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RĪGAS STRADIŅA UNIVERSITĀTE

Elga Sidhoma

MORPHOLOGICAL CHARACTERIZATION OF LOCAL DEFENCE SYSTEM IN THE SKIN OF PSORIASIS PATIENTS

Summary of the Doctoral Thesis
for obtaining the degree of Doctor of Medicine
Speciality – Morphology

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Scientific supervisors:
Dr. med., Dr. habil. med., Professor **Māra Pilmane**
Dr. med., Associate Professor **Jānis Ķīsis**

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The Doctoral Thesis was carried out at the Department of Morphology, Institute of Anatomy and Anthropology, Rīga Stradiņš University, Latvia

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The Doctoral Thesis is available in the library of RSU and RSU homepage: www.rsu.lv

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Secretary of the Doctoral Council of Medicine:

Dr. med., Assistant Professor **Andrejs Vanags**

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LIST OF ABBREVIATIONS

AAI – Institute of Anatomy and Anthropology

CGRP – Calcitonin gene-related peptide

hBD-2 – Human beta defensin-2

IL-1α – Interleukin-1alpha

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IMH – Immunohistochemistry

µl – Microliter

µm – Micrometer

MMP-2 – Matrix metalloproteinase-2

PASI – Psoriasis Area Severity Index

PGP 9.5 – Protein gene product 9.5

RSU – Rīga Stradiņš University

Th – T helper lymphocytes

TIMP-2 – Tissue inhibitor of metalloproteinase-2

TIMP-4 – Tissue inhibitor of metalloproteinase-4

TNF-α – Tumor necrosis factor alpha

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling

INTRODUCTION

Psoriasis is a chronic and incurable skin disease with microbial superantigens playing a role in the pathogenesis and significant impact on reducing patient's quality of life. In recent years, new and yet incompletely studied data on the natural antimicrobial peptides presence in the skin has been obtained. Despite the increased amount of microorganisms in the skin of psoriasis patients, these patients rarely develop secondary bacterial skin infections. Therefore, the presence of natural antimicrobial peptides in the skin of psoriasis patient is essential.

Increased antimicrobial peptide production in psoriasis correlates with a low proportion of secondary infections compared with other inflammatory dermatoses, in which case the antimicrobial peptide production is not increased and patients are highly susceptible to bacterial and viral infections. The differences (decrease) of antimicrobial peptide expression of patients with various other dermatoses and the subsequent (more frequent) skin infections show the antimicrobial peptide immunological value (Braff *et al*., 2005; Jongh *et al.*, 2005; Schauber and Gallo, 2007).

Skin neuroimmunoendocrine system consists of skin macrophages, neuropeptide-containing innervation and neuroendocrine cells (Roosterman *et al*., 2006). Thus, this system affecting factors in the context with the changes of antimicrobial peptides and their interaction, such as degeneration, apoptosis and cell death markers, neuropeptides and interleukins, which together provide local skin protection, is essential. Overall, despite some research directions, a series of questions remain unanswered about the antimicrobial peptide correlation with local skin neuroimmunoendocrine system in patients with psoriasis of different age and different course of disease.

The **aim** of this research was a morphological study of local skin defense system in psoriasis patients with various progress of the disease in ontogenetic aspect.

To conduct the research, the following **objectives** were created:

1. To study the distribution and relative amount of the factors affecting the progress of the disease: antimicrobial peptides, degeneration, apoptosis and cell death markers, neuropeptides and interleukins in psoriasis affected skin.
2. To study the distribution and relative amount of antimicrobial peptides, degeneration, apoptosis and cell death markers, neuropeptides and interleukins in intact, i. e., healthy skin.
3. To mutually correlate the data obtained from both psoriasis patients and control patients, and with each other.
4. To correlate morphological data with patient's disease progress and clinical picture.
5. To create morphological diagnostic prognostic psoriasis criteria in patients with psoriasis in the course of the disease progress and treatment.

Scientific hypothesis or assumption:

Natural antimicrobial peptides and their secretion affecting factors are important in the morphopathogenesis of psoriasis, which generally correlates with the local neuroimmunoendocrine system of skin and variably manifests in various progresses of the disease.

Novelty of the study:

In this research, for the first time skin tissue obtained from untreated skin inflammation area in 40 *Psoriasis vulgaris* patients has been studied by immunohistochemistry and TUNEL methods. It is documented in

microphotographs and statistically significantly proved the hBD-2, PGP 9.5, CGRP, substance P, TNF-α, IL-1α, IL-6, IL-8, MMP-2, TIMP-2 and TIMP-4 relative amount of immunoreactive cells and nerve fibers, as well as the presence of apoptosis in psoriasis inflammation affected skin. So far, in the published literature such **complex** data cannot be found, as mostly a single factor or group of factors is being investigated.

Personal contribution

The author of this research has taken part in carrying out all stages of the study, performed the immunohistochemical evaluation and visualization, as is the author of all microphotographs.

Ethical aspects

The research was approved by the Committee of Ethics at Rīga Stradiņš University on 1st of September, 2009 and complies with the Declaration of Helsinki.

Structure and volume of the Doctoral Thesis

The Doctoral Thesis is written in Latvian. It has a classical structure and consists of 8 parts: introduction, review of literature, materials and methods, results, discussion, conclusions, references and appendix. The volume of the Doctoral Thesis covers 126 pages, including 12 tables, 10 graphs and 72 figures (67 microphotographs). The list of references consists of 201 sources.

1. MATERIALS AND METHODS

1.1. Morphologically investigated material

For patient inclusion in the study were established selection criteria. The inclusion criteria were following:

1. The patient is 18 years and older;
2. Visible characteristic for psoriasis eruptions in typical locations;
3. The patient is suffering from psoriasis for at least 4 weeks, first-time onset and has not been previously diagnosed or with a history of failed drug treatment;
4. The patient has not received any treatment for psoriasis in the past month, did not use topical medications containing corticosteroids in the damaged skin areas (with the exception of moisturizing skin care products);
5. The skin does not have a fierce tan;
6. There aren't other skin diseases or other illnesses.

From all patients during the visit data on disease duration, course, medication, family's history was collected. Further PASI (Psoriasis Area Severity Index) was calculated. Three signs – erythema, induration and desquamation with the affected body surface area percentage were assessed in four areas of the body – head, trunk, hands and feet. PASI can range from 0 to 72 points (Ashcroft *et al*., 1999).

Overall, in our study we included 40 *Psoriasis vulgaris* patients (9 female and 31 male), in the age group from 18 to 70, with a history of disease from 4 weeks to 44 years and PASI in the range of 1.2 to 60.0. The most common rash localizations were scalp, elbows, knees, and also scattered on the body.

As control group in our study we included 10 patients. The selected patient group consisted of 4 adults (3 female and 1 male, aged from 31 to 54) and 6 children (aged from 7 to 12) without a history of inflammatory skin diseases and existing visual changes on the skin. Adult control skin tissue material was obtained during benign nevi excision operations of operative wound borders. Child control skin tissue material was obtained from RSU AAI archival material collected 1998–2003.

1.2. Methods of investigation

1.2.1. Skin biopsy

Skin biopsy was performed using Punch technique. Skin biopsies were obtained from new untreated psoriasis lesions using routine 3 mm punch biopsy under local infiltrative anesthesia using 