insight about the extent to which alterations are manifest in lesions and in non-lesional skin. Strikingly, nearly 80% of the 249 proteins exhibiting differential expression in lesional and healthy skin exhibited an intermediate expression level in the non-lesional skin, suggesting the possible presence of early, lesional-like alterations in the non-lesional skin. Divergence from this trend was only observed in two small protein groups. 10 proteins — CHCHD6, CHMP5, COLEC12, FLOT2, ITGA7, LEMD2, NOP56, PLVAP, RRAS and SMARCA5 — differed in relative protein amounts in non-lesional and lesional skin, but the amounts of these proteins were similar in healthy and lesional samples. These 10 proteins are likely to represent a group of non-lesional characteristic alterations. For 9 proteins — CD207, COLEC12, CTSV, ITGA7, ITGA8, PLVAP, PSAPL1, SMARCA5 and XP32 — the direction of the expressional changes was different in non-lesional and lesional samples compared to healthy skin. It might represent proteins that contribute to maintaining the non-lesional state.

Next, with the proteins in these two groups, we performed an extensive literature search to suggest potential mechanisms by which they may influence disease pathogenesis. Interestingly, all the identified proteins may play a role in signaling at different levels starting from the cell surface all the way to the nucleus or mitochondria. The identified cell surface receptors include two integrins (ITGA7 and ITGA8) that are important in external signal recognition. Decreased ITGA7 levels — as observed in lesional vs. non-lesional skin — might be associated with delayed autophagy[91], differentiation[92] and increased migration[93], all known to be affected in psoriatic lesions. In contrast, elevation of ITGA7 may induce growth suppression[94]. However, ITGA7 is characteristically expressed mainly by smooth muscle cells[95] in the skin, suggesting that its involvement in keratinocyte-related events is limited or none. Instead, it may suggest alterations in (vascular) smooth muscle cell adhesion-related

processes[96]. Alternatively, ITGA7 may influence neurite outgrowth[97]. Therefore, further studies are required to confirm the observed ITGA7 expression alteration and to identify the cell types of source.

FLOT2[98], CHMP5[99] and COLEC12[100] participate in endocytic pathways, regulating the levels of cell surface receptors and, thereby, signaling. FLOT2 is a known component of the raft microdomain complex that represents the major unit regulating STAT signaling pathways according to the raft-STAT signaling hypothesis[101]. The alteration of FLOT2 expression could suggest a high relevance since STAT3 is a key regulator in psoriasis[102]. Reduction of the scavenger receptor COLEC12 could trigger psoriasis by trastuzumab treatment[103]. Moreover, COLEC12 may influence the mitochondrial respiratory chain[104], and this property is in agreement with the decreased level of the mitochondrial MICOS complex subunit CHCHD6[105] observed in lesions compared to non-lesional skin. CHCHD6 regulates oxygen consumption and thereby may influence cell growth[106]. Reduced levels of CHCHD6 were shown previously to lead to a shift from oxidative metabolism to glycolytic metabolism[107] that influences keratinocyte differentiation[108] negatively, and both types of mechanisms are known to be affected in psoriasis[109,110].

The altered expression of LEMD2 may suggest that signal transduction is also altered at the level of the nucleus. LEMD2, located in the inner nuclear membrane, regulates nuclear import/export processes[111] and thereby intranuclear signaling[106]. During this regulation, LEMD2 is associated with the same complex as CHMP5, which was also identified in our studies. In the nucleus, the STAT-regulated protein NOP56[112], a core protein of the box C/D small nucleolar ribonucleoprotein (snoRNP) complex, participates in the biogenesis of rRNAs[113]. Increased rRNA biogenesis is suggested to be necessary for high proliferation rate[114], a process that is crucial for the development of psoriatic lesions.

Overall, our results indicate that dysregulation of cellular signaling — from signal detection through endocytosis of receptors and transduction of signal from the cell surface to the nucleus — may be affected during the disease. The alteration of these systems is likely to lead to increased reaction to external signals that could contribute to the maintenance of psoriatic plaques.

During psoriatic plaque formation, abnormal proliferation[70], differentiation[108] and thereby, skin barrier function are key processes. Among proteins identified with altered amount in the

proteomic analysis, SMARCA5 is a component of the nucleosome remodelling factor complex[115]. Decreasing SMARCA5 levels are required for basal keratinocytes to shift from proliferation toward differentiation[116]. XP32 is also a component of the epidermal differentiation complex[117] and it is associated with skin barrier function[118]. Further proteins related to mechanical stress (for example SYNM[81]) and disturbances of osmoregulation (such as ATP1B1[83]) were also identified. These alterations with the abnormal barrier function are characteristic not only of psoriasis, but also of irritant contact dermatitis (ICD).

ICD is a frequent occupational disorder[119], which is characterised by impaired barrier function leading to increased TEWL. Water evaporation exposes keratinocytes to a condition of high osmotic pressure[52]. In our previous animal experiments, sodium lauryl sulfate (SLS)-induced skin irritation resulted in the reduction of epidermal thickness[61] that can be considered as an indirect evidence for osmotic stress. Therefore, the present study was aimed at the investigation of osmotic challenge potentially accompanying ICD. In response to hyperosmotic stress, organic osmolytes are accumulated by the cells. In the skin, betaine, myoinositol and taurine are important osmolytes and the expression of their transporters (betaine/GABA transporter, sodium/myoinositol transporter and taurine transporter) is induced by osmotic stress[120].

The anti-irritant and anti-inflammatory effects of glycerol and xylitol have already been demonstrated in animal experiments[61]. Joint application of glycerol and xylitol increases skin hydration, decreases TEWL, improves biomechanical properties of the skin and induces a higher filaggrin production in the epidermis after 2 weeks of application[62]. However, their cellular mechanism of action has not been revealed in details. We assumed that these polyols may act as organic osmolytes, this way they may have a role in osmoregulation.

Effects of polyols may depend on the applied concentration[61,121]. Hence, two different concentrations were tested in our *in vivo* studies[60,61] and also two different concentrations were chosen for the present experiments, based on previous *in vitro* results[63]. According to the results of the effect of polyols on Ca^{2+} concentration in our preliminary experiments, only the higher polyol concentrations were applied in the measurement of cellular viability, cytotoxicity and cytokine expression.

In order to induce osmotic stress, instead of the ionic sodium chloride and the detergent SLS, sorbitol was chosen, which is a metabolically inactive, inert agent. Sorbitol is also a

polyol osmolyte such as glycerol and xylitol, however, it has no known protective effect in inflammatory skin conditions, and it is used to induce osmotic stress[51].

Osmotic challenge influences cellular viability in an osmolarity- and time-dependent manner[47–50]. Such an effect is also characteristic of ICD: severe irritation of the skin (24 h exposure to SLS) can induce tissue necrosis *in vivo*[122] but milder irritant challenges do not lead to necrosis[123]. In our experiments, 24 h exposure to 450 and 500 mOsm, sorbitol-containing medium did not influence cellular viability and cytotoxicity, but 600 mOsm resulted in a significant decrease in viability as compared to untreated control cells.

However, instead of 600 mOsm, 450 mOsm osmotic stress was applied to measure intracellular Ca^{2+}concentration, in order to examine the protective effects of polyols, with the elimination of cell death. According to our pilot study, 450 mOsm, sorbitol-containing hyperosmotic stimulus was sufficient to induce a short elevation of intracellular Ca^{2+} with kinetics similar to that measured by *Dascalu et al.* who applied 500 mOsm, sucrose-containing solution[49]. Although the exact molecular mechanism is still not fully clarified, dihydropiridine-sensitive Ca^{2+} channels have been described not to be affected[49]. The transient receptor potential vanilloid 1b (TRPV1b) non-selective cation channel, which is expressed also by HaCaT cells[124], can be induced by cell shrinking in hypertonicity[125] and therefore may have a potential role in Ca^{2+} response. In osmotic stress, increased inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) synthesis were observed, as well[126]. TRPV1 can also be activated by Ca^{2+} [127] and DAG[128]. Moreover, IP3 receptors may contribute to Ca^{2+} influx, too[129].

Based on literature data[63], the applied polyols alone did not influence intracellular Ca^{2+} concentration. Our results show that xylitol prevented the elevation of intracellular Ca^{2+} concentration induced by the hyperosmotic sorbitol solution, while glycerol did not influence this parameter. Further investigations are necessary to reveal the reason for this difference and the exact mechanism via which xylitol inhibits Ca^{2+} signal.

In addition to the rapid Ca^{2+} response, the applied osmotic challenge has longer effects, as well. 2 h of exposure to 450 mOsm, sorbitol-containing medium increased the expression of IL-1α, IL-1β and IL-8 in HaCaT cells. *Terunuma and colleagues* have described that cytokine expression elevates in keratinocytes when 500 mOsm of osmotic stimulus is present for 6 h[52]. Cytokine production may originate from the Ca^{2+} response[130]. However, in a recent study,

pro-inflammatory responses evoked with ATP- or bradykinin-induced elevation of the intracellular Ca^{2+} concentration could not be suppressed by glycerol or xylitol. Moreover, none of these polyols influenced the subcellular translocation of the Ca^{2+}-dependent protein kinase C-α (PKC-α). However, xylitol but not glycerol translocated the Ca^{2+}-independent PKC-δ[131]. TRPV1 activation can also lead to enhanced cytokine production via direct or indirect NF-κB activation[132]. Independently from the Ca^{2+} response, hyperosmotic stress also induces the expression of the transcription factor NFAT5[48], which regulates TNF-α[133] and can bind the promoter of IL-1 and IL-6[57]. Thus, elevated expression of NFAT5 might have contributed to the increased cytokine response in the present study. Our previous *in vivo* investigations have already shown the anti-inflammatory effect of glycerol and xylitol[60–62]. However, differences can be found as compared to the present *in vitro* results. Glycerol and xylitol decreased the mRNA expression of IL-1β and TNF-α, but had no effect on the IL-1α levels in a murine model of ICD[61]. Several factors may explain the beneficial effects of polyols on cytokine expression. In addition to the prevention of Ca^{2+} signal and the inhibition of NFAT5 expression, glycerol and xylitol may affect the inflammatory process via stabilising protein structure. As chaperon osmolytes, these polyols are able to enhance protein folding, thereby assisting in the development of the final structure essential for the optimal enzyme function, and they promote protein-protein and protein-DNA interactions[134].

Our findings indicate that glycerol provides protection not only against acute inflammation but also against a more serious damage, which occurs after a relatively longer time-span. The protective effect of glycerol was found to appear after a 24 h exposure to osmotic stress when aquaporin-3 (AQP-3) gene expression shows a peak[135]. If AQP-3 expression increases at protein level, as well, it provides an enhanced intracellular transport of glycerol. This theory is supported by our observation that cellular viability was ameliorated only when glycerol was continuously available (data not shown).

SUMMARY

In this study, a complex comparison of the psoriatic lesional, non-lesional and healthy skin was performed by semi-quantitative proteomic analysis. In addition, the effects of glycerol and xylitol were tested under hyperosmotic conditions as an *in vitro* model of osmotic stress accompanying psoriasis, ICD and other xerotic skin diseases.

- Our comparative proteomic approach of healthy, non-lesional and lesional skin led to the identification of various proteins, which may function in psoriasis pathogenesis, providing a strong base for future studies.
- Proteins exhibiting opposite expression changes in lesional and non-lesional samples compared to healthy skin may function in the maintenance of the non-lesional stage and may represent future targets for therapeutic purposes.
- Glycerol supported cell viability, while xylitol prevented the hyperosmosis-induced Ca^{2+} signal, and both polyol protected against the increased expression of some inflammatory cytokines in hyperosmotic stress.
- Despite the similar chemical structure of glycerol and xylitol, their effects displayed some differences. Hence, joint application of glycerol and xylitol may be a useful therapeutic approach for different skin disorders.

ACKNOWLEDGEMENT

First of all, I would like to thank Prof. Dr. Lajos Kemény for the opportunity to participate in the PhD program of the Doctoral School of Clinical Medicine and to perform my experiments in the laboratory of the Department of Dermatology and Allergology, University of Szeged.

I am thankful to Prof. Dr. Zsuzsanna Bata-Csörgő, Prof. Dr. Márta Széll, Dr. Kornélia Szabó and Hilda Polyánka for their excellent suggestions and invaluable comments related to my laboratory work and during the preparation of the manuscripts.

I am grateful to Prof. Katalin F. Medzihradszky, Dr. Éva Hunyadi-Gulyás and all members of the Proteomic Research Group of the Biological Research Centre (Hungarian Academy of Sciences) for the matterful consultations and performing the proteomic analysis.

It also gives me a great pleasure to acknowledge the ideas and support of Prof. Dr. Shabtay Dikstein.

I owe a large debt of gratitude to my supervisor, Dr. Gergely Groma for his constant availability, inspiration and endless patience.

I would like to express my special thanks to my other supervisor, Dr. Gábor Erős for his valuable advice and ideas during my studies. His colleagues, Dr. Erzsébet Csányi, Dr. József Kaszaki, Dr. Győző Szolnoky, Dr. Petra Hartmann, Dr. Csilla Korponyai and Dr. Döníz Degovics also contributed to the success of publication.

Dr. Károly Acsai, Dr. János Prorok and Dániel Tóth provided the opportunity and assistance to perform experiments at the laboratory of the Department of Pharmacology and Pharmacotherapy, University of Szeged, for which I am very grateful.

I am thankful to Dr. Máté Manczinger who helped with the statistical analysis of the proteomic results.

Dr. Judit Danis merits my thanks for participating in the performance and evaluation of the *in vitro* experiments.

I considered it an honour to introduce two undergraduate students to the laboratory work and research, Renáta Bozó and Evelin Sőrés. They both have considerably contributed to the manual work of this thesis.

Fort the skin sample collection, Dr. Róbert Kui, Dr. Nóra Belső and Dr. Brigitta Gál, for the histological procession of the samples, Dr. István Németh and Róbertné Függ deserve my warmest thanks.

I am most grateful to Mónika Kohajda for her tireless assistance.

I am thankful to all of my colleagues for my having had the opportunity to work with them.

Last but not least I am also very thankful to all members of my family, especially to my husband, for their acceptance, patience and encouragement.

This study was supported by NKFI (formal OTKA) PD116992, K111885 and GINOP-2.2.1-15-2016-00007, GINOP-2.3.2-15-2016-00020 research grants and co-financed by the European Social Fund in the framework of TAMOP-4.2.4.A/2-11-1/2012-0001 “National Excellence Program” A2-SZGYA-FOK-13-0001. I was supported by the National Talent Program (NTP-EFÖ-P-15-0473) provided by the Hungarian Ministry of Human Capacities.

REFERENCES

1. Slominski, A. T., Zmijewski, M. A., Plonka, P. M., Szaflarski, J. P. & Paus, R. How UV Light Touches the Brain and Endocrine System Through Skin, and Why. *Endocrinology* **159**, 1992–2007 (2018).
2. Slominski, A. T. *et al.* Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. *Adv. Anat. Embryol. Cell Biol.* **212**, v, vii, 1-115 (2012).
3. Chiricozzi, A., Romanelli, P., Volpe, E., Borsellino, G. & Romanelli, M. Scanning the Immunopathogenesis of Psoriasis. *Int. J. Mol. Sci.* **19**, (2018).
4. Cristophers, E. & Mrowietz, U. Psoriasis. in *Braun-Falco's Dermatology* (eds. Burgdorf,W.H.C. & Plewig, G.) 506–526 (Springer, 2009).
5. van de Kerkhof, P.C.M. & Nestle, F.O. Psoriasis. in *Dermatology* (eds. Bolognia, J.L. & Jorizzo, J.L.) 135–156 (Elsevier, 2012).
6. Mobini, N., Toussaint, S. & Kamino, H. Noninfectious Erythematous, Papular, and Squamous Diseases. in *Lever's Histopathology of the Skin* 186–187 (Lippincott Williams & Wilkins, 2005).
7. Harden, J. L., Krueger, J. G. & Bowcock, A. M. The immunogenetics of Psoriasis: A comprehensive review. *J. Autoimmun.* **64**, 66–73 (2015).
8. Davidovici, B. B. *et al.* Psoriasis and systemic inflammatory diseases: potential mechanistic links between skin disease and co-morbid conditions. *J. Invest. Dermatol.* **130**, 1785–1796 (2010).
9. Boehncke, W.-H. & Schön, M. P. Psoriasis. *Lancet Lond. Engl.* **386**, 983–994 (2015).
10. Suárez-Fariñas, M. *et al.* Expanding the psoriasis disease profile: interrogation of the skin and serum of patients with moderate-to-severe psoriasis. *J. Invest. Dermatol.* **132**, 2552–2564 (2012).
11. Lundberg, K. C. *et al.* Proteomics of skin proteins in psoriasis: from discovery and verification in a mouse model to confirmation in humans. *Mol. Cell. Proteomics MCP* **14**, 109–119 (2015).
12. Williamson, J. C. *et al.* A proteomics approach to the identification of biomarkers for psoriasis utilising keratome biopsy. *J. Proteomics* **94**, 176–185 (2013).
13. Gudjonsson, J. E. *et al.* Assessment of the Psoriatic Transcriptome in a Large Sample: Additional Regulated Genes and Comparisons with In Vitro Models. *J. Invest. Dermatol.* **130**, 1829–1840 (2010).
14. Piruzian, E. *et al.* Integrated network analysis of transcriptomic and proteomic data in psoriasis. *BMC Syst. Biol.* **4**, 41 (2010).
15. Tsoi, L. C. *et al.* Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nat. Genet.* **44**, 1341–1348 (2012).
16. Jiang, S., Hinchliffe, T. E. & Wu, T. Biomarkers of An Autoimmune Skin Disease--Psoriasis. *Genomics Proteomics Bioinformatics* **13**, 224–233 (2015).
17. Schonthaler, H. B. *et al.* S100A8-S100A9 protein complex mediates psoriasis by regulating the expression of complement factor C3. *Immunity* **39**, 1171–1181 (2013).
18. Oestreicher, J. L. *et al.* Molecular classification of psoriasis disease-associated genes through pharmacogenomic expression profiling. *Pharmacogenomics J.* **1**, 272–287 (2001).
19. Matsuura, T. *et al.* Serum peptides as putative modulators of inflammation in psoriasis. *J. Dermatol. Sci.* **87**, 36–49 (2017).
20. Wang, J. *et al.* Identification of unique proteomic signatures in allergic and non-allergic skin disease. *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* **47**, 1456–1467 (2017).
21. Reindl, J. *et al.* Proteomic biomarkers for psoriasis and psoriasis arthritis. *J. Proteomics* **140**, 55–61 (2016).
22. Chularojanamontri, L., Charoenpipatsin, N., Silpa-Archa, N., Wongpraparut, C. & Thongboonkerd, V. Proteomics in Psoriasis. *Int. J. Mol. Sci.* **20**, 1141 (2019).
23. Ryu, J. *et al.* Proteomic analysis of psoriatic skin tissue for identification of differentially expressed proteins: up-regulation of GSTP1, SFN and PRDX2 in psoriatic skin. *Int. J. Mol. Med.* **28**, 785–792 (2011).

24. Lysvand, H., Helland, R., Hagen, L., Slupphaug, G. & Iversen, O.-J. Psoriasis pathogenesis - Pso p27 constitutes a compact structure forming large aggregates. *Biochem. Biophys. Rep.* **2**, 132–136 (2015).
25. Swindell, W. R. *et al.* Proteogenomic analysis of psoriasis reveals discordant and concordant changes in mRNA and protein abundance. *Genome Med.* **7**, (2015).
26. Méhul, B. *et al.* Noninvasive proteome analysis of psoriatic stratum corneum reflects pathophysiological pathways and is useful for drug profiling. *Br. J. Dermatol.* **177**, 470–488 (2017).
27. Kang, H. *et al.* Exploration of candidate biomarkers for human psoriasis based on gas chromatography-mass spectrometry serum metabolomics. *Br. J. Dermatol.* **176**, 713–722 (2017).
28. Gubán, B. *et al.* Abnormal regulation of fibronectin production by fibroblasts in psoriasis. *Br. J. Dermatol.* **174**, 533–541 (2016).
29. Eyre, R. W. & Krueger, G. G. Response to injury of skin involved and uninvolved with psoriasis, and its relation to disease activity: Koebner and 'reverse' Koebner reactions. *Br. J. Dermatol.* **106**, 153–159 (1982).
30. Ye, L. *et al.* Abnormal epidermal barrier recovery in uninvolved skin supports the notion of an epidermal pathogenesis of psoriasis. *J. Invest. Dermatol.* **134**, 2843–2846 (2014).
31. Gudjonsson, J. E. *et al.* Global Gene Expression Analysis Reveals Evidence for Decreased Lipid Biosynthesis and Increased Innate Immunity in Uninvolved Psoriatic Skin. *J. Invest. Dermatol.* **129**, 2795–2804 (2009).
32. Szabó, K. *et al.* Regulatory networks contributing to psoriasis susceptibility. *Acta Derm. Venereol.* **94**, 380–385 (2014).
33. Henno, A. *et al.* Altered expression of angiogenesis and lymphangiogenesis markers in the uninvolved skin of plaque-type psoriasis. *Br. J. Dermatol.* **160**, 581–590 (2009).
34. Chiricozzi, A. *et al.* Increased expression of interleukin-17 pathway genes in nonlesional skin of moderate-to-severe psoriasis vulgaris. *Br. J. Dermatol.* **174**, 136–145 (2016).
35. Ting, K. M. *et al.* Overexpression of the oncofetal Fn variant containing the EDA splice-in segment in the dermal-epidermal junction of psoriatic uninvolved skin. *J. Invest. Dermatol.* **114**, 706–711 (2000).
36. Szlavicz, E. *et al.* Splicing factors differentially expressed in psoriasis alter mRNA maturation of disease-associated EDA+ fibronectin. *Mol. Cell. Biochem.* **436**, 189–199 (2017).
37. Lee, S. E. & Lew, W. The Increased Expression of Matrix Metalloproteinase-9 Messenger RNA in the Non-lesional Skin of Patients with Large Plaque Psoriasis Vulgaris. *Ann. Dermatol.* **21**, 27–34 (2009).
38. Danis, J., Göblös, A., Bata-Csörgő, Z., Kemény, L. & Széll, M. PRINS Non-Coding RNA Regulates Nucleic Acid-Induced Innate Immune Responses of Human Keratinocytes. *Front. Immunol.* **8**, 1053 (2017).
39. Szegedi, K. *et al.* The anti-apoptotic protein G1P3 is overexpressed in psoriasis and regulated by the non-coding RNA, PRINS. *Exp. Dermatol.* **19**, 269–278 (2010).
40. Göblös, A. *et al.* Keratinocytes express functional CARD18, a negative regulator of inflammasome activation, and its altered expression in psoriasis may contribute to disease pathogenesis. *Mol. Immunol.* **73**, 10–18 (2016).
41. Farkas, A., Kemény, L., Széll, M., Dobozy, A. & Bata-Csörgo, Z. Ethanol and acetone stimulate the proliferation of HaCaT keratinocytes: the possible role of alcohol in exacerbating psoriasis. *Arch. Dermatol. Res.* **295**, 56–62 (2003).
42. Farkas, A. & Kemény, L. Psoriasis and alcohol: is cutaneous ethanol one of the missing links? *Br. J. Dermatol.* **162**, 711–716 (2010).
43. Farkas, A. & Kemény, L. The alcohol metabolite acetaldehyde and psoriasis: another trigger factor? *Clin. Exp. Dermatol.* **35**, 923–925 (2010).
44. Farkas, A. & Kemény, L. Alcohol, liver, systemic inflammation and skin: a focus on patients with psoriasis. *Skin Pharmacol. Physiol.* **26**, 119–126 (2013).

45. Tan, C.-H., Rasool, S. & Johnston, G. A. Contact dermatitis: allergic and irritant. *Clin. Dermatol.* **32**, 116–124 (2014).
46. Li, D.-Q. *et al.* JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp. Eye Res.* **82**, 588–596 (2006).
47. Veltmann, M. *et al.* Osmotic Induction of Angiogenic Growth Factor Expression in Human Retinal Pigment Epithelial Cells. *PloS One* **11**, e0147312 (2016).
48. Lee, J. H. *et al.* NFAT5 induction and its role in hyperosmolar stressed human limbal epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **49**, 1827–1835 (2008).
49. Dascalu, A., Matithyou, A., Oron, Y. & Korenstein, R. A hyperosmotic stimulus elevates intracellular calcium and inhibits proliferation of a human keratinocyte cell line. *J. Invest. Dermatol.* **115**, 714–718 (2000).
50. Zhang, S. *et al.* Hyperosmotic stress enhances interleukin-1beta expression in Helicobacter pylori-infected murine gastric epithelial cells in vitro. *J. Gastroenterol. Hepatol.* **21**, 759–766 (2006).
51. Rodríguez, I. *et al.* Hyperosmotic stress induces phosphorylation of cytosolic phospholipase A(2) in HaCaT cells by an epidermal growth factor receptor-mediated process. *Cell. Signal.* **14**, 839–848 (2002).
52. Terunuma, A., Aiba, S. & Tagami, H. Cytokine mRNA profiles in cultured human skin component cells exposed to various chemicals: a simulation model of epicutaneous stimuli induced by skin barrier perturbation in comparison with that due to exposure to haptens or irritant. *J. Dermatol. Sci.* **26**, 85–93 (2001).
53. Magnúsdóttir, E. *et al.* Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 14988–14993 (2007).
54. Wang, H. *et al.* PKC-α contributes to high NaCl-induced activation of NFAT5 (TonEBP/OREBP) through MAPK ERK1/2. *Am. J. Physiol. Renal Physiol.* **308**, F140-148 (2015).
55. Hiyama, A. *et al.* Activation of TonEBP by calcium controls {beta}1,3-glucuronosyltransferase-I expression, a key regulator of glycosaminoglycan synthesis in cells of the intervertebral disc. *J. Biol. Chem.* **284**, 9824–9834 (2009).
56. Feske, S. Calcium signalling in lymphocyte activation and disease. *Nat. Rev. Immunol.* **7**, 690–702 (2007).
57. Neuhofer, W. Role of NFAT5 in inflammatory disorders associated with osmotic stress. *Curr. Genomics* **11**, 584–590 (2010).
58. Wiig, H. *et al.* Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. *J. Clin. Invest.* **123**, 2803–2815 (2013).
59. Fluhr, J. W., Darlenski, R. & Surber, C. Glycerol and the skin: holistic approach to its origin and functions. *Br. J. Dermatol.* **159**, 23–34 (2008).
60. Korponyai, C., Kovács, R. K., Erös, G., Dikstein, S. & Kemény, L. Antiirritant properties of polyols and amino acids. *Dermatitis* **22**, 141–146 (2011).
61. Szél, E. *et al.* Anti-irritant and anti-inflammatory effects of glycerol and xylitol in sodium lauryl sulphate-induced acute irritation. *J. Eur. Acad. Dermatol. Venereol. JEADV* **29**, 2333–2341 (2015).
62. Korponyai, C. *et al.* Effects of Locally Applied Glycerol and Xylitol on the Hydration, Barrier Function and Morphological Parameters of the Skin. *Acta Derm. Venereol.* **97**, 182–187 (2017).
63. Szabó-Papp J, Sós K, Oláh A, Szöllösi AG, Tóth BI, Czifra G and Bíró T. Differential effects of common moisturizer polyols on normal human epidermal keratinocytes. *J Invest Dermatol* **132**, S58 (2012).
64. Han, S.-J. *et al.* Xylitol inhibits inflammatory cytokine expression induced by lipopolysaccharide from Porphyromonas gingivalis. *Clin. Diagn. Lab. Immunol.* **12**, 1285–1291 (2005).
65. Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. & Somero, G. N. Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222 (1982).
66. Wiśniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **6**, 359–362 (2009).

67. Prorok, J. *et al.* Herpesvirus-mediated delivery of a genetically encoded fluorescent Ca(2+) sensor to canine cardiomyocytes. *J. Biomed. Biotechnol.* **2009**, 361795 (2009).
68. Paredes, R. M., Etzler, J. C., Watts, L. T., Zheng, W. & Lechleiter, J. D. Chemical calcium indicators. *Methods San Diego Calif* **46**, 143–151 (2008).
69. Schwämmle, V., León, I. R. & Jensen, O. N. Assessment and improvement of statistical tools for comparative proteomics analysis of sparse data sets with few experimental replicates. *J. Proteome Res.* **12**, 3874–3883 (2013).
70. Jiang, M. *et al.* TGFβ/SMAD/microRNA-486-3p Signaling Axis Mediates Keratin 17 Expression and Keratinocyte Hyperproliferation in Psoriasis. *J. Invest. Dermatol.* **137**, 2177–2186 (2017).
71. Mali, J. W. Psoriasis: a dynamic disease. *Br. J. Dermatol.* **101**, 725–730 (1979).
72. Schopf, R. E. & Straussfeld, E. Stimulus-dependent increased generation of oxygen intermediates in monocytes and polymorphonuclear leukocytes in psoriasis. *J. Invest. Dermatol.* **84**, 73–76 (1985).
73. Dou, J. *et al.* Integrative analyses reveal biological pathways and key genes in psoriasis. *Br. J. Dermatol.* **177**, 1349–1357 (2017).
74. Shi, X. *et al.* IL-17A upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. *J. Invest. Dermatol.* **131**, 2401–2408 (2011).
75. Owen, H. R. *et al.* MYBBP1a is a novel repressor of NF-kappaB. *J. Mol. Biol.* **366**, 725–736 (2007).
76. Imani-Saber, Z. & Ghafouri-Fard, S. Promyelocytic leukemia gene functions and roles in tumorigenesis. *Asian Pac. J. Cancer Prev. APJCP* **15**, 8021–8028 (2014).
77. Lee, S.-H. & Kim, C.-H. DNA-dependent protein kinase complex: a multifunctional protein in DNA repair and damage checkpoint. *Mol. Cells* **13**, 159–166 (2002).
78. An, J. *et al.* DNA-dependent protein kinase catalytic subunit modulates the stability of c-Myc oncoprotein. *Mol. Cancer* **7**, 32 (2008).
79. Gurley, K. E., Ashley, A. K., Moser, R. D. & Kemp, C. J. Synergy between Prkdc and Trp53 regulates stem cell proliferation and GI-ARS after irradiation. *Cell Death Differ.* **24**, 1853–1860 (2017).
80. Goehringer, C. *et al.* Prevention of cardiomyopathy in delta-sarcoglycan knockout mice after systemic transfer of targeted adeno-associated viral vectors. *Cardiovasc. Res.* **82**, 404–410 (2009).
81. Izmiryan, A. *et al.* Different expression of synemin isoforms in glia and neurons during nervous system development. *Glia* **54**, 204–213 (2006).
82. Smith, A. S. *et al.* Myosin IIA interacts with the spectrin-actin membrane skeleton to control red blood cell membrane curvature and deformability. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E4377–E4385 (2018).
83. Mladinov, D., Liu, Y., Mattson, D. L. & Liang, M. MicroRNAs contribute to the maintenance of cell-type-specific physiological characteristics: miR-192 targets Na+/K+-ATPase β1. *Nucleic Acids Res.* **41**, 1273–1283 (2013).
84. Kim, S. *et al.* Schwann Cell O-GlcNAc Glycosylation Is Required for Myelin Maintenance and Axon Integrity. *J. Neurosci. Off. J. Soc. Neurosci.* **36**, 9633–9646 (2016).
85. Schäfer, M. K. E. & Tegeder, I. NG2/CSPG4 and progranulin in the posttraumatic glial scar. *Matrix Biol. J. Int. Soc. Matrix Biol.* **68–69**, 571–588 (2018).
86. Müller, U., Bossy, B., Venstrom, K. & Reichardt, L. F. Integrin alpha 8 beta 1 promotes attachment, cell spreading, and neurite outgrowth on fibronectin. *Mol. Biol. Cell* **6**, 433–448 (1995).
87. Johar, K., Priya, A. & Wong-Riley, M. T. T. Regulation of Na(+)/K(+)-ATPase by neuron-specific transcription factor Sp4: implication in the tight coupling of energy production, neuronal activity and energy consumption in neurons. *Eur. J. Neurosci.* **39**, 566–578 (2014).
88. Zhu, T. H. *et al.* The Role of the Nervous System in the Pathophysiology of Psoriasis: A Review of Cases of Psoriasis Remission or Improvement Following Denervation Injury. *Am. J. Clin. Dermatol.* **17**, 257–263 (2016).

89. Clarkin, C. E. *et al.* Regulation of UDP-glucose dehydrogenase is sufficient to modulate hyaluronan production and release, control sulfated GAG synthesis, and promote chondrogenesis. *J. Cell. Physiol.* **226**, 749–761 (2011).
90. Malaisse, J. *et al.* Hyaluronan Does Not Regulate Human Epidermal Keratinocyte Proliferation and Differentiation. *J. Biol. Chem.* **291**, 6347–6358 (2016).
91. Tang, Y. *et al.* ART1 promotes starvation-induced autophagy: a possible protective role in the development of colon carcinoma. *Am. J. Cancer Res.* **5**, 498–513 (2015).
92. Ozeki, N. *et al.* Bone morphogenetic protein-induced cell differentiation involves Atg7 and Wnt16 sequentially in human stem cell-derived osteoblastic cells. *Exp. Cell Res.* **347**, 24–41 (2016).
93. Laszlo, V. *et al.* Epigenetic down-regulation of integrin α7 increases migratory potential and confers poor prognosis in malignant pleural mesothelioma. *J. Pathol.* **237**, 203–214 (2015).
94. Tan, L.-Z., Song, Y., Nelson, J., Yu, Y. P. & Luo, J.-H. Integrin α7 binds tissue inhibitor of metalloproteinase 3 to suppress growth of prostate cancer cells. *Am. J. Pathol.* **183**, 831–840 (2013).
95. Wilson, E. Alpha 7 beta 1 integrin: putting the brakes on smooth muscle cell proliferation. *Circ. Res.* **101**, 651–653 (2007).
96. de Rezende, F. F. *et al.* Integrin α7β1 is a redox-regulated target of hydrogen peroxide in vascular smooth muscle cell adhesion. *Free Radic. Biol. Med.* **53**, 521–531 (2012).
97. Plantman, S. *et al.* Integrin-laminin interactions controlling neurite outgrowth from adult DRG neurons in vitro. *Mol. Cell. Neurosci.* **39**, 50–62 (2008).
98. Solis, G. P. *et al.* Reggies/flotillins regulate E-cadherin-mediated cell contact formation by affecting EGFR trafficking. *Mol. Biol. Cell* **23**, 1812–1825 (2012).
99. Shim, J.-H. *et al.* CHMP5 is essential for late endosome function and down-regulation of receptor signaling during mouse embryogenesis. *J. Cell Biol.* **172**, 1045–1056 (2006).
100. Graham, S. A. *et al.* Identification of neutrophil granule glycoproteins as Lewis(x)-containing ligands cleared by the scavenger receptor C-type lectin. *J. Biol. Chem.* **286**, 24336–24349 (2011).
101. Lalazar, G. *et al.* Beta-glycoglycosphingolipid-induced alterations of the STAT signaling pathways are dependent on CD1d and the lipid raft protein flotillin-2. *Am. J. Pathol.* **174**, 1390–1399 (2009).
102. Calautti, E., Avalle, L. & Poli, V. Psoriasis: A STAT3-Centric View. *Int. J. Mol. Sci.* **19**, (2018).
103. Kim, D. H. *et al.* Psoriasis induced by trastuzumab (herceptin®). *Ann. Dermatol.* **25**, 229–231 (2013).
104. Degenhardt, F. *et al.* Genome-wide association study of serum coenzyme Q10 levels identifies susceptibility loci linked to neuronal diseases. *Hum. Mol. Genet.* **25**, 2881–2891 (2016).
105. Ott, C., Dorsch, E., Fraunholz, M., Straub, S. & Kozjak-Pavlovic, V. Detailed analysis of the human mitochondrial contact site complex indicate a hierarchy of subunits. *PloS One* **10**, e0120213 (2015).
106. An, J. *et al.* CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. *J. Biol. Chem.* **287**, 7411–7426 (2012).
107. Chella Krishnan, K. *et al.* Integration of Multi-omics Data from Mouse Diversity Panel Highlights Mitochondrial Dysfunction in Non-alcoholic Fatty Liver Disease. *Cell Syst.* **6**, 103–115.e7 (2018).
108. Monteleon, C. L. *et al.* Lysosomes Support the Degradation, Signaling, and Mitochondrial Metabolism Necessary for Human Epidermal Differentiation. *J. Invest. Dermatol.* **138**, 1945–1954 (2018).
109. Lambert, S., Swindell, W. R., Tsoi, L. C., Stoll, S. W. & Elder, J. T. Dual Role of Act1 in Keratinocyte Differentiation and Host Defense: TRAF3IP2 Silencing Alters Keratinocyte Differentiation and Inhibits IL-17 Responses. *J. Invest. Dermatol.* **137**, 1501–1511 (2017).

110. Zhang, Z. *et al.* Differential glucose requirement in skin homeostasis and injury identifies a therapeutic target for psoriasis. *Nat. Med.* **24**, 617–627 (2018).
111. Gu, M. *et al.* LEM2 recruits CHMP7 for ESCRT-mediated nuclear envelope closure in fission yeast and human cells. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E2166–E2175 (2017).
112. Cowling, V. H., Turner, S. A. & Cole, M. D. Burkitt's lymphoma-associated c-Myc mutations converge on a dramatically altered target gene response and implicate Nol5a/Nop56 in oncogenesis. *Oncogene* **33**, 3519–3527 (2014).
113. Lykke-Andersen, S., Ardal, B. K., Hollensen, A. K., Damgaard, C. K. & Jensen, T. H. Box C/D snoRNP Autoregulation by a cis-Acting snoRNA in the NOP56 Pre-mRNA. *Mol. Cell* **72**, 99–111.e5 (2018).
114. Marcel, V. *et al.* Expression Profiling of Ribosome Biogenesis Factors Reveals Nucleolin as a Novel Potential Marker to Predict Outcome in AML Patients. *PloS One* **12**, e0170160 (2017).
115. Vermeulen, M. *et al.* Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. *Cell* **142**, 967–980 (2010).
116. Mulder, K. W. *et al.* Diverse epigenetic strategies interact to control epidermal differentiation. *Nat. Cell Biol.* **14**, 753–763 (2012).
117. Zhao, X. P. & Elder, J. T. Positional cloning of novel skin-specific genes from the human epidermal differentiation complex. *Genomics* **45**, 250–258 (1997).
118. Toulza, E. *et al.* Large-scale identification of human genes implicated in epidermal barrier function. *Genome Biol.* **8**, R107 (2007).
119. Schwensen, J. F., Friis, U. F., Menné, T. & Johansen, J. D. One thousand cases of severe occupational contact dermatitis. *Contact Dermatitis* **68**, 259–268 (2013).
120. Warskulat, U., Reinen, A., Grether-Beck, S., Krutmann, J. & Häussinger, D. The osmolyte strategy of normal human keratinocytes in maintaining cell homeostasis. *J. Invest. Dermatol.* **123**, 516–521 (2004).
121. Atrux-Tallau, N. *et al.* Effects of glycerol on human skin damaged by acute sodium lauryl sulphate treatment. *Arch. Dermatol. Res.* **302**, 435–441 (2010).
122. Erős G, Kurgyis Z, Németh IB, Csizmazia E, Berkó Sz, Szabó-Révész P, Kemény L, Csányi E. The irritant effects of pharmaceutically applied surfactants. *J Surfact Deterg* **17**, 67–70 (2014).
123. Jibry, N. & Murdan, S. In vivo investigation, in mice and in man, into the irritation potential of novel amphiphilogels being studied as transdermal drug carriers. *Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV* **58**, 107–119 (2004).
124. Pecze, L. *et al.* Human keratinocytes are vanilloid resistant. *PloS One* **3**, e3419 (2008).
125. Sharif Naeini, R., Witty, M.-F., Séguéla, P. & Bourque, C. W. An N-terminal variant of Trpv1 channel is required for osmosensory transduction. *Nat. Neurosci.* **9**, 93–98 (2006).
126. Rodriguez, I. *et al.* Activation of phospholipase C-gamma1 in human keratinocytes by hyperosmolar shock without enzyme phosphorylation. *Arch. Dermatol. Res.* **295**, 490–497 (2004).
127. Zitt, C. *et al.* Expression of TRPC3 in Chinese hamster ovary cells results in calcium-activated cation currents not related to store depletion. *J. Cell Biol.* **138**, 1333–1341 (1997).
128. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H.-T. & Gill, D. L. The cellular and molecular basis of store-operated calcium entry. *Nat. Cell Biol.* **4**, E263-272 (2002).
129. Boulay, G. *et al.* Modulation of Ca(2+) entry by polypeptides of the inositol 1,4, 5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca(2+) entry. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14955–14960 (1999).
130. Szöllősi, A. G. *et al.* Activation of TRPV3 Regulates Inflammatory Actions of Human Epidermal Keratinocytes. *J. Invest. Dermatol.* **138**, 365–374 (2018).
131. Páyer, E. *et al.* Beyond the physico-chemical barrier: Glycerol and xylitol markedly yet differentially alter gene expression profiles and modify signalling pathways in human epidermal keratinocytes. *Exp. Dermatol.* **27**, 280–284 (2018).

132. Huang, J. *et al.* Transient receptor potential vanilloid-1 participates in the inhibitory effect of ginsenoside Rg1 on capsaicin-induced interleukin-8 and prostaglandin E_2 production in HaCaT cells: GRg1 inhibits TRPV1 activation in HaCaT cells. *J. Pharm. Pharmacol.* **64**, 252–258 (2012).
133. López-Rodríguez, C. *et al.* Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. *Immunity* **15**, 47–58 (2001).
134. Brocker, C., Thompson, D. C. & Vasiliou, V. The role of hyperosmotic stress in inflammation and disease. *Biomol. Concepts* **3**, 345–364 (2012).
135. Sugiyama, Y., Ota, Y., Hara, M. & Inoue, S. Osmotic stress up-regulates aquaporin-3 gene expression in cultured human keratinocytes. *Biochim. Biophys. Acta* **1522**, 82–88 (2001).

I.

SCIENTIFIC REPORTS
nature research

OPEN

Comprehensive Proteomic Analysis Reveals Intermediate Stage of Non-Lesional Psoriatic Skin and Points out the Importance of Proteins Outside this Trend

Received: 6 February 2019
Accepted: 28 June 2019
Published online: 06 August 2019

Edit Szél[1], Renáta Bozó[1], Éva Hunyadi-Gulyás[2], Máté Manczinger[1,3], Kornélia Szabó[1,3], Lajos Kemény[1,3], Zsuzsanna Bata-Csörgő[1,3] & Gergely Groma[1,3]

To better understand the pathomechanism of psoriasis, a comparative proteomic analysis was performed with non-lesional and lesional skin from psoriasis patients and skin from healthy individuals. Strikingly, 79.9% of the proteins that were differentially expressed in lesional and healthy skin exhibited expression levels in non-lesional skin that were within twofold of the levels observed in healthy and lesional skin, suggesting that non-lesional skin represents an intermediate stage. Proteins outside this trend were categorized into three groups: I. proteins in non-lesional skin exhibiting expression similar to lesional skin, which might be predisposing factors (i.e., CSE1L, GART, MYO18A and UGDH); II. proteins that were differentially expressed in non-lesional and lesional skin but not in healthy and lesional skin, which might be non-lesional characteristic alteration (i.e., CHCHD6, CHMP5, FLOT2, ITGA7, LEMD2, NOP56, PLVAP and RRAS); and III. proteins with contrasting differential expression in non-lesional and lesional skin compared to healthy skin, which might contribute to maintaining the non-lesional state (i.e., ITGA7, ITGA8, PLVAP, PSAPL1, SMARCA5 and XP32). Finally, proteins differentially expressed in lesions may indicate increased sensitivity to stimuli, peripheral nervous system alterations, furthermore MYBBP1A and PRKDC were identified as potential regulators of key pathomechanisms, including stress and immune response, proliferation and differentiation.

To date, all therapies available for psoriasis only manage symptoms. Understanding alterations that cause the disease is highly important for developing new therapies to better manage the disease.

Our skin connects, and at the same time separates internal the external environment. It is constantly subjected to many different stimuli that requires proper response, through which the skin can influences the function of other organs, like the brain and the endocrine system in a mutual way[1,2]. In psoriasis, the macroscopically healthy looking non-lesional skin harbors alterations that might cause symptoms[3]. One of the most characteristic properties of non-lesional skin is an altered response to mechanical stress or injury[4] leading to barrier disruption[5], which leads to an elevated innate immune response[6,7]. Alterations in non-lesional skin are not restricted to keratinocytes. Angiogenesis is also among those mechanisms that is already affected in non-lesional skin, resulting in altered quantity and quality of microvessels[8]. In addition, it is becoming clear that some adaptive immune responses are also altered[9]. Abnormalities in the dermal extracellular matrix composition — such as elevated expression of the oncofetal splice variant of fibronectin[10], due to altered splicing events[11] indicate the involvement of dermal fibroblasts[3]. Several matrix metalloproteinases (MMPs), such as MMP-9, previously thought to be increased only in lesions, are now known to be elevated in non-lesional skin compared to healthy skin[12]. There is also evidence for mechanisms in non-lesional skin that contribute to the maintenance of its state. The PRINS long non-coding RNA is induced by stress and nucleic acids, and it is anticipated to have a protective

[1]Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary. [2]Laboratory of Proteomics Research, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary. [3]MTA-SZTE Dermatological Research Group, Szeged, Hungary. Correspondence and requests for materials should be addressed to G.G. (email: groma.gergely@med.u-szeged.hu)

function in psoriasis. PRINS in the non-lesional skin not only decreases inflammatory responses[13] by inhibiting IL-6 and CCL-5 mRNA translation, but also influences anti-apoptotic mechanisms[14]. Elevated expression of the anti-inflammatory regulator caspase recruitment domain family member 18 (CARD18) in non-lesional skin compared to healthy skin was found to aid the inhibition of inflammatory events[15]. These mechanisms, among many others, highlight the relevance of comparing non-lesional skin to healthy skin.

One of the most effective ways to study different diseases with such a high complexity and to elucidate related mechanisms is to perform a comparative proteomic analysis of protein extracts derived from affected tissues. Previous large-scale treatises including genomic, transcriptomic and proteomic studies have identified psoriasis-related markers playing key roles in the pathomechanism, such as AKR1B10[16], CSTA[17], FABP5[18], PI3[19], SCCA2[20], STAT1[16], STAT3[21], S100A7[18–20], S100A8[19,22] and S100A9[19,20,22,23], among others, thereby contributing greatly to the better understanding of the disease. However, none of the full scale proteomic studies[17,18,22,24–27] to the best of our knowledge, compared lesional and non-lesional psoriatic full thickness skin regions, with the inclusion of biopsies from healthy individuals as a reference in the comparison. The inclusion of healthy skin could provide several important additional information. I. Alterations that are similar in non-lesional and lesional skin, but differ from healthy skin, can be detected and used to identify potential novel disease markers or predisposing factors already present in the non-lesional skin. II. The comparison of non-lesional skin to healthy skin might facilitate the identification of inherent characteristics of psoriatic patients that are already present in their healthy-looking skin prior to lesion development. III. Information could be gained about the extent to which the non-lesional skin is affected in respect to lesional alterations. IV. Altered processes in the non-lesional skin that are contrary to the changes of lesional skin could be identified, some of which may contribute to the maintenance of the non-lesional state and serve as novel intervention points for disease management. We aimed to extend previous proteomic studies, in order to get more information regarding the putative alterations mentioned above. Therefore, a complex comparison was performed, where in addition to non-lesional and lesional skin, samples from healthy skin were also included, in a label-free, semi-quantitative proteomic analysis.

Results

Proteomic workflow and information on involved donors. Three biological replicas of our proteomic approach were performed following sequential protein extraction of total skin biopsies. Each proteomic replica contained samples from three healthy donors as well as non-lesional and lesional biopsies from three psoriatic patients. The schematic overview of the applied proteomic strategy is summarized in Fig. 1 (also see Supplementary Information: Materials and Methods), and basic demographic and clinical characteristics of psoriatic patients and healthy donors are listed in Table 1. (Criteria for inclusion of patients in the study and skin sample collection are described at Supplementary Material: Materials and Methods section).

Biological processes associated with differential expression in healthy and lesional skin. As an initial step, proteomic results of lesional and healthy skin samples were compared and the relative abundance of 249 proteins was found to be different (Fig. 2a and Supplementary Table 1). A protein–protein interaction-based enrichment analysis was performed with these proteins. We screened for interaction networks and biological processes related to the observed differences in expression using Gene Ontology (GO) analysis of the STRING database (version 10.5). Based on the GO nomenclature and protein composition, the identified biological processes could be classified into the following categories: development, proliferation, regulation of expression and response to stimulus related processes. The ten most significantly different biological processes of each category are listed in Fig. 2b,c and Supplementary Table 2.

Since the major characteristics of psoriatic alterations include altered stress and immune responses as well as dysregulation of proliferation and differentiation, we screened among proteins expressed differentially in lesion compared to healthy skin for central regulators participating in all four of these mechanisms (Fig. 2d). As a result, four central proteins — MYBBP1A, PML, PRKDC and STAT1 — were identified (Fig. 2e).

Differential protein expression in non-lesional and lesional skin and the biological processes associated with these proteins. Comparison of non-lesional and lesional skin proteomes led to the identification of 56 proteins exhibiting at least 2-fold differences in relative abundances. Of these proteins, 32 exhibited higher protein abundance in non-lesional skin compared to lesions, whereas 24 exhibited lower abundance (Fig. 3a and Supplementary Table 3). Functional enrichment analysis of these 56 proteins revealed several biological processes identified in psoriasis pathomechanism, including development, and response to stimulus (Fig. 3b and Supplementary Table 4).

We also found a subset of proteins to be differentially expressed in non-lesional and lesional skin that were not differentially expressed in healthy skin and lesions (Fig. 3c).

The levels of eight proteins were greater in non-lesional skin and lower in lesional skin compared to the levels in healthy skin (non-lesional $<$ healthy $<$ lesional), and one protein exhibited the opposite trend (non-lesional $>$ healthy $>$ lesional). Although the non-lesional and lesional differences in the abundance of these proteins were not statistically significant when compared to healthy skin, the difference in abundance between non-lesional and lesional samples differed significantly by more than two-fold (Fig. 3d).

We also identified 44 proteins that had altered expression only in the comparison of lesional skin to either non-lesional or healthy skin; it is anticipated that these proteins play a role in manifestation and/or maintenance of lesions. The results of a computer-aided, keyword-based literature search suggests that, of these 44 proteins, 23 are already associated with the disease (Fig. 3e), whereas 21 have not yet been associated with psoriasis pathogenesis (Fig. 3f).

Proteins without previous association to psoriasis that exhibited decreased expression in lesions compared to expression in to both non-lesional and healthy skin include modulators of apoptosis, signaling, endothelial cell

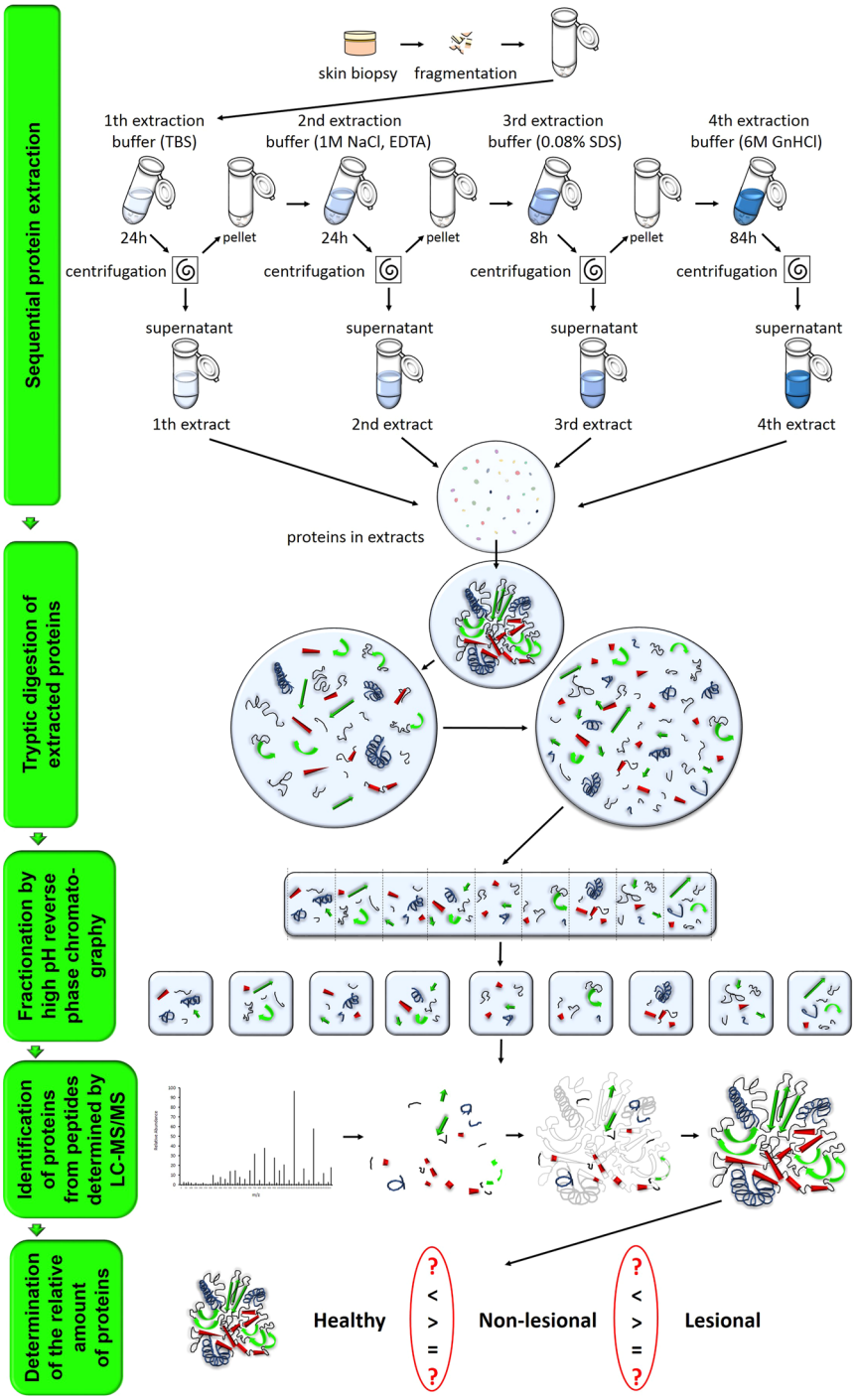


Figure 1. Schematic illustration of the applied proteomic workflow.

proliferation, neurite outgrowth, migration, resistance to mechanical stress, cell–cell and extracellular matrix interactions, myelination of peripheral nerves, osmotic and membrane-potential regulation (Supplementary Table 5). In contrast, proteins with increased expression in lesions compared to both non-lesional and healthy skin are involved in cell death, cell proliferation, transcription and translation, calcium sensing (neuronal) and processing of class I MHC peptides (Supplementary Table 5). To further elucidate the significance in psoriasis of the differential expression of these latter three groups of proteins (Fig. 3c,d,f), a detailed automated literature analysis was conducted for associated known functions.

Comparison of protein expression in non-lesional skin compared to healthy skin. Proteins that were differentially expressed in non-lesional skin compared to healthy were also identified. Seven proteins exhibited higher expression levels in non-lesional skin compared to healthy skin and one with lower expression

Proteomic experiment	Group of donors	Donors	Age	Gender	PASI score
No. 1.	Healthy	H I.	46	Male	n/a
		H II.	59		
		H III.	51		
	Plaque type psoriasis	P I.	65		17.1
		P II.	63		9.9
		P III.	50		5.5
No. 2.	Healthy	H IV.	23	Female	n/a
		H V.	48		
		H VI.	51		
	Plaque type psoriasis	P IV.	25		9.2
		P V.	62		21.5
		P VI.	70		17.5
No. 3.	Healthy	H VII.	37	Male	n/a
		H VIII.	39		
		H IX.	61		
	Plaque type psoriasis	P VII.	49		22.4
		P VIII.	55		12.1
		P IX.	61		12

Table 1. Basic demographic and clinical characteristics of donors involved in the proteomic analysis. (H: healthy donor, P: plaque-type psoriatic patient).

(Fig. 4a). Among these, the relative amount of four proteins (GART, CSE1L, GBP1 and UGDH) was similar in the non-lesional and lesional skin samples. Out of the eight proteins that are differentially expressed in non-lesional skin compared to healthy GBP1, KLK10 and S100A7 have already been associated with psoriasis pathogenesis; the other five are potential novel, early markers of the disease.

To verify our proteomic results, immunofluorescent staining was performed to gain additional information regarding protein localization, deposition and distribution. UGDH had the largest expression differences in non-lesional and healthy skin. As UGDH has not been linked to psoriasis previously, this protein was chosen for further analysis. UGDH staining showed similar epidermal distribution in all three sample types, with the highest protein levels detected in basal keratinocytes (n = 10 different individuals in each group, listed in Supplementary Table 6). Despite the similarities in the UGDH localization pattern, clear differences in staining intensities were observed. The non-lesional and lesional psoriatic samples displayed more robust intensities compared to that of healthy samples, confirming our proteomic results (Fig. 4b,c).

To determine which lesional alterations and to what extent are manifest in non-lesional skin, we selected the 249 proteins that exhibited differential expression in healthy and lesional skin and their expression levels was compared to those in non-lesional skin. In non-lesional skin, the expression of 199 (79.9%) of the proteins differed from the expression in healthy and lesional skin by less than two-fold. Therefore, this category was termed as intermediate, as they may represent a discrete step in the healthy-to-lesional transition (Supplementary Table 7).

Psoriatic biomarkers, biological functions, canonical pathways and annotation of diseases associated with the detected alterations in protein amounts. To examine the validity of our experimental approach, we further screened our proteomic dataset (lesional vs. healthy) for known, major biomarkers characteristic for psoriasis. These were identified previously in by large scale genomic, transcriptomic and/or proteomic studies. Out of these biomarkers AKR1B10, CSTA, FABP5, PI3, SCCA2, STAT1, STAT3 and members of the S100 family, including S100A7, S100A8, S100A9 were also found in our study. These molecules exhibited elevated expression levels in psoriatic lesions, compared to healthy control skin (Table 2).

Further analysis was performed to identify the cellular mechanisms that may be associated with the proteins that were detected in altered amounts in a proteomic approach, using the Ingenuity Pathway Analysis software (IPA). Diseases annotation revealed 'psoriasis' as the first hit when lesional and healthy (Table 3), or lesional and non-lesional differences (Table 3) were compared.

Annotation of biological functions by IPA highlighted 'initiation of protein translation' (Table 3) and 'killing of Staphylococcus aureus' as the main functions likely to be affected, respectively. Ingenuity canonical pathway screening identified the 'role of IL-17A in psoriasis' among the top ten most significant canonical pathways, when either lesional, or non-lesional protein expression was compared to healthy samples (Table 4). In addition, several cancer, neurological and neuromuscular canonical pathways were also highlighted.

Discussion

To expand knowledge about the pathomechanism of psoriasis, many extensive, large-scale comparative proteomic approaches have been performed[17,24,26]. However, the comparison of healthy, non-lesional and lesional skin at the proteomic level has been missing from these studies. To fill this gap, our comparative proteomic analysis included healthy skin as well as non-lesional and lesional psoriatic samples.

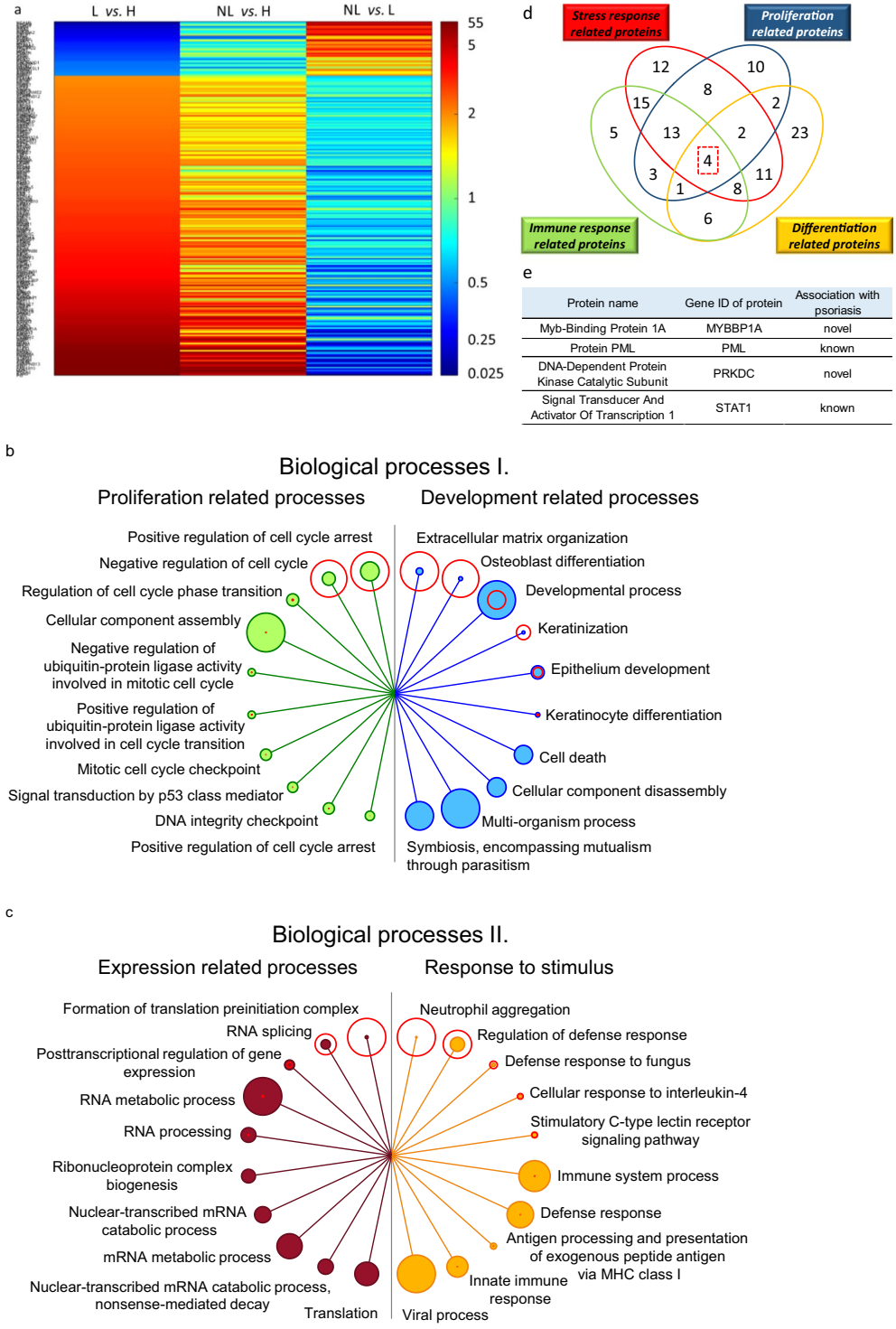
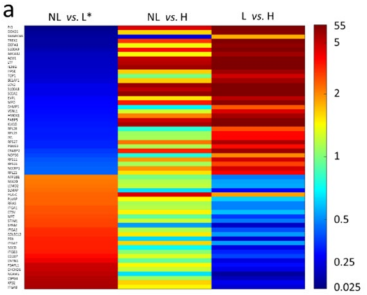


Figure 2. Characterization of altered protein expression of lesional (L) skin compared to healthy (H) skin. Heatmap of relative expression of proteins differentially expressed in L and H skin (**a**, left column), and their expression in non-lesional (NL) and L skin (**a**, middle column) and NL and H skin (a, right column) (**a**). Biological processes for which proteins were differentially expressed in L and H are listed. The top ten processes are depicted for proliferation (**b** left, green circles), development (**b** right, blue circles), expression (**c** left, filled red circles) and response to stimulus (c right, orange circles). False detection rate (FDR) values are indicated with unfilled red circles around the filled circles for the various biological processes. The size of each circle is proportional to FDR values (unfilled circles) or to the number of proteins (filled circles). Four proteins differentially expressed in H and L skin are believed to participate in all four mechanisms of stress, immune response, proliferation and differentiation (**d**) and are listed in (**e**). (*Significant difference in relative protein expression at least by two-fold in L and H comparison).

a



d

Protein name	Gene ID of protein	NL vs. L*	L vs. H	NL vs. H	Limma (NL vs. L)	Rankprod (NL vs. L)
Integrin alpha-7	ITGA7	2.983*	0.532	1.586	0.329	0.022
Plasmalemma vesicle-associated protein	PLVAP	2.284*	0.615	1.406	0.319	0.021
Cathepsin L2	CTSV¥	2.431*	0.596	1.450	0.213	0.020
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMARCA5	0.131*	1.768	0.232*	0.050	0.006
Collectin-12	COLEC12	2.842*	0.524	1.489	0.240	0.018
Skin-specific protein 32	XP32¥	6.118*	0.262	1.601	0.010	0.005
Proactivator polypeptide-like 1	PSAPL1¥	4.776*	0.300*	1.432	0.012	0.005
Integrin alpha-8	ITGA8	6.435*	0.222*	1.43	0.035	0.026
C-type lectin domain family 4 member K	CD207¥	3.335*	0.387*	1.292	0.051	0.039

e

Known psoriasis associated trigger proteins

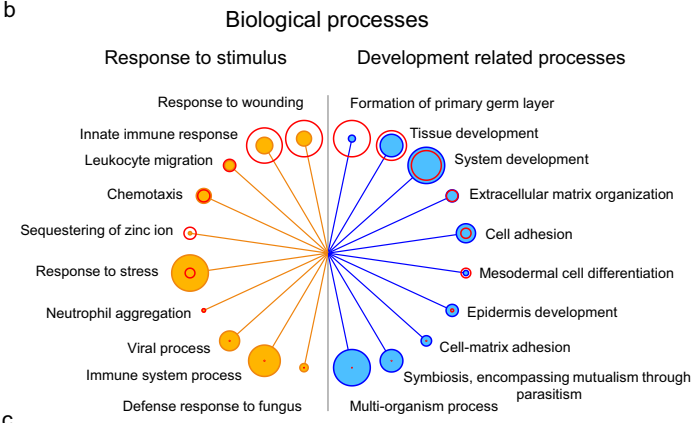
ABCA12	ITGA2	TOP1
AZU1	IVL	TREX2
CD207	LCN2	XP32
CRABP2	LTF	
DEFA1	MPO	
EVPL	NCAM1	
FABP5	PI3	
HMOX1	PSAPL1	
HPSE	S100A8	
IL36G	S100A9	

f

Novel psoriasis associated trigger proteins

NCCRP1	ATP1B1	SYNM
RPL28	CNTN1	
PRX	MPZ	
RPS11	VSNL1	
ITGA8	RPL21	
DDX21	PSME3	
RPL23	SGCD	
RPS15	MYH11	
SLMAP	RPS27	
CSPG4	BCLAF1	

b



c

Protein name	Gene ID of protein	NL vs. L*	L vs. H	NL vs. H	Limma (NL vs. L)	Rankprod (NL vs. L)	Protein function and functional complexes
MICOS complex subunit MIC25	CHCHD6	4.887*	0.248	1.213	0.281	0.014	MICOS complex
Charged multivesicular body protein 5	CHMP5	0.293*	2.376	0.695	0.194	0.044	ESCRT-III complex
Collectin-12	COLEC12	2.842*	0.524	1.489	0.240	0.018	Scavenger receptor
Flotillin-2	FLOT2	2.043*	0.607	1.240	0.394	0.037	CSRM and CIE complex
Integrin alpha-7	ITGA7	2.983*	0.532	1.586	0.329	0.022	Cell surface receptor
LEM domain-containing protein 2	LEMD2	2.163*	0.552	1.194	0.099	0.039	Nuclear lamina and nuclear pore complexes
Nucleolar protein 56	NOP56	0.390*	1.994	0.778	0.281	0.045	snoRNP complex
Plasmalemma vesicle-associated protein	PLVAP	2.285*	0.615	1.406	0.319	0.021	Endothelial diaphragm
Ras-related protein R-Ras	RRAS	2.293*	0.488	1.120	0.258	0.037	Lysosomal degradation
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMARCA5	0.131*	1.767	0.232*	0.050	0.006	Nucleosome remodeling factor complex

Figure 3. Differential protein expression in lesional (L) and non-lesional (NL) skin and affected biological processes. Heatmap of relative expression for proteins differentially expressed in L and NL skin (**a**, left column) and the relative expression of these proteins NL and L skin (middle column) and L and healthy (H) skin (right column) (**a**). Biological processes for which proteins were differentially expressed in L and NL are listed. The top ten processes depicted to be affected in response to stimulus (**b** left, filled orange circles) and development (b right, filled blue circles). False detection rate (FDR) values are indicated with unfilled red circles around the filled circles for the various biological processes. The size of each circle is proportional to FDR values (unfilled red circles) or to the number of proteins (filled circles) (**b**). Proteins differentially expressed in L and NL but not in H and L are listed (**c**). Proteins for which the changes in NL and L compared to H are in different directions (increased vs. decreased and vice versa) are listed (**d**). Proteins that exhibited altered expression only in lesions (potentially trigger proteins) with known (**e**) and novel (**f**) association with psoriasis are listed. (*Significant difference in relative protein expression at least by two-fold in L and NL comparison).

a

Protein description	Gene ID of protein	NL vs. H*	L vs. H	NL vs. L	Limma (NL vs. H)	Rankprod (NL vs. H)
Trifunctional purine biosynthetic protein adenosine-3	GART	4.451*	4.325	1.029	0.389	0.047
Guanylate-binding protein 1	GBP1¥	5.925*	4.745*	1.249	0.161	0.046
Kallikrein-10	KLK10¥	6.549*	20.793*	0.315	0.060	0.047
Exportin-2	CSE1L	7.204*	5.799	1.242	0.253	0.024
Protein S100-A7	S100A7¥	7.519*	14.74*	0.510	0.027	0.024
Unconventional myosin-XVIIIa	MYO18A	8.191*	14.777*	0.554	0.027	0.024
UDP-glucose 6-dehydrogenase	UGDH	10.175*	8.269*	1.231	0.027	0.024
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMARCA5	0.232*	1.767	0.131*	0.050	0.006

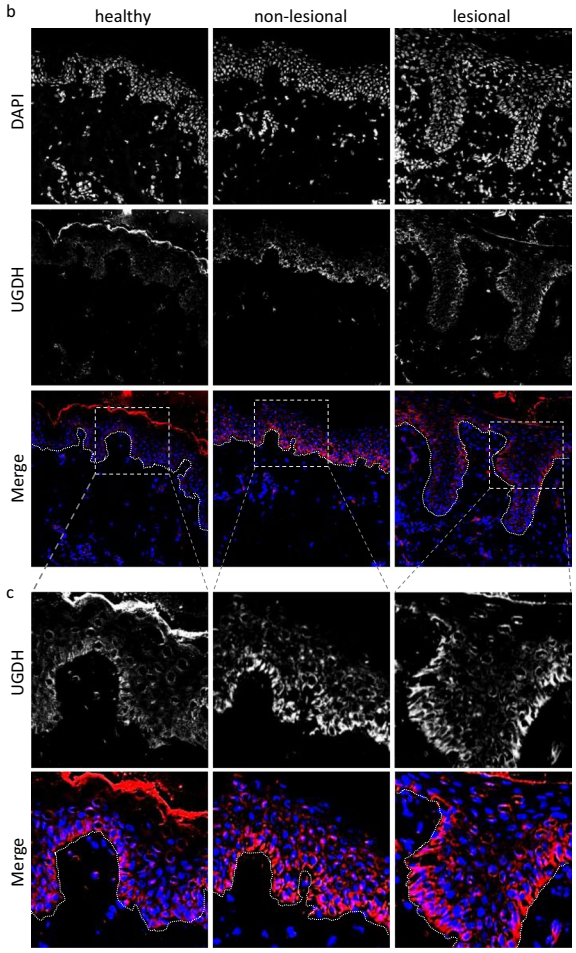


Figure 4. Differentially expressed proteins in non-lesional (NL) and healthy (H) skin. Proteins with expression that differs by at least 2-fold in non-lesional skin and healthy skin are listed (**a**). UGDH protein expression is similarly increased in NL and lesional psoriatic skin, compared to H controls. The highest difference in expression for NL and H was seen with immunohistochemical characterization of UGDH (n = 10), which indicated similar patterns of distribution in the three sample types. The strongest staining was observed in basal keratinocytes, and weaker staining was observed in the upper parts of the epidermis. Higher intensity staining UGDH was observed in non-lesional and lesional skin compared to healthy skin (**b**). A higher magnification of the epidermis is provided (**c**). (In merged figures, DAPI nuclear staining and UGDH are shown in blue and red, respectively; *: indicates statistical significance, ¥: indicates proteins with known association with psoriasis).

Gene ID of protein	L *vs.* H	NL *vs.* H	NL *vs.* L
AKR1B10	32.769*	25.318	0.773
CSTA	2.335*	1.752	0.75
FABP5	15.076*	4.678	0.31*
PI3	52.616*	4.105	0.078
S100A2	5.878*	2.527	0.43
S100A7	14.74*	7.519	0.51
S100A8	20.639*	5.234	0.254*
S100A9	19.679*	3.306	0.168*
SCCA2	35.468*	9.221	0.26*
STAT1	20.504*	14.478	0.706
STAT3	3.766*	2.309	0.613

Table 2. Detected expressional differences of classic protein biomarkers for psoriasis.

In order to check the validity of our proteomic approach we compared major known psoriatic biomarkers published in previous genetic (genome-wide association studies)[21], transcriptomic[19,21,23] and proteomic studies[17,18,20,22] with our proteomic dataset. Known psoriatic lesional biomarkers also found in our study includes AKR1B10[16], CSTA[17], FABP5[18], PI3[19], SCCA2[20], STAT1[16], STAT3[21], S100A7[18–20], S100A8[19,22] and S100A9[19,20,22,23]. Moreover, annotation of diseases resulted in the identification of psoriasis with the strongest correlation based on differentially expressed proteins in either lesional vs. healthy or in lesional vs. non-lesional comparison. Canonical pathway analysis of non-lesional differences compared to healthy skin resulted in the identification of 'Role of IL-17A in Psoriasis'. However, these annotations also highlighted cancer, neurological, neuromuscular or muscular disease-related mechanism, suggesting their potential involvement in disease pathomechanism, or some similarities between these diseases.

Since our proteomic and *in silico* analysis cannot distinguish between cell-types, and provide information whether mechanistically linked alterations are taking place within the same, or different cell types, further experiments are required in this direction to clarify the exact relevance of these predicted connections to psoriasis pathomechanism.

We performed literature a search for known functions of proteins found to be altered in amounts in our study to suggest mechanism through which they may potentially participate in the pathomechanism of the disease. The detected differences in the expression of proteins in healthy and lesional skin highlighted involvement in psoriasis of cell proliferation[28], development[29], response to stimulus[30], expression[31] related processes. In the comparison of non-lesional and lesional skin, we identified 56 proteins with differential expression, which represents only 22.5% of the number of proteins which showed altered expression in the comparison of healthy and lesional skin (56 vs. 249). This highlights the importance of studying healthy skin in comparisons using patient samples for pinpointing disease-associated alterations. Qualitative literature-based analysis of these 56 proteins led to the identification of several mechanism for which association with psoriasis has already been described, including processes related to development[29], response to stimulus[26] and expression[31].

Further analysis focused on gaining insight about the extent to which alterations are manifest in lesions and in non-lesional skin. Strikingly, nearly 80% of the 249 proteins exhibiting differential expression in lesional and healthy skin exhibited an intermediate expression level in the non-lesional skin, suggesting the possible presence of early, lesional-like alterations in non-lesional skin. Divergence from this trend was only observed for two small protein groups. Ten proteins — CHCHD6, CHMP5, COLEC12, FLOT2, ITGA7, LEMD2, NOP56, PLVAP, RRAS and SMARCA5 — differed in relative protein amounts in non-lesional and lesional skin, but the amounts of these proteins were similar in healthy and lesional samples. These ten proteins are likely to represent a group of non-lesional characteristic alteration. For nine proteins — CD207, COLEC12, CTSV, ITGA7, ITGA8, PLVAP, PSAPL1, SMARCA5 and XP32 — the direction of the expressional changes was different in non-lesional and lesional samples compared to healthy skin and might represent proteins that contribute to maintaining the non-lesional state.

Next, with the proteins in these two groups, we performed an extensive literature search to suggest potential mechanisms by which they may influence disease pathogenesis. Interestingly, all the identified proteins may play a role in signaling at different levels starting from the cell surface all away to the nucleus or mitochondria. The identified cell surface receptors include two integrins (ITGA7 and ITGA8) that are important in external signal recognition. Decreased ITGA7 levels — as observed in lesional vs. non-lesional skin — could be associated with delayed autophagy[32], differentiation[33] and increased migration[34], all known to be affected in psoriatic lesions. In contrast, elevation of ITGA7 may induce growth suppression[35]. However, ITGA7 is characteristically expressed mainly by smooth muscle cells[36] in the skin, suggesting that its involvement in keratinocyte related events are at least limited, or none. Instead, may suggest alterations in (vascular) smooth muscle cell adhesion-related processes[37]. Alternatively, ITGA7 may influence neurite outgrowth[38]. Therefore, further studies are required to confirm the observed TGA7 expression alteration and to identify the cell-types of source. Another identified cell-surface molecule, MYO18A, through recognizing microorganism lipopolysaccharides, may increase innate immune responses[39] promoting cytokine production towards Th1 direction[40]; known to be important in psoriasis[41].

Categories (L *vs.* H)	Diseases or disease related processes	p-value	Predicted Activation State	Activation z-score	Number of Proteins
Disease annotation of protein expressional differences between lesional (L) and healthy (H) skin					
Dermatological Diseases and Conditions, Organismal Injury and Abnormalities	Psoriasis	6.89E-32	—	—	61
	Chronic psoriasis	2.15E-23	—	—	27
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities, Tumor Morphology	Cell death of osteosarcoma cells	4.04E-22	Decreased	−4.899	24
Dermatological Diseases and Conditions, Organismal Injury and Abnormalities	Chronic skin disorder	7.47E-22	—	—	28
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities, Tumor Morphology	Cell death of cancer cells	4.24E-15	Decreased	−4.64	33
Infectious Diseases	Viral Infection	1.89E-12	Increased	3.883	69
	Replication of virus	3.58E-11	—	1.819	34
	Replication of RNA virus	1.93E-10	—	1.799	31
Dermatological Diseases and Conditions, Organismal Injury and Abnormalities	Plaque psoriasis	4.05E-10	—	—	15
Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease, Organismal Injury and Abnormalities	Lichen planus	4.86E-10	—	—	13
Categories (NL vs. L)	**Diseases or disease related processes**	**p-value**	**Predicted Activation State**	**Activation z-score**	**Number of Proteins**
Disease annotation of protein expressional differences between non-lesional (NL) and lesional (L) skin					
Dermatological Diseases and Conditions, Organismal Injury and Abnormalities	Psoriasis	3.72E-15	—	—	21
	Plaque psoriasis	1.78E-11	—	—	10
	Chronic skin disorder	3.06E-10	—	—	10
	Chronic psoriasis	8.58E-10	—	—	9
Immunological Disease	Allergy	8.57E-08	—	—	12
Immunological Disease	Hypersensitive reaction	1.30E-07	—	—	12
Immunological Disease	Immediate hypersensitivity	4.94E-07	—	—	10
Dermatological Diseases and Conditions, Inflammatory Disease, Inflammatory Response, Organismal Injury and Abnormalities	Dermatitis	9.38E-07	—	−1.067	11
Cardiovascular Disease, Organismal Injury and Abnormalities, Renal and Urological Disease	Ischemic acute renal failure	2.99E-06	—	—	3
Organismal Injury and Abnormalities, Reproductive System Disease	Endometriosis	3.13E-06	—	—	10
Categories (L vs.H)	**Biological Function**	**p-value**	**Predicted Activation State**	**Activation z-score**	**Number of Proteins**
Biological function annotation of protein expressional differences between lesional (L) and healthy (H) skin					
Protein Synthesis	Initiation of translation of protein	3.11E-46	—	—	42
	Translation	3.28E-40	—	0.737	57
	Translation of protein	1.09E-38	—	0.555	55
	Synthesis of protein	3.18E-36	Increased	2.691	64
	Expression of protein	6.76E-36	—	0.527	57
RNA Damage and Repair	Nonsense-mediated mRNA decay	3.06E-35	—	—	32
Protein Synthesis	Metabolism of protein	6.39E-31	Increased	2.92	85
Cell Death and Survival	Necrosis	6.99E-18	Decreased	−2.168	109
RNA Post-Transcriptional Modification	Processing of RNA	4.39E-14	—	−0.577	32
Cellular Movement	Migration of cells	1.1E-12	Increased	2.067	83
Categories (NL vs. L)	**Biological Function**	**p-value**	**Predicted Activation State**	**Activation z-score**	**Number of Proteins**

Continued

Categories (L *vs.* H)	Diseases or disease related processes	p-value	Predicted Activation State	Activation z-score	Number of Proteins
Biological function annotation of protein expressional differences between non-lesional (NL) and lesional (L) skin					
Cell Death and Survival	Killing of Staphylococcus aureus	8.53E-10	—	−0.655	5
Cellular Movement,Immune Cell Trafficking	Leukocyte migration	7.62E-08	—	−0.509	18
Cell Death and Survival	Killing of bacteria	1.10E-07	—	−1.608	6
Cell-To-Cell Signaling and Interaction, Reproductive System Development and Function	Binding of gonadal cell lines	1.19E-07	—	1.964	6
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	Cell movement of leukocytes	1.55E-07	—	−0.429	16
Cell Death and Survival	Necrosis	2.20E-07	—	−1.927	30
Antimicrobial Response, Inflammatory Response	Antimicrobial response	2.42E-07	Decreased	−2	10
Cell Death and Survival	Killing of Staphylococcus aureus subsp. aureus	5.03E-07	—	—	3
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Cell movement of phagocytes	5.87E-07	—	−0.902	13
Cellular Compromise, Inflammatory Response	Degranulation of cells	6.21E-07	—	−0.87	13

Table 3. Disease and biological function annotation of differentially expressed proteins.

FLOT2[42], CHMP5[43] and COLEC12[44] participate in endocytic pathways, regulating the levels of cell-surface receptors and, thereby, signaling. FLOT2 is a known component of the raft microdomain complex that represents the major unit regulating STAT signaling pathways according to the raft-STAT signaling hypothesis[45]. The alteration of FLOT2 expression could suggest a high relevance since STAT3 is a key regulator in psoriasis[46]. Reduction of the scavenger receptor COLEC12 could trigger psoriasis by trastuzumab treatment[47]. Moreover, COLEC12 may influence the mitochondrial respiratory chain[48], and this property is in agreement with the decreased level of mitochondrial MICOS complex subunit CHCHD6[49] observed in lesions compared to non-lesional skin. CHCHD6 regulates oxygen consumption and thereby may influence cell growth[50]. Reduced levels of CHCHD6 were shown previously to lead to a shift from oxidative metabolism to glycolytic metabolism[51] that negatively influences keratinocyte differentiation[52], and both types of mechanisms are known to be affected in psoriasis[53,54].

The altered expression of LEMD2 may suggest that signal transduction is also altered at the level of the nucleus. LEMD2, located in the inner nuclear membrane, regulates nuclear import/export processes[55] and thereby intranuclear signaling[50]. During this regulation, LEMD2 is associated with the same complex as CHMP5, which was also identified in our studies. In the nucleus, the STAT-regulated protein NOP56[56], a core protein of the box C/D small nucleolar ribonucleoprotein (snoRNP) complex, participates in the biogenesis of rRNAs[57]. Increased rRNA biogenesis is suggested to be necessary for high proliferation rate[58], a process that is crucial for the development of psoriatic lesions.

Abnormal proliferation[28], differentiation[52] and, thereby, skin barrier function are key processes during psoriatic plaque formation. SMARCA5 is a component of the nucleosome remodeling factor complex[59]. Decreasing SMARCA5 levels are required for basal keratinocytes to shift form proliferation toward differentiation[60]. XP32 is also a component of the epidermal differentiation complex[61] and associated with skin barrier function[62]. The observed contrasting expressional differences of these two proteins in the non-lesional and lesional skin may contribute to our understanding of lesion formation and how non-lesional skin maintains its state.

Overall, our results indicate that dysregulation of cellular signaling — from signal detection, through endocytosis of receptors and transduction of signal from the cell surface to the nucleus — may be affected during the disease. The alteration of these systems is likely to lead to increased reaction to external signals that could contribute to the maintenance of psoriatic plaques.

By comparing non-lesional and healthy skin, differential expression was observed for eight proteins (CSE1L, GART, GBP1, KLK10, MYO18A, S1007A, SMARCA5 and UGDH). Four of these proteins (CSE1L, GART, GBP1, UGDH) might be predisposing factors, as their expression was similar in non-lesional and lesional skin, and their significance would have been missed in comparisons for which healthy samples were not included. Of these, UGDH was detected with the highest relative difference. UGDH has not been highlighted previously in association with psoriasis. We therefore decided to analyze it further. Immunohistochemical analysis confirmed our proteomic results: higher UGDH levels were found in non-lesional and lesional skin compared to healthy skin, that was mainly associated with keratinocytes. Elevated UGDH levels may increase chondrocytes proliferation indirectly, likely through increased hyaluronan production that binds different cytokines[63]. However, *in vitro* downregulation of UGDH and consequently decreased hyaluronan amounts did not influence keratinocyte proliferation[64]. These results are in line with our observation, suggesting that elevated UGDH levels observed in non-lesional keratinocytes are not sufficient to modify their proliferation.

Ingenuity Canonical Pathways (L vs. H)	−log (p-value)
EIF2 Signaling	3.69E + 01
Regulation of eIF4 and p70S6K Signaling	1.95E + 01
mTOR Signaling	1.16E + 01
FAT10 Signaling Pathway	4.22E + 00
tRNA Charging	4.01E + 00
Role of IL-17A in Psoriasis	3.32E + 00
RAN Signaling	2.96E + 00
Intrinsic Prothrombin Activation Pathway	2.80E + 00
Polyamine Regulation in Colon Cancer	2.62E + 00
Neuroprotective Role of THOP1 in Alzheimer's Disease	2.56E + 00
Ingenuity Canonical Pathways (NL vs. L)	**−log(p-value)**
Caveolar-mediated Endocytosis Signaling	8.77E + 00
Paxillin Signaling	5.79E + 00
EIF2 Signaling	5.35E + 00
Virus Entry via Endocytic Pathways	4.51E + 00
IL-8 Signaling	4.41E + 00
Integrin Signaling	4.31E + 00
Agrin Interactions at Neuromuscular Junction	4.11E + 00
Regulation of eIF4 and p70S6K Signaling	3.88E + 00
NF-κB Activation by Viruses	3.68E + 00
mTOR Signaling	3.35E + 00
Ingenuity Canonical Pathways (NL vs. H)	**−log(p-value)**
UDP-D-xylose and UDP-D-glucuronate Biosynthesis	3.10E + 00
5-aminoimidazole Ribonucleotide Biosynthesis I	2.92E + 00
Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate	2.70E + 00
Purine Nucleotides *De Novo* Biosynthesis II	2.36E + 00
Role of IL-17A in Psoriasis	2.29E + 00
Colanic Acid Building Blocks Biosynthesis	2.25E + 00
RAN Signaling	2.17E + 00
Intrinsic Prothrombin Activation Pathway	1.79E + 00
SPINK1 Pancreatic Cancer Pathway	1.66E + 00
MSP-RON Signaling Pathway	1.54E + 00

Table 4. Canonical pathways predicted to be affected in psoriasis based on detected expressional differences of proteins.

Proteins for which expression was affected only in lesions are often considered "trigger" proteins, as changes in the expression of these proteins are linked to the shift of the disease state. The proteins that have not previously been associated with psoriasis were categorized into two groups. The first group of proteins might contribute to the mechanosensitivity of the tissue (SGCD[65], SYNM[66], MYH11[67], ATP1B1[68]). The second group functions within the nervous system (MPZ, PRX[69], CSPG4[70], CNTN1 and ITGA8[71], ATP1B1[72]), could suggest the involvement of the peripheral nerve system in psoriasis[73].

Finally, we searched for potentially central proteins in disease pathogenesis participating in key mechanisms of psoriasis including regulation of stress and immune response, proliferation and differentiation. Some of the identified proteins, such as PML[74] and STAT1[75], have already been linked to psoriasis. We also identified two proteins, PRKDC and MYBBP1A, which have not previously been highlighted in context with the disease. The PRKDC may plays a role in the detection and repair of breaks in double-stranded DNA[76] and mediates the phosphorylation of c-MYC[77] and p53[78] suggesting a potentially important role in psoriasis. The suggested altered expression by our results of the transcription factor MYBBP1A may also be among the potentially important proteins in psoriasis implicated in the pathogenesis since it functions as a co-repressor of NF-κB that may regulate responses to stress and cytokines[79].

Taken together, our comparative proteomic approach of healthy, non-lesional and lesional skin led to the identification of various proteins which may function in psoriasis pathogenesis, providing a strong base for future studies. Proteins exhibiting opposite expression changes in lesional and non-lesional samples compared to healthy skin may function in the maintenance of the non-lesional stage and may represent future targets for therapeutic purposes.

Materials and Methods

Ethics. Skin biopsy collection from donors, the procedure of collection and all experimental protocols were approved by the Regional and Institutional Research Ethics Committee and by the Human Investigation Review Board of the University of Szeged (SOEDAFN-002, IF-562-5/2016 and IF-15056/2015; 157/2015; 3638 and 2799, 3517), strictly following the guidelines and regulations of the Declaration of Helsinki. Prior to surgical intervention and following a detailed description of the skin biopsy donation procedure, participants provided written informed consent. No donor under the age of 18 was included in our study.

Criteria for inclusion of patients in the study and skin sample collection. To identify alterations that are general in chronic plaque psoriasis and keep the number of volunteers for skin biopsy collection to a minimum, for our proteomic approach, we randomly engaged individuals (I) of different age to minimize possible age-related differences; (II) with various Psoriasis Area Severity Index (PASI) scores between 5 and 25, since the score for an individual patient varies over time and with relapse; (III) of both genders to avoid possible gender-associated differences; and (IV) with both early and late onset. A total of 9 (3×3) patients suffering from chronic plaque psoriasis and the same number of healthy donors were involved in our study. The data of individuals involved in the study are summarized in Table 1. All psoriatic patients had not received any kind of treatment for the condition for at least 6 months. The 6 mm skin punch biopsies containing the epidermis and the dermis were collected from an area of the upper-middle gluteal region that is not exposed to sunlight. Both lesional and non-lesional samples were collected from patients. Non-lesional samples were taken at least 7 cm from the edge of the lesion subjected for biopsy. The presence of psoriasis was clinically verified for all patients, and clinical as well as demographic data of the donors are presented in Table 1.

Comprehensive and comparative proteomics of healthy, non-lesional and lesional skin. *Sample preparation from skin biopsy and sequential protein extraction.* Samples were cut with a razor blade. Skin proteins were extracted sequentially in four consecutive solubility-based extraction steps. Extraction buffers were used in increasing order of their solubilizing properties for a better separation of proteins. Samples were initially incubated in extraction buffer I. (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4) for 24 h at 4 °C in the presence of protease inhibitors. Protein extracts were then clarified by centrifugation and separated from the pellet. This step was repeated by resuspending the pellet in extraction buffer II, which contained 1 M NaCl, 25 mM EDTA, 50 mM Tris-HCl, pH 7.4. Following extraction with 250 mM SDS-containing extraction buffer III (8 h at room temperature), guanidine hydrochloride containing extraction buffer IV (4 M GuHCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.4) was applied for 48 h at 4 °C. The same protein extracts of the three donors were pooled in each investigated group (healthy, non-lesional, lesional). Extraction procedure was carried out three times and each contained extracted proteins of three donors following the pooling of the samples which were than subjected for downstream proteomic analysis.

Protein identification by 2D LC-MSMS. A total 35 µg protein from each sample was applied for mass spectrometry analysis. A modified filter-aided sample preparation method was used for tryptic digestion of the protein extracts[80]. High-pH reversed-phase chromatography was performed on a C18 column (Phenomenex, Kinetex 5 µ EVO C18 100 A, 2.1 × 100 mm; cat. no. 00D-4622-AN, flow rate: 150 µl/min). Forty-eight fractions were collected from 1 to 25 minutes (half minute/fraction) and 4-4 fractions were combined (1,13,24,37; 2,14,25,38 and so on) to get 12 final fractions. Each fraction was subjected to nano LC-MSMS analysis on an Orbitrap Elite hybrid mass spectrometer (Thermo) coupled with a Waters nanoAcquity UPLC system, using a gradient elution after trapping the samples onto the trap column. Data-dependent analyses were applied; the 20 most intense peaks were selected for ion-trap collision-induced dissociation after each survey scan measured in the Orbitrap. Proteome Discoverer (ver.: 1.3) was used to generate MS/MS peak-list files and our in-cloud ProteinProspector (ver.: 5.16.0) database search engine was used for protein identification against the human sequences from the UniProtKB.2015.12.14.random.concat (149781/55820795 entries searched) database. Detailed protocols and applied counting for semi-quantitative analysis is described as Supplementary Information.

Immunofluorescence staining of skin sections for UGDH. For immunofluorescence analysis, 5 µm sections of frozen embedded skin biopsies from psoriatic patients (non-lesional and lesional skin) and healthy individuals were used. After fixation and permeabilization (Foxp3 staining buffer set, fixation/permeabilization kit, Miltenyi Biotec, used according to the description of the manufacturer), samples were blocked in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 1% normal goat serum (NGS) for 1 h at room temperature. Samples were incubated overnight at 4 °C in TBS with 1% NGS and primary antibodies against UGDH (rabbit polyclonal antibody, ab155005, Abcam), diluted to 1:100. Following washing in TBS, AF546 secondary antibodies (Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546, A-11035, Invitrogen), diluted in TBS containing 1% NGS to 1:500, were applied for 1 h at room temperature.

Literature search to identify novel psoriasis-associated proteins. To identify proteins not yet linked with the pathomechanism of psoriasis, literature mining was carried out using protein names or the encoding gene's HUGO Gene Nomenclature Committee (HGNC) symbol(s), applying the following strategy: each protein or gene name was searched together with "psoriasis" as a keyword using the RISmed R package.

Statistical analysis. To compare protein abundance from healthy, lesional and non-lesional skin extracts, significant differences were determined based on relative peptide ion chromatograms and spectrum counting and evaluated using two different approaches: (1) modified t-test (limma) and (2) rank product test (as described by Schwämmle *et al.*[81]) following t-test. We considered a protein amount to be different between two samples if at least one of the three tests were significant (test <0.05) and the absolute fold change was at least two or higher.

References

1. Slominski, A. T., Zmijewski, M. A., Plonka, P. M., Szaflarski, J. P. & Paus, R. How UV Light Touches the Brain and Endocrine System Through Skin, and Why. *Endocrinology* **159**, 1992–2007 (2018).
2. Slominski, A. T. *et al*. Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. *Adv. Anat. Embryol. Cell Biol.* **212**, v, vii, 1–115 (2012).
3. Gubán, B. *et al*. Abnormal regulation of fibronectin production by fibroblasts in psoriasis. *Br. J. Dermatol.* **174**, 533–541 (2016).
4. Eyre, R. W. & Krueger, G. G. Response to injury of skin involved and uninvolved with psoriasis, and its relation to disease activity: Koebner and 'reverse' Koebner reactions. *Br. J. Dermatol.* **106**, 153–159 (1982).
5. Ye, L. *et al*. Abnormal epidermal barrier recovery in uninvolved skin supports the notion of an epidermal pathogenesis of psoriasis. *J. Invest. Dermatol.* **134**, 2843–2846 (2014).
6. Gudjonsson, J. E. *et al*. Global gene expression analysis reveals evidence for decreased lipid biosynthesis and increased innate immunity in uninvolved psoriatic skin. *J. Invest. Dermatol.* **129**, 2795–2804 (2009).
7. Szabó, K. *et al*. Regulatory networks contributing to psoriasis susceptibility. *Acta Derm. Venereol.* **94**, 380–385 (2014).
8. Henno, A. *et al*. Altered expression of angiogenesis and lymphangiogenesis markers in the uninvolved skin of plaque-type psoriasis. *Br. J. Dermatol.* **160**, 581–590 (2009).
9. Chiricozzi, A. *et al*. Increased expression of interleukin-17 pathway genes in nonlesional skin of moderate-to-severe psoriasis vulgaris. *Br. J. Dermatol.* **174**, 136–145 (2016).
10. Ting, K. M. *et al*. Overexpression of the oncofetal Fn variant containing the EDA splice-in segment in the dermal-epidermal junction of psoriatic uninvolved skin. *J. Invest. Dermatol.* **114**, 706–711 (2000).
11. Szlavicz, E. *et al*. Splicing factors differentially expressed in psoriasis alter mRNA maturation of disease-associated EDA+ fibronectin. *Mol. Cell. Biochem.* **436**, 189–199 (2017).
12. Lee, S. E. & Lew, W. The Increased Expression of Matrix Metalloproteinase-9 Messenger RNA in the Non-lesional Skin of Patients with Large Plaque Psoriasis Vulgaris. *Ann. Dermatol.* **21**, 27–34 (2009).
13. Danis, J., Göblös, A., Bata-Csörgő, Z., Kemény, L. & Széll, M. PRINS Non-Coding RNA Regulates Nucleic Acid-Induced Innate Immune Responses of Human Keratinocytes. *Front. Immunol.* **8**, 1053 (2017).
14. Szegedi, K. *et al*. The anti-apoptotic protein G1P3 is overexpressed in psoriasis and regulated by the non-coding RNA, PRINS. *Exp. Dermatol.* **19**, 269–278 (2010).
15. Göblös, A. *et al*. Keratinocytes express functional CARD18, a negative regulator of inflammasome activation, and its altered expression in psoriasis may contribute to disease pathogenesis. *Mol. Immunol.* **73**, 10–18 (2016).
16. Suárez-Fariñas, M. *et al*. Expanding the psoriasis disease profile: interrogation of the skin and serum of patients with moderate-to-severe psoriasis. *J. Invest. Dermatol.* **132**, 2552–2564 (2012).
17. Lundberg, K. C. *et al*. Proteomics of skin proteins in psoriasis: from discovery and verification in a mouse model to confirmation in humans. *Mol. Cell. Proteomics MCP* **14**, 109–119 (2015).
18. Williamson, J. C. *et al*. A proteomics approach to the identification of biomarkers for psoriasis utilising keratome biopsy. *J. Proteomics* **94**, 176–185 (2013).
19. Gudjonsson, J. E. *et al*. Assessment of the psoriatic transcriptome in a large sample: additional regulated genes and comparisons with *in vitro* models. *J. Invest. Dermatol.* **130**, 1829–1840 (2010).
20. Piruzian, E. *et al*. Integrated network analysis of transcriptomic and proteomic data in psoriasis. *BMC Syst. Biol.* **4**, 41 (2010).
21. Tsoi, L. C. *et al*. Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nat. Genet.* **44**, 1341–1348 (2012).
22. Schonthaler, H. B. *et al*. S100A8-S100A9 protein complex mediates psoriasis by regulating the expression of complement factor C3. *Immunity* **39**, 1171–1181 (2013).
23. Oestreicher, J. L. *et al*. Molecular classification of psoriasis disease-associated genes through pharmacogenomic expression profiling. *Pharmacogenomics J.* **1**, 272–287 (2001).
24. Wang, J. *et al*. Identification of unique proteomic signatures in allergic and non-allergic skin disease. *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* **47**, 1456–1467 (2017).
25. Kang, H. *et al*. Exploration of candidate biomarkers for human psoriasis based on gas chromatography-mass spectrometry serum metabolomics. *Br. J. Dermatol.* **176**, 713–722 (2017).
26. Méhul, B. *et al*. Noninvasive proteome analysis of psoriatic stratum corneum reflects pathophysiological pathways and is useful for drug profiling. *Br. J. Dermatol.* **177**, 470–488 (2017).
27. Ryu, J. *et al*. Proteomic analysis of psoriatic skin tissue for identification of differentially expressed proteins: up-regulation of GSTP1, SFN and PRDX2 in psoriatic skin. *Int. J. Mol. Med.* **28**, 785–792 (2011).
28. Jiang, M. *et al*. TGFβ/SMAD/microRNA-486-3p Signaling Axis Mediates Keratin 17 Expression and Keratinocyte Hyperproliferation in Psoriasis. *J. Invest. Dermatol.* **137**, 2177–2186 (2017).
29. Mali, J. W. Psoriasis: a dynamic disease. *Br. J. Dermatol.* **101**, 725–730 (1979).
30. Schopf, R. E. & Straussfeld, E. Stimulus-dependent increased generation of oxygen intermediates in monocytes and polymorphonuclear leukocytes in psoriasis. *J. Invest. Dermatol.* **84**, 73–76 (1985).
31. Dou, J. *et al*. Integrative analyses reveal biological pathways and key genes in psoriasis. *Br. J. Dermatol.* **177**, 1349–1357 (2017).
32. Tang, Y. *et al*. ART1 promotes starvation-induced autophagy: a possible protective role in the development of colon carcinoma. *Am. J. Cancer Res.* **5**, 498–513 (2015).
33. Ozeki, N. *et al*. Bone morphogenetic protein-induced cell differentiation involves Atg7 and Wnt16 sequentially in human stem cell-derived osteoblastic cells. *Exp. Cell Res.* **347**, 24–41 (2016).
34. Laszlo, V. *et al*. Epigenetic down-regulation of integrin α7 increases migratory potential and confers poor prognosis in malignant pleural mesothelioma. *J. Pathol.* **237**, 203–214 (2015).
35. Tan, L.-Z., Song, Y., Nelson, J., Yu, Y. P. & Luo, J.-H. Integrin α7 binds tissue inhibitor of metalloproteinase 3 to suppress growth of prostate cancer cells. *Am. J. Pathol.* **183**, 831–840 (2013).
36. Wilson, E. Alpha 7 beta 1 integrin: putting the brakes on smooth muscle cell proliferation. *Circ. Res.* **101**, 651–653 (2007).
37. de Rezende, F. F. *et al*. Integrin α7β1 is a redox-regulated target of hydrogen peroxide in vascular smooth muscle cell adhesion. *Free Radic. Biol. Med.* **53**, 521–531 (2012).
38. Plantman, S. *et al*. Integrin-laminin interactions controlling neurite outgrowth from adult DRG neurons *in vitro*. *Mol. Cell. Neurosci.* **39**, 50–62 (2008).
39. Yang, L. *et al*. SP-R210 (Myo18A) Isoforms as Intrinsic Modulators of Macrophage Priming and Activation. *PloS One* **10**, e0126576 (2015).
40. Hohlfeld, J. M., Erpenbeck, V. J. & Krug, N. Surfactant proteins SP-A and SP-D as modulators of the allergic inflammation in asthma. *Pathobiol. J. Immunopathol. Mol. Cell. Biol.* **70**, 287–292 (2002).
41. Jiang, W. *et al*. A Toll-like receptor 7, 8, and 9 antagonist inhibits Th1 and Th17 responses and inflammasome activation in a model of IL-23-induced psoriasis. *J. Invest. Dermatol.* **133**, 1777–1784 (2013).
42. Solis, G. P. *et al*. Reggies/flotillins regulate E-cadherin–mediated cell contact formation by affecting EGFR trafficking. *Mol. Biol. Cell* **23**, 1812–1825 (2012).
43. Shim, J.-H. *et al*. CHMP5 is essential for late endosome function and down-regulation of receptor signaling during mouse embryogenesis. *J. Cell Biol.* **172**, 1045–1056 (2006).
44. Graham, S. A. *et al*. Identification of neutrophil granule glycoproteins as Lewis(x)-containing ligands cleared by the scavenger receptor C-type lectin. *J. Biol. Chem.* **286**, 24336–24349 (2011).

45. Lalazar, G. *et al*. Beta-glycoglycosphingolipid-induced alterations of the STAT signaling pathways are dependent on CD1d and the lipid raft protein flotillin-2. *Am. J. Pathol.* **174**, 1390–1399 (2009).
46. Calautti, E., Avalle, L. & Poli, V. Psoriasis: A STAT3-Centric View. *Int. J. Mol. Sci.* **19** (2018).
47. Kim, D. H. *et al*. Psoriasis induced by trastuzumab (herceptin®). *Ann. Dermatol.* **25**, 229–231 (2013).
48. Degenhardt, F. *et al*. Genome-wide association study of serum coenzyme Q10 levels identifies susceptibility loci linked to neuronal diseases. *Hum. Mol. Genet.* **25**, 2881–2891 (2016).
49. Ott, C., Dorsch, E., Fraunholz, M., Straub, S. & Kozjak-Pavlovic, V. Detailed analysis of the human mitochondrial contact site complex indicate a hierarchy of subunits. *PloS One* **10**, e0120213 (2015).
50. An, J. *et al*. CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. *J. Biol. Chem.* **287**, 7411–7426 (2012).
51. Chella Krishnan, K. *et al*. Integration of Multi-omics Data from Mouse Diversity Panel Highlights Mitochondrial Dysfunction in Non-alcoholic Fatty Liver Disease. *Cell Syst.* **6**, 103–115.e7 (2018).
52. Monteleon, C. L. *et al*. Lysosomes Support the Degradation, Signaling, and Mitochondrial Metabolism Necessary for Human Epidermal Differentiation. *J. Invest. Dermatol.* **138**, 1945–1954 (2018).
53. Lambert, S., Swindell, W. R., Tsoi, L. C., Stoll, S. W. & Elder, J. T. Dual Role of Act1 in Keratinocyte Differentiation and Host Defense: TRAF3IP2 Silencing Alters Keratinocyte Differentiation and Inhibits IL-17 Responses. *J. Invest. Dermatol.* **137**, 1501–1511 (2017).
54. Zhang, Z. *et al*. Differential glucose requirement in skin homeostasis and injury identifies a therapeutic target for psoriasis. *Nat. Med.* **24**, 617–627 (2018).
55. Gu, M. *et al*. LEM2 recruits CHMP7 for ESCRT-mediated nuclear envelope closure in fission yeast and human cells. *Proc. Natl. Acad. Sci. USA* **114**, E2166–E2175 (2017).
56. Cowling, V. H., Turner, S. A. & Cole, M. D. Burkitt's lymphoma-associated c-Myc mutations converge on a dramatically altered target gene response and implicate Nol5a/Nop56 in oncogenesis. *Oncogene* **33**, 3519–3527 (2014).
57. Lykke-Andersen, S., Ardal, B. K., Hollensen, A. K., Damgaard, C. K. & Jensen, T. H. Box C/D snoRNP Autoregulation by a cis-Acting snoRNA in the NOP56 Pre-mRNA. *Mol. Cell* **72**, 99–111.e5 (2018).
58. Marcel, V. *et al*. Expression Profiling of Ribosome Biogenesis Factors Reveals Nucleolin as a Novel Potential Marker to Predict Outcome in AML Patients. *PloS One* **12**, e0170160 (2017).
59. Vermeulen, M. *et al*. Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. *Cell* **142**, 967–980 (2010).
60. Mulder, K. W. *et al*. Diverse epigenetic strategies interact to control epidermal differentiation. *Nat. Cell Biol.* **14**, 753–763 (2012).
61. Zhao, X. P. & Elder, J. T. Positional cloning of novel skin-specific genes from the human epidermal differentiation complex. *Genomics* **45**, 250–258 (1997).
62. Toulza, E. *et al*. Large-scale identification of human genes implicated in epidermal barrier function. *Genome Biol.* **8**, R107 (2007).
63. Clarkin, C. E. *et al*. Regulation of UDP-glucose dehydrogenase is sufficient to modulate hyaluronan production and release, control sulfated GAG synthesis, and promote chondrogenesis. *J. Cell. Physiol.* **226**, 749–761 (2011).
64. Malaisse, J. *et al*. Hyaluronan Does Not Regulate Human Epidermal Keratinocyte Proliferation and Differentiation. *J. Biol. Chem.* **291**, 6347–6358 (2016).
65. Goehringer, C. *et al*. Prevention of cardiomyopathy in delta-sarcoglycan knockout mice after systemic transfer of targeted adeno-associated viral vectors. *Cardiovasc. Res.* **82**, 404–410 (2009).
66. Izmiryan, A. *et al*. Different expression of synemin isoforms in glia and neurons during nervous system development. *Glia* **54**, 204–213 (2006).
67. Smith, A. S. *et al*. Myosin IIA interacts with the spectrin-actin membrane skeleton to control red blood cell membrane curvature and deformability. *Proc. Natl. Acad. Sci. USA* **115**, E4377–E4385 (2018).
68. Mladinov, D., Liu, Y., Mattson, D. L. & Liang, M. MicroRNAs contribute to the maintenance of cell-type-specific physiological characteristics: miR-192 targets Na+/K+-ATPase β1. *Nucleic Acids Res.* **41**, 1273–1283 (2013).
69. Kim, S. *et al*. Schwann Cell O-GlcNAc Glycosylation Is Required for Myelin Maintenance and Axon Integrity. *J. Neurosci. Off. J. Soc. Neurosci.* **36**, 9633–9646 (2016).
70. Schäfer, M. K. E. & Tegeder, I. NG2/CSPG4 and progranulin in the posttraumatic glial scar. *Matrix Biol. J. Int. Soc. Matrix Biol*, https://doi.org/10.1016/j.matbio.2017.10.002 (2017).
71. Müller, U., Bossy, B., Venstrom, K. & Reichardt, L. F. Integrin alpha 8 beta 1 promotes attachment, cell spreading, and neurite outgrowth on fibronectin. *Mol. Biol. Cell* **6**, 433–448 (1995).
72. Johar, K., Priya, A. & Wong-Riley, M. T. T. Regulation of Na(+)/K(+)-ATPase by neuron-specific transcription factor Sp4: implication in the tight coupling of energy production, neuronal activity and energy consumption in neurons. *Eur. J. Neurosci.* **39**, 566–578 (2014).
73. Zhu, T. H. *et al*. The Role of the Nervous System in the Pathophysiology of Psoriasis: A Review of Cases of Psoriasis Remission or Improvement Following Denervation Injury. *Am. J. Clin. Dermatol.* **17**, 257–263 (2016).
74. Stoppe, M. *et al*. Cerebellar manifestation of PML under fumarate and after efalizumab treatment of psoriasis. *J. Neurol.* **261**, 1021–1024 (2014).
75. Shi, X. *et al*. IL-17A upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. *J. Invest. Dermatol.* **131**, 2401–2408 (2011).
76. Lee, S.-H. & Kim, C.-H. DNA-dependent protein kinase complex: a multifunctional protein in DNA repair and damage checkpoint. *Mol. Cells* **13**, 159–166 (2002).
77. An, J. *et al*. DNA-dependent protein kinase catalytic subunit modulates the stability of c-Myc oncoprotein. *Mol. Cancer* **7**, 32 (2008).
78. Gurley, K. E., Ashley, A. K., Moser, R. D. & Kemp, C. J. Synergy between Prkdc and Trp53 regulates stem cell proliferation and GI-ARS after irradiation. *Cell Death Differ.* **24**, 1853–1860 (2017).
79. Owen, H. R. *et al*. MYBBP1a is a novel repressor of NF-kappaB. *J. Mol. Biol.* **366**, 725–736 (2007).
80. Wiśniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **6**, 359–362 (2009).
81. Schwämmle, V., León, I. R. & Jensen, O. N. Assessment and improvement of statistical tools for comparative proteomics analysis of sparse data sets with few experimental replicates. *J. Proteome Res.* **12**, 3874–3883 (2013).

Acknowledgements

The MTA Cloud (https://cloud.mta.hu/) and Peter Baker are acknowledged for the use of ProteinProspector database search, which significantly helped us achieving the results published in this paper. The study was supported by NKFI (formal OTKA) PD116992, K111885 and GINOP-2.2.1-15-2016-00007, GINOP-2.3.2-15-2016-00020 research grants and co-financed by the European Social Fund in the framework of TAMOP-4.2.4.A/2-11-1/2012-0001 "National Excellence Program" A2-SZGYA-FOK-13-0001. K.S. is a recipient of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences, and also supported by the UNKP-18-4 New National Excellence Program of the Ministry of Human Capacities. Publication costs were financed by the 'University of Szeged Open Access Fund' (Grant number: 4151).

Author Contributions

G.G. conceived the study; G.G., Z.B.C. and L.K. supervised the project; É.H.-G. and G.G. designed the experiments; E.S., R.B., É.H.-G. and G.G. performed the experiments; statistical analysis was done by M.M.; E.S., R.B., M.M. and G.G. analyzed the proteomic data; experiments were performed in the laboratory of Z.B.C., K.L. and K.S.; E.S., K.S. and G.G. wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-47774-5.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

II.

Clinical, Cosmetic and Investigational Dermatology

Dovepress

open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Protective effects of glycerol and xylitol in keratinocytes exposed to hyperosmotic stress

This article was published in the following Dove Press journal:
Clinical, Cosmetic and Investigational Dermatology

Edit Szél[1]
Judit Danis[2]
Evelin Sőrés[1]
Dániel Tóth[3]
Csilla Korponyai[1]
Döníz Degovics[1]
János Prorok[3]
Károly Acsai[3]
Shabtay Dikstein[4]
Lajos Kemény[1,2]
Gábor Erős[1]

[1]Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary; [2]MTA-SZTE Dermatological Research Group, Szeged, Hungary; [3]Department of Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary; [4]Unit of Cell Pharmacology, Hebrew University, Jerusalem, Israel

Purpose: Our goal was to study whether glycerol and xylitol provide protection against osmotic stress in keratinocytes.

Methods: The experiments were performed on HaCaT keratinocytes. Hyperosmotic stress was induced by the addition of sorbitol (450, 500 and 600 mOsm). Both polyols were applied at two different concentrations (glycerol: 0.027% and 0.27%, xylitol: 0.045% and 0.45%). Cellular viability and cytotoxicity were assessed, intracellular Ca^{2+} concentration was measured, and the RNA expression of inflammatory cytokines was determined by means of PCR. Differences among groups were analyzed with one-way ANOVA and Holm-Sidak post-hoc test. When the normality test failed, Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison was performed.

Results: The higher concentrations of the polyols were effective. Glycerol ameliorated the cellular viability while xylitol prevented the rapid Ca^{2+} signal. Both polyols suppressed the expression of IL-1α but only glycerol decreased the expression of IL-1β and NFAT5.

Conclusions: Glycerol and xylitol protect keratinocytes against osmotic stress. Despite their similar chemical structure, the effect of these polyols displayed differences. Hence, joint application of glycerol and xylitol may be a useful therapeutic approach for different skin disorders.

Keywords: hyperosmotic stress, glycerol, xylitol, intracellular calcium concentration

Introduction

Local hyperosmotic condition is revealed to be associated with several inflammatory disorders (eg, corneal inflammation in dry eyes syndrome, inflammatory bowel disease, etc.).[1] Presumably, a local hyperosmotic challenge may contribute to the development of irritant contact dermatitis (ICD). ICD is a non-immunologic, non-specific inflammatory skin disease induced by physical, mechanical or chemical factors and accompanied by impaired barrier function. This results in increased skin permeability and transepidermal water loss (TEWL).[2] Water evaporation can lead to a higher osmotic pressure in the superficial layer of the skin. Furthermore, hyperosmolarity induces various intracellular responses in different types of cells and reduces cellular viability.[3–7] It has been described that a hyperosmotic stimulus elevates intracellular calcium (Ca^{2+}) concentration in HaCaT keratinocytes.[6,8] Although its exact molecular mechanism has not yet been revealed, it has been shown that Ca^{2+} is derived from both intra- and extracellular compartments.[6,8]

Keratinocytes are known to produce pro-inflammatory cytokines when exposed to osmotic stress.[9] At mRNA level, expression of tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β), IL-8,[3] IL-6[4] and nuclear factor of activated

Correspondence: Edit Szél
Department of Dermatology and Allergology, University of Szeged, Korányi fasor 6., Szeged 6720, Hungary
Tel +36 70 776 1104
Fax +36 62 545 954
Email szeledit@hotmail.com

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/CCID.S197946

T cells 5 (NFAT5)[5] was found to be higher in epithelial cells. In normal human epidermal keratinocytes, elevated mRNA expression of TNF-α, IL-1β, IL-6 and IL-8 was observed.[9]

NFAT5 is a principal transcription factor involved in water homeostasis during normal cornification[10] and activated by hyperosmotic stress;[5] however, its regulation by Ca^{2+} signals is equivocal.[11–13] Local hyperosmotic stress of the skin activates NFAT5 in macrophages[1] thereby intensifying the electrolyte clearance via lymphatic vessels.[14]

Under experimental conditions, different methods are used to induce hyperosmotic stress. Sodium chloride is usually used for this aim[3–5] and non-ionic organic agents such as sorbitol[9] and sucrose[4] can also be applied on keratinocyte cultures.

Glycerol and xylitol have well-known beneficial effects on the skin[15–18] but their role as osmolytes has not yet been fully clarified. It has been revealed that glycerol composes the principal osmolyte system of several bacterial species[19] while xylitol inhibits inflammatory cytokine expression.[20] Previously, we have shown the anti-inflammatory and anti-irritant effects of glycerol and xylitol.[16–18] Other in vitro experiments have revealed that glycerol suppresses human leukocyte antigen-DR (HLA-DR) mRNA level and xylitol upregulates filaggrin mRNA expression.[21]

The aim of the present study was to investigate whether glycerol and xylitol provide protection against hyperosmotic stress in vitro. Their effects on cellular viability and cytotoxicity, intracellular Ca^{2+} concentration, expression of pro-inflammatory cytokines and cellular viability were studied.

Materials and methods

Cell culture

HaCaT cells kindly provided by Dr N. E. Fusenig (Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 1.8 mM Ca^{2+} (DMEM-HG) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B and grown at 37°C in a humidified atmosphere containing 5% CO_2. Three to four days after plating, cells were collected from semi-confluent cultures by 5–10 mins trypsinization (0.25% trypsin-EDTA solution). The proteolic digestion was stopped by FBS and the cells were sedimented (10 mins, 4°C, 1,500 g) and resuspended in the medium for further use. Cellular viability was determined by the trypan blue exclusion test. The experiments were performed on cultures, where trypan blue was excluded from 95% of the cells.

Preparation of the treating media

For osmolarity measurement, Model 5600 Vapro® Vapor Pressure Osmometer (Dieren, The Netherlands) was used. The basal osmolarity of the serum-free DMEM-HG medium was 338 mOsm. Media contained 3 or 30 mM polyols, respectively, which was equivalent to 0.027 or 0.27 w/w% glycerol and 0.045 or 0.45 w/w % xylitol.[21] The osmolarity of these solutions was also measured. The final osmolarity of 450, 500 and 600 mOsm was reached by the addition of the appropriate amount of 1.83M sorbitol stock solution. Serum-free DMEM-HG media containing only glycerol, xylitol or sorbitol in concentrations mentioned above, respectively, were also applied.

Cellular viability and cytotoxicity

Cells were seeded into 96-well culture plates at a density of 10^4 cells/well in DMEM-HG supplemented with the appropriate agents detailed above, and serum-starved 24 hrs before any treatments. For 60 mins, cells were incubated with or without 0.27% glycerol or 0.45% xylitol in serum-free DMEM-HG, followed by incubation with 450, 500 or 600 mOsm culture medium with or without 0.27% glycerol or 0.45% xylitol for 24 hrs. Experiments were carried out in triplicates, and data are presented as the means of three experiments.

For the cell viability assay, 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to the cells at 37°C for 4 hrs, and then the formazan crystals were solubilized and the optical density (OD) was measured at 540 nm by a Multiscan Ex spectrophotometer (Thermo Labsystems, Beverly, Massachusetts, USA) and Ascent Software (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The absorbance rate was compared to untreated samples and represented as percentage rate of living cells.

Cytotoxicity was assessed by using the Cytotoxicity Detection Kit PLUS (Roche Diagnostics, Risch, Switzerland) according to the manufacturer's instructions. Briefly, released lactate dehydrogenase (LDH) was quantified from the supernatant by a colorimetric method and optical density was measured at 492 nm. Measured values

were corrected with the background values of the cell-free culture medium. Results are presented of % in cytotoxicity, where lysed cells correspond to 100% cytotoxicity.

Determination of changes in $[Ca^{2+}]_i$

HaCaT keratinocytes were plated onto 13 mm diameter uncoated sterile coverslips (VWR, Radnor, Pennsylvania, USA) and were left to attach and proliferate for 24 hrs. Coverslips with the attached cells were transferred and incubated in Tyrode's solution (144 mM NaCl, 0.4 mM NaH_2PO_4, 4 mM KCl, 0.53 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5.5 mM glucose and 5 mM HEPES, pH=7.4). HaCaT cells were loaded by incubation for 20 mins with the acetoxymethyl ester (AM) form of a single wavelength calcium-sensitive fluorescent dye (Fluo-4, Molecular Probes Inc., Eugene, Oregon, USA, 5 μM from a stock of 1 mM in DMSO +20% pluronic acid Pluronic F-127 Sigma-Aldrich, Saint Louis, Missouri, USA and 6.25 nM/mL Probenecid, Molecular Probes Inc., Eugene, Oregon, USA) at room temperature in dark. After incubation period, the cells were washed in indicator-free Tyrode's solution to remove any dye. The technique for intracellular calcium detection was based on established procedures described earlier.[22] Subsequently, cells were incubated for 30 mins with 0.027% or 0.27% glycerol and 0.045% or 0.45% xylitol[21] in Tyrode solution, respectively.

Optical measurements were performed using a Zeiss Axiovert 100 microscope (Zeiss, Oberkochen, Germany) equipped with a xenon lamp and used in epifluorescent mode at 100x magnitude. The coverslips were placed into a low volume imaging chamber (Warner Instruments, Hamden, Connecticut, USA) (37°C) at the microscope stage and cells were superfused with Tyrode alone for at least 10 mins (control period). Hyperosmotic stimulus was added to the cells in rapid perfusion (2–3 μL/sec) of 450 mOsm sorbitol, followed by the addition of 30 nmol/mL A23187 ionophor in Tyrode. Cells in the 75×75 μm frame were illuminated at 485 nm and the emitted light was recorded at 535 nm. Images of relative fluorescence intensity of a cell group (3–5 cells/group) were collected in 8–10 independent experiments per treatment group with the Mintron 7266pd Color CCD Camera (New Taipei City, Taiwan). Raw data were analyzed with WinWCP V4.5.0 (Glasgow, Scotland) and Clampfit 10.6 (San Jose, California, USA) softwares.

Calibration was performed by the $[Ca^{2+}]_i$ = Kd (F-Fmin)/(Fmax-F) method[23] where $[Ca^{2+}]_i$ is the intracellular Ca^{2+} concentration, Kd is the dissociation constant (345 nM), F is the fluorescence intensity evoked with the hyperosmotic stimulus, Fmin values were developed from corrected fluorescence intensity before osmotic stress and Fmax values were derived from the maximal fluorescent response after the addition of the ionophor solution. Background correction was calculated with the autofluorescence of unloaded cells and the decrease of fluorescence intensity in untreated cells caused by bleaching or dye efflux. For representative figures, $\Delta Ca^{2+} = \Delta F/F = (F - Frest)/Frest)$ was calculated.[23]

Real-time polymerase chain reaction (RT-PCR)

Cells were seeded into 6-well plates at a density of 2×10^5 cells/mL and serum-starved overnight. Cells were pre-incubated for 60 mins with 0.27% glycerol or 0.45% xylitol, followed by incubation with 450 mOsm sorbitol for 2 and 6 hrs.[3–5] Untreated or sorbitol-treated cells served as negative or positive controls, respectively. Total RNA was isolated using TRIzol reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. To avoid DNA contamination, DNAse treatment was performed and intron-spanning assays were used. cDNA was synthesized from 1 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (Roche Diagnostics, Risch, Switzerland) using TaqMan probe-based assays and a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The applied primers are listed in Table 1. The expression of each gene was normalized to the 18S rRNA gene, and relative mRNA levels were calculated by the ΔΔCt method, compared to untreated, time-matched control samples.

Statistical analysis

Data analysis was performed with SigmaStat for Windows 11.0 software (Jandel Scientific, San Rafael, California, USA). Differences among groups were analyzed with one-way ANOVA and Holm-Sidak post-hoc test and data are presented as mean (m) values with standard deviation (SD). When the normality test failed, Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison was performed. In such cases, median values (M) with 25th and 75th percentiles (25p and 75p, respectively) are given. $P<0.05$ was considered statistically significant.

Table 1 Known sequences of primer pairs and probes used for RT-PCR

	Sense	Antisense
TNF-α	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
IL-1α	AACCAGTGCTGCTGAAGGA	TTCTTAGTGCCGTGAGTTTCC
IL-1β	AAAGCTTGGTGATGTCTGGTC	AAAGGACATGGAGAACACCACT
IL-6	CAGGAGCCCAGCTATGAACT	GAAGGCAGCAGGCAACAC
NFAT5	TCAGACAAGCGGTGGTGA	AGGGAGCTGAAGAAGCATCA
IL-8	TaqMan® Gene Expression Assay, Thermo Scientific	

Results

Glycerol of 0.27% but not xylitol ameliorated the viability of HaCaT cells exposed to hyperosmotic stress

During a period of 24 hrs, osmotic stresses of 450 and 500 mOsm did not influence cellular viability, and the average cytotoxicity values were negligible. However, 600 mOsm sorbitol significantly reduced viability. The reduction was also considerable in the additional polyol-treated groups, but glycerol treatment resulted in significantly higher viability as compared to the positive control (DMEM-HG +600 mOsm sorbitol) group. 0.45% xylitol failed to improve the survival of the cells. The average cytotoxicity value of the 0.27% glycerol +600 mOsm sorbitol-treated group was somewhat lower but did not differ significantly from that of its control (DMEM-HG+600 mOsm sorbitol) group. Viability and cytotoxicity in groups exposed to 0.27% glycerol and 0.45% xylitol alone did not differ significantly from that of their matched DMEM-HG control group (Figure 1A, B).

Xylitol of 0.45% provides protection against the hyperosmotic stimulus-induced increase in intracellular Ca^{2+} concentration

Hyperosmotic stress induced by 450 mOsm sorbitol was accompanied by a short elevation of intracellular Ca^{2+} concentration. This elevation (M=328.6 nM, 25p=232.0, 75p=602.4) was prevented neither by glycerol nor the lower concentration of xylitol, but it was suppressed by the higher concentration (0.45%) of xylitol (M=78.76 nM, 25p=45.92, 75p=140.72) (Figure 2A). Figure 2 also shows

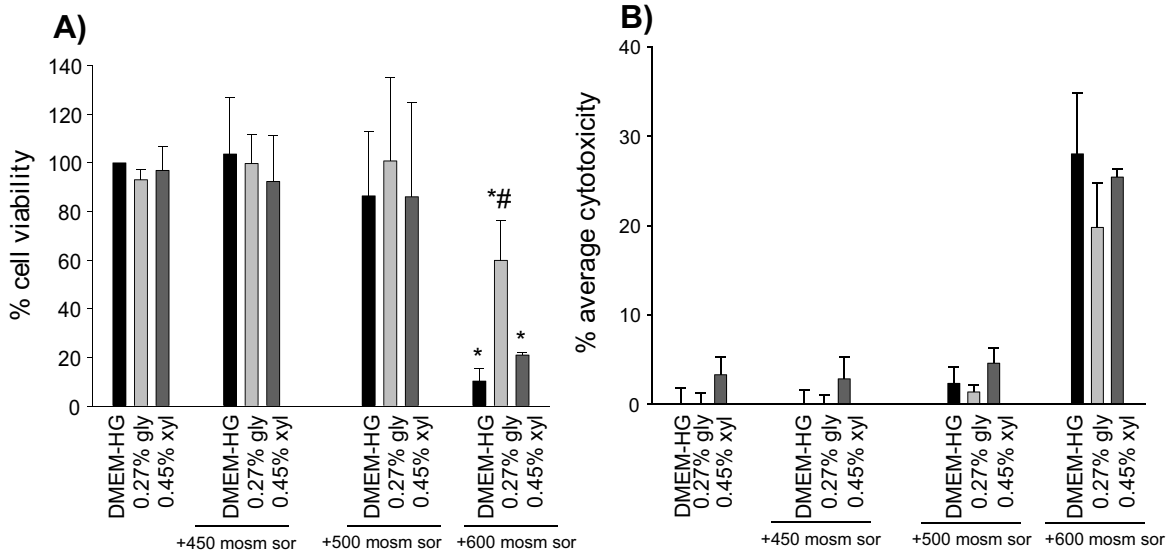


Figure 1 (**A**) The effects of glycerol and xylitol on cellular viability in hyperosmotic stress. **(B)** The effects of polyols in osmotic stress-induced cytotoxicity. Statistical analysis was performed with one-way ANOVA and Holm-Sidak post-hoc test (mean+SD), *p<0.05 vs DMEM-HG., #p<0.05 vs DMEM-HG +600 mOsm sor, n=3. **Abbreviations:** DMEM-HG, Dulbecco's modified eagle's medium containing 4.5 g/L glucose; sor, sorbitol; gly, glycerol; xyl, xylitol.

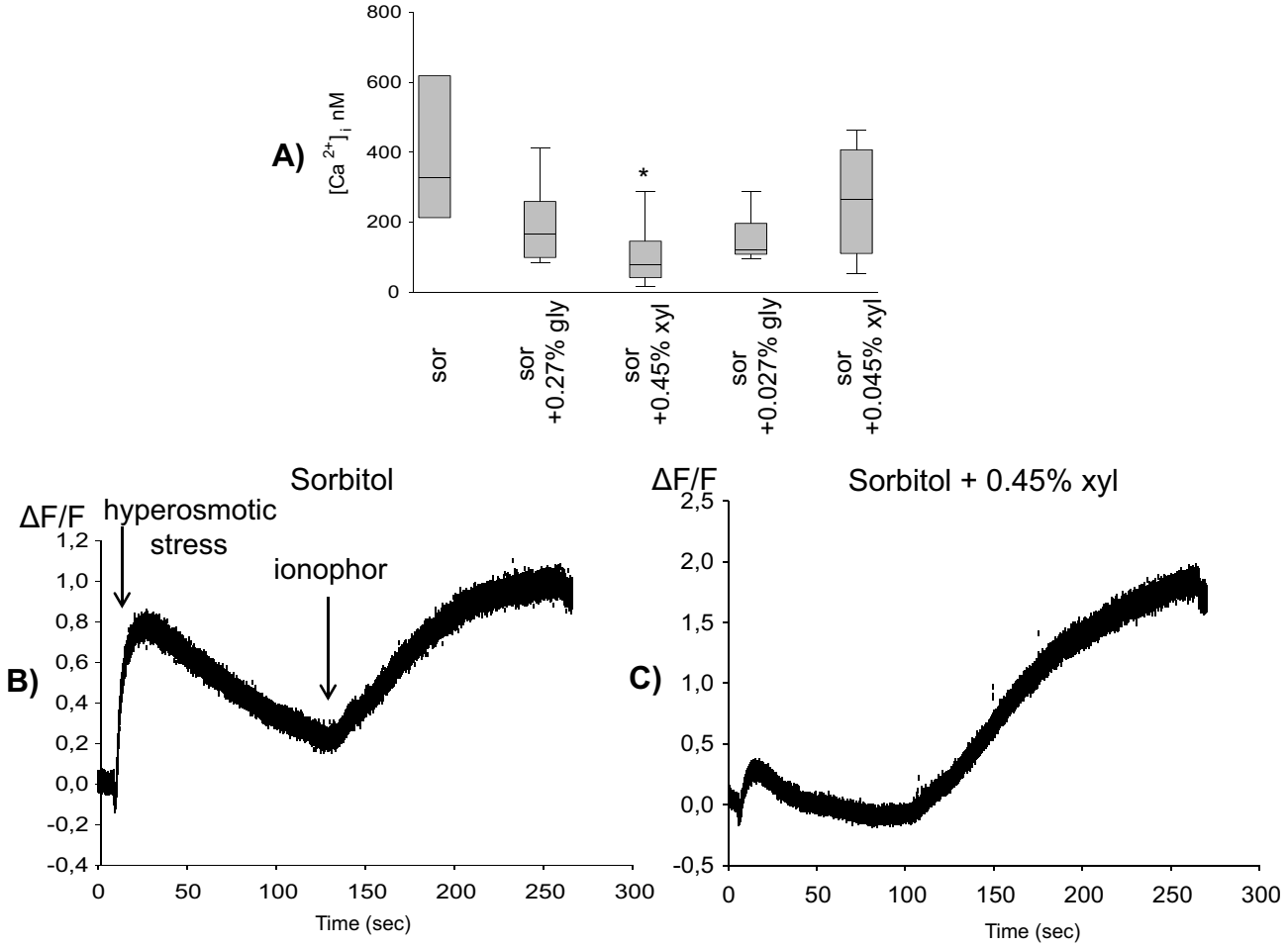


Figure 2 (**A**) Xylitol protected against the hyperosmotic stimulus-induced increase in intracellular Ca^{2+} concentration. Statistical analysis was performed with Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method (median, 25th and 75th percentile), *p<0.05 vs sor, n=8–10. (**B** and **C**) A representative curve of the relative fluorescence on 450 mOsm sorbitol (**B**) and additional xylitol-treated group (**C**).
Abbreviations: sor, sorbitol; gly, glycerol; xyl, xylitol.

two representative curves of this response from the sorbitol-treated (Figure 2B) and the sorbitol and 0.45% xylitol-treated groups (Figure 2C).

Polyols prevented the elevation in the mRNA expression of inflammatory cytokines and NFAT5 induced by osmotic stress

The mRNA expression of inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6 and IL-8) and the osmosis-related NFAT5 was measured after 2 and 6 hrs hyperosmotic stress. After 6 hrs, no significant changes were detected (data not presented). Following 2 hrs treatment, the cytokine levels of glycerol or xylitol-treated groups were similar to that of the untreated control group. 450 mOsm sorbitol induced considerable increase in IL-1α, IL-1β, IL-8 and NFAT5 expression. Both 0.27% glycerol and 0.45% xylitol prevented the elevation in the expression of IL-1α (Figure 3A). Furthermore, both 0.27% glycerol and 0.45% xylitol led to considerably lower expression of IL-8, and the expression levels did not differ significantly from those of untreated control group (Figure 3D). As concerns IL-1β and NFAT5, only 0.27% glycerol diminished considerably their expression (Figure 3B, C). No significant difference was found among the six study groups in TNF-α and IL-6 expression (data not shown).

Discussion

ICD is a frequent occupational disorder[24] which is characterized by impaired barrier function leading to increased TEWL. Water evaporation exposes keratinocytes to a condition of high osmotic pressure.[9] In our previous animal experiments, sodium lauryl sulfate (SLS)-induced skin irritation resulted

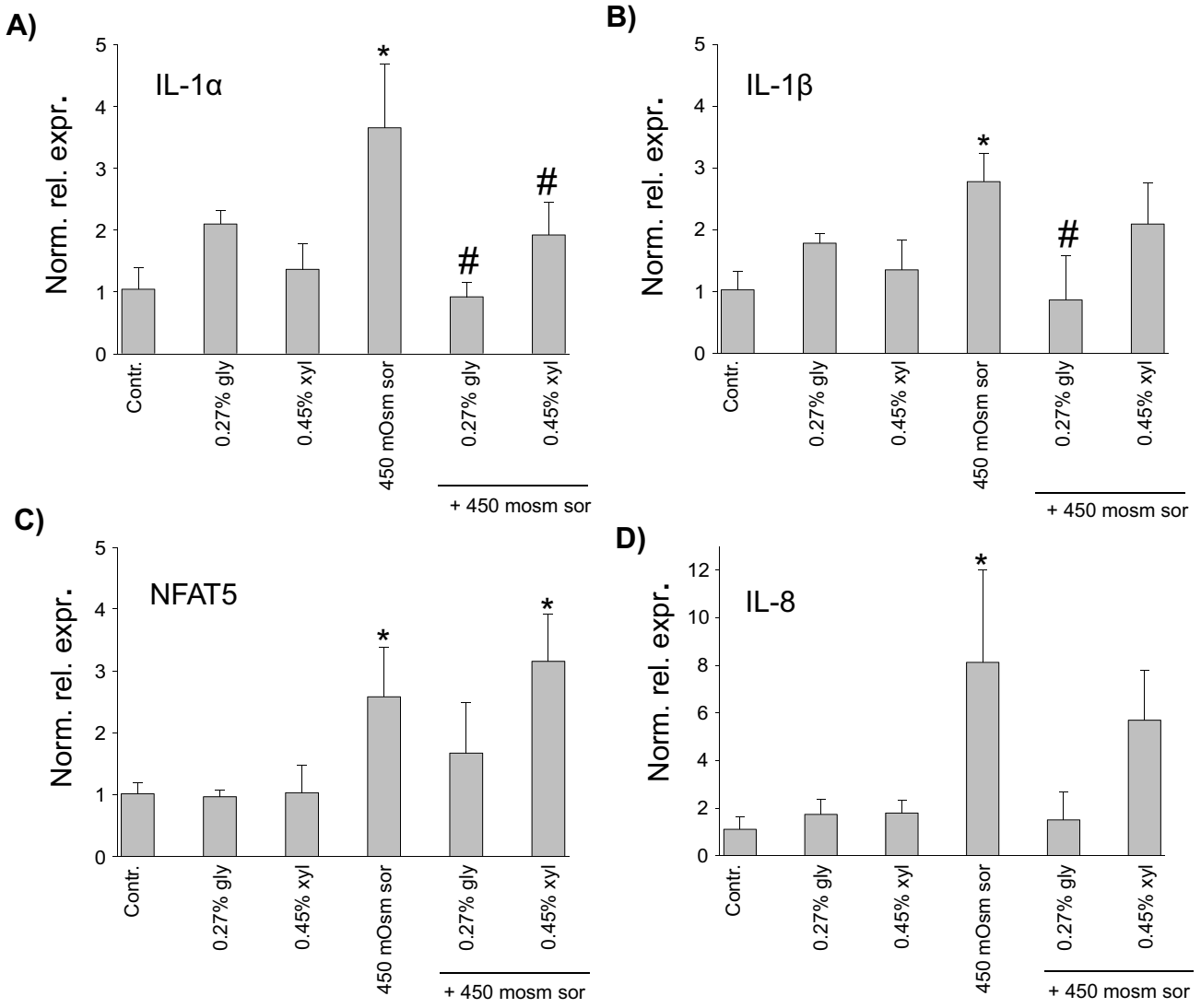


Figure 3 In vitro effects of polyols on (**A**) IL-1α, (**B**) IL-1β, (**C**) NFAT5 and (**D**) IL-8 expression. The expression of each gene was normalized to the 18S rRNA gene and relative mRNA levels were calculated by the ΔΔCt method, compared to the untreated, time-matched control samples. Statistical analysis was performed with one-way ANOVA and Holm-Sidak post-hoc test (mean + SD), *p<0.05 vs Contr., #p<0.05 vs 450 mOsm sor, n=3 (a-c) and Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method (median, 25th and 75th percentile), *p<0.05 vs Contr, n=3 (d) Despite the difference in the statistical tests, mean + SD values are shown in all subfigures for uniform presentation.
Abbreviations: Norm. rel. expr, normalized relative expression; Contr, control; sor, sorbitol; gly, glycerol; xyl, xylitol.

in the reduction of epidermal thickness[17] that can be considered as an indirect evidence for osmotic stress. Thus, the present study aimed at the investigation of osmotic challenge potentially accompanying ICD. In response to hyperosmotic stress, organic osmolytes are accumulated by the cells. In the skin, betaine, myoinositol and taurine are important osmolytes and the expression of their transporters (betaine/GABA transporter, sodium/myoinositol transporter and taurine transporter) is induced by osmotic stress.[25]

The anti-irritant and anti-inflammatory effects of glycerol and xylitol have already been demonstrated in animal experiments.[17] Joint application of glycerol and xylitol increases skin hydration, decreases TEWL, improves biomechanical properties of the skin and induces a higher filaggrin production in the epidermis after 2 weeks of application.[18] However, their cellular mechanism of action has not been revealed in details. We assumed that these polyols may act as organic osmolytes and therefore may have a role in osmoregulation.

It has been described that the effects of polyols may depend on the applied concentration.[17,26] Hence, two different concentrations were tested in our in vivo studies[16,17] and also two different concentrations were chosen for the present experiments, based on previous in vitro results.[21] According

to the results of the effect of polyols on Ca^{2+} concentration in our preliminary experiments, only the higher polyol concentrations were applied in the measurement of cellular viability, cytotoxicity and cytokine expression.

In order to induce osmotic stress, instead of the ionic sodium chloride and the detergent SLS, sorbitol was chosen, which is a metabolically inactive, inert agent. Sorbitol is also a polyol osmolyte such as glycerol and xylitol; however, it has no known protective effect in inflammatory skin conditions, and it is used to induce osmotic stress.[8]

Osmotic challenge influences cellular viability in an osmolarity- and time-dependent manner.[4–7] Such effect is also characteristic of ICD: severe irritation of the skin (24 hrs exposure to SLS) can induce tissue necrosis in vivo[27] but milder irritant challenges do not lead to necrosis.[28] In our experiments, 24 hrs exposure to 450 and 500 mOsm sorbitol did not influence cellular viability and cytotoxicity, but 600 mOsm resulted in a significant decrease in viability as compared to untreated control cells.

However, instead of 600 mOsm, 450 mOsm osmotic stress was applied to measure intracellular calcium concentration, in order to examine the protective effects of polyols, with the elimination of cell death. According to our pilot study, 450 mOsm hyperosmotic stimulus with sorbitol was sufficient to induce a short elevation of intracellular Ca^{2+} with a kinetics similar to that of measured by Dascalu et al who applied 500 mOsm sucrose.[6] Although the exact molecular mechanism is still not fully clarified, it has been described that dihydropiridine-sensitive Ca^{2+} channels are not affected.[6] The transient receptor potential vanilloid 1b (TRPV1b) non-selective cation channel, which is expressed also by HaCaT cells,[29] can be induced by cell shrinking in hypertonicity[30] and therefore may have a potential role in Ca^{2+} response. In osmotic stress, increased inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) synthesis were observed, as well.[31] TRPV1 can also be activated by Ca^{2+}[32] and DAG.[33] Moreover, IP3 receptors may contribute to Ca^{2+} influx, too.[34]

Based on literature data,[21] the applied polyols alone did not influence intracellular Ca^{2+} concentration. According to our results, xylitol prevented the elevation of intracellular Ca^{2+} concentration induced by the hyperosmotic sorbitol solution while glycerol did not influence this parameter. Further investigations are necessary to reveal the reason for this difference and the exact mechanism via which xylitol inhibits Ca^{2+} signal.

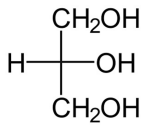
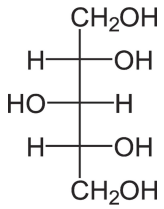
In addition to the rapid Ca^{2+} response, the applied osmotic challenge has longer effects, as well. 2 hrs of exposure to 450 mOsm sorbitol increased the expression of IL-1α, IL-1β and IL-8 in HaCaT cells. It has been described that cytokine expression elevates in keratinocytes when 500 mOsm of osmotic stimulus is present for 6 hrs.[9] Cytokine production may originate from the Ca^{2+} response.[35] However, in a recent study, pro-inflammatory responses evoked with ATP- or bradykinin-induced elevation of the intracellular Ca^{2+} concentration could not be suppressed by glycerol or xylitol. Moreover, none of these polyols influenced the subcellular translocation of the Ca^{2+}-dependent protein kinase C (PKC)α. However, xylitol but not glycerol translocated the Ca^{2+}-independent PKCδ.[36] TRPV1 activation can also lead to enhanced cytokine production via direct or indirect NF-κB activation.[37] Independently from the Ca^{2+} response, hyperosmotic stress also activates the transcription factor NFAT5[5] which regulates TNF-α[38] and can bind the promoter of IL-1 and IL-6.[1] Thus, elevated expression of NFAT5 might have contributed to the increased cytokine expression in the present study. Our previous in vivo investigations have already shown the anti-inflammatory effect of glycerol and xylitol.[16–18] However, differences can be found as compared to the present in vitro results. Glycerol and xylitol decreased the mRNA expression of IL-1β and TNF-α, but had no effect on the IL-1α levels in a murine model of ICD.[17] Several factors may explain the beneficial effects of polyols on cytokine expression. In addition to the prevention of Ca^{2+} signal and the inhibition of NFAT5 expression, glycerol and xylitol may affect the inflammatory process via stabilizing protein structure. As chaperon osmolytes, these polyols are able to enhance protein folding, thereby assisting in the development of the final structure essential for the optimal enzyme function, and they promote protein–protein and protein–DNA interactions.[39]

Our findings indicate that glycerol provides protection not only against acute inflammation but also against a more serious damage which occurs after a relatively longer timespan. The protective effect of glycerol was found to appear after a 24 hrs exposure to osmotic stress when aquaporin-3 (AQP-3) gene expression shows a peak.[40] If AQP-3 expression increases at protein level, as well, it provides an enhanced intracellular transport of glycerol. This theory is supported by our observation that cellular viability was ameliorated only when glycerol was continuously available (data not shown).

Conclusion

The effects of glycerol and xylitol were tested under hyperosmotic condition as an in vitro model of osmotic

Table 2 The chemical structure, known and novel properties of glycerol and xylitol

	Glycerol	Xylitol
Chemical structure		
Known properties	*in vivo* anti-inflammatory and anti-irritant effects[16–18]	
	Composition of the bacterial osmolyte system[19]	Inhibition of TNF-α and IL-1β expression *in vitro*[20]
	HLA-DR mRNA suppression[21]	Filaggrin mRNA upregulation[21]
Novel *in vitro* features in HaCaT keratinocytes exposed to hyperosmotic stress	Suppression of IL-1α	
	Amelioration of cell viability	Prevention of rapid intracellular Ca^{2+} signal
	Decrease in the expression of IL-1β and NFAT5	

stress accompanying ICD and other xerotic skin diseases. The applied polyols supported cell viability, prevented hyperosmosis-induced Ca^{2+} signal and the expression of inflammatory cytokines. Despite their similar chemical structure, the effect of these polyols displayed differences (Table 2). Hence, joint application of glycerol and xylitol may be a useful therapeutic approach for different skin disorders.

Acknowledgment

The authors are grateful to the assistants for their valuable contribution to the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Neuhofer W. Role of NFAT5 in inflammatory disorders associated with osmotic stress. *Curr Genomics*. 2010;11:584–590. doi:10.2174/138920210793360961
2. Tan CH, Rasool S, Johnston GA. Contact dermatitis: allergic and irritant. *Clin Dermatol*. 2014;32:116–124. doi:10.1016/j.clindermatol.2013.05.033
3. Li DQ, Luo L, Chen Z, Kim HS, Song XJ, Pflugfelder SC. JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res*. 2006;82:588–596. doi:10.1016/j.exer.2005.08.019
4. Veltmann M, Hollborn M, Reichenbach A, Wiedemann P, Kohen L, Bringmann A. Osmotic induction of angiogenic growth factor expression in human retinal pigment epithelial cells. *PloS One*. 2016;11:e0147312. doi:10.1371/journal.pone.0147312
5. Lee JH, Kim M, Im YS, Choi W, Byeon SH, Lee HK. NFAT5 induction and its role in hyperosmolar stressed human limbal epithelial cells. *Invest Ophthalmol Vis Sci*. 2008;49:1827–1835. doi:10.1167/iovs.07-0624
6. Dascalu A, Matithyou A, Oron Y, Korenstein R. A hyperosmotic stimulus elevates intracellular calcium and inhibits proliferation of a human keratinocyte cell line. *J Invest Dermatol*. 2000;115:714–718. doi:10.1046/j.1523-1747.2000.00099.x
7. Zhang S, Yanaka A, Tauchi M, et al. Hyperosmotic stress enhances interleukin-1beta expression in helicobacter pylori-infected murine gastric epithelial cells in vitro. *J Gastroenterol Hepatol*. 2006;21:759–766. doi:10.1111/j.1440-1746.2006.04293.x
8. Rodríguez I, Kaszkin M, Holloschi A, et al. Hyperosmotic stress induces phosphorylation of cytosolic phospholipase A(2) in HaCaT cells by an epidermal growth factor receptor-mediated process. *Cell Signal*. 2002;14:839–848. doi:10.1016/S0898-6568(02)00031-1
9. Terunuma A, Aiba S, Tagami H. Cytokine mRNA profiles in cultured human skin component cells exposed to various chemicals: a simulation model of epicutaneous stimuli induced by skin barrier perturbation in comparison with that due to exposure to haptens or irritant. *J Dermatol Sci*. 2001;26:85–93. doi:10.1016/S0923-1811(00)00165-1
10. Magnúsdóttir E, Kalachikov S, Mizukoshi K, et al. Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1. *Proc Natl Acad Sci U S A*. 2007;104:14988–14993. doi:10.1073/pnas.0707323104
11. Wang H, Ferraris JD, Klein JD, Sands JM, Burg MB, Zhou X. PKC-α contributes to high NaCl-induced activation of NFAT5 (TonEBP/OREBP) through MAPK ERK1/2. *Am J Physiol Renal Physiol*. 2015;308:F140–148. doi:10.1152/ajprenal.00167.2014

12. Hiyama A, Gajghate S, Sakai D, Mochida J, Shapiro IM, Risbud MV. Activation of TonEBP by calcium controls {beta}1,3-glucuronosyltransferase-I expression, a key regulator of glycosaminoglycan synthesis in cells of the intervertebral disc. *J Biol Chem*. 2009;284:9824–9834. doi:10.1074/jbc.M807081200
13. Feske S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol*. 2007;7:690–702. doi:10.1038/nri2152
14. Wiig H, Schröder A, Neuhofer W, et al. Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. *J Clin Invest*. 2013;123:2803–2815. doi:10.1172/JCI60113
15. Fluhr JW, Darlenski R, Surber C. Glycerol and the skin: holistic approach to its origin and functions. *Br J Dermatol*. 2008;159:23–34. doi:10.1111/j.1365-2133.2008.08643.x
16. Korponyai C, Kovács RK, Erös G, Dikstein S, Kemény L. Antiirritant properties of polyols and amino acids. *Dermatitis*. 2011;22:141–146.
17. Szél E, Polyánka H, Szabó K, et al. Anti-irritant and anti-inflammatory effects of glycerol and xylitol in sodium lauryl sulphate-induced acute irritation. *J Eur Acad Dermatol Venereol*. 2015;29:2333–2341. doi:10.1111/jdv.12669
18. Korponyai C, Szél E, Behány Z, et al. The effects of locally applied glycerol and xylitol on the hydration, barrier function and morphological parameters of the skin. *Acta Derm Venereol*. 2017;97:182–187. doi:10.2340/00015555-2493
19. Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: evolution of osmolyte systems. *Science*. 1982;217:1214–1222.
20. Han SJ, Jeong SY, Nam YJ, Yang KH, Lim HS, Chung J. Xylitol inhibits inflammatory cytokine expression induced by lipopolysaccharide from Porphyromonas gingivalis. *Clin Diagn Lab Immunol*. 2005;12:1285–1291. doi:10.1128/CDLI.12.2.280-286.2005
21. Szabó-Papp J, Sós K, Oláh A, et al. Differential effects of common moisturizer polyols on normal human epidermal keratinocytes. *J Invest Dermatol*. 2012;132:S58.
22. Prorok J, Kovács PP, Kristóf AA, et al. Herpesvirus-mediated delivery of a genetically encoded fluorescent Ca(2+) sensor to canine cardiomyocytes. *J Biomed Biotechnol*. 2009;2009:361795. doi:10.1155/2009/361795
23. Paredes RM, Etzler JC, Watts LT, Zheng W, Lechleiter JD. Chemical calcium indicators. *Methods San Diego Calif*. 2008;46:143–151. doi:10.1016/j.ymeth.2008.09.025
24. Schwensen JF, Friis UF, Menné T, Johansen JD. One thousand cases of severe occupational contact dermatitis. *Contact Dermatitis*. 2013;68:259–268. doi:10.1111/cod.2013.68.issue-5
25. Warskulat U, Reinen A, Grether-Beck S, Krutmann J, Häussinger D. The osmolyte strategy of normal human keratinocytes in maintaining cell homeostasis. *J Invest Dermatol*. 2004;123:516–521. doi:10.1111/j.0022-202X.2004.23313.x
26. Atrux-Tallau N, Romagny C, Padois K, et al. Effects of glycerol on human skin damaged by acute sodium lauryl sulphate treatment. *Arch Dermatol Res*. 2010;302:435–441. doi:10.1007/s00403-009-1021-z
27. Erös G, Kurgyis Z, Németh IB, et al. The irritant effects of pharmaceutically applied surfactants. *J Surfact Deterg*. 2014;17:67–70. doi:10.1007/s11743-013-1444-6
28. Jibry N, Murdan S. In vivo investigation, in mice and in man, into the irritation potential of novel amphiphilogels being studied as transdermal drug carriers. *Eur J Pharm Biopharm Off J Arbeitsgemeinschaft Pharm Verfahrenstechnik EV*. 2004;58:107–119. doi:10.1016/j.ejpb.2004.02.013
29. Pecze L, Szabó K, Széll M, et al. Human keratinocytes are vanilloid resistant. *PLoS One*. 2008;3:e3419. doi:10.1371/journal.pone.0003419
30. Sharif Naeini R, Witty MF, Séguéla P, Bourque CW. An N-terminal variant of Trpv1 channel is required for osmosensory transduction. *Nat Neurosci*. 2006;9:93–98. doi:10.1038/nn1614
31. Rodriguez I, Holloschi A, Kaszkin M, et al. Activation of phospholipase C-gamma1 in human keratinocytes by hyperosmolar shock without enzyme phosphorylation. *Arch Dermatol Res*. 2004;295:490–497. doi:10.1007/s00403-004-0457-4
32. Zitt C, Obukhov AG, Strübing C, et al. Expression of TRPC3 in Chinese hamster ovary cells results in calcium-activated cation currents not related to store depletion. *J Cell Biol*. 1997;138:1333–1341.
33. Venkatachalam K, van Rossum DB, Patterson RL, Ma HT, Gill DL. The cellular and molecular basis of store-operated calcium entry. *Nat Cell Biol*. 2002;4:E263–272. doi:10.1038/ncb1102-e263
34. Boulay G, Brown DM, Qin N, et al. Modulation of Ca(2+) entry by polypeptides of the inositol 1,4, 5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca(2+) entry. *Proc Natl Acad Sci U S A*. 1999;96:14955–14960. doi:10.1073/pnas.96.26.14955
35. Szöllősi AG, Vasas N, Angyal Á, et al. Activation of TRPV3 regulates inflammatory actions of human epidermal keratinocytes. *J Invest Dermatol*. 2018;138:365–374. doi:10.1016/j.jid.2017.11.021
36. Páyer E, Szabó-Papp J, Ambrus L, et al. Beyond the physico-chemical barrier: glycerol and xylitol markedly yet differentially alter gene expression profiles and modify signalling pathways in human epidermal keratinocytes. *Exp Dermatol*. 2018;27:280–284. doi:10.1111/exd.13493
37. Huang J, Ding L, Shi D, et al. Transient receptor potential vanilloid-1 participates in the inhibitory effect of ginsenoside Rg1 on capsaicin-induced interleukin-8 and prostaglandin E 2 production in HaCaT cells: GRg1 inhibits TRPV1 activation in HaCaT cells. *J Pharm Pharmacol*. 2012;64:252–258. doi:10.1111/j.2042-7158.2011.01392.x
38. López-Rodríguez C, Aramburu J, Jin L, Rakeman AS, Michino M, Rao A. Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. *Immunity*. 2001;15:47–58.
39. Brocker C, Thompson DC, Vasiliou V. The role of hyperosmotic stress in inflammation and disease. *Biomol Concepts*. 2012;3:345–364. doi:10.1515/bmc-2012-0001
40. Sugiyama Y, Ota Y, Hara M, Inoue S. Osmotic stress up-regulates aquaporin-3 gene expression in cultured human keratinocytes. *Biochim Biophys Acta*. 2001;1522:82–88. doi:10.1016/S0167-4781(01)00320-7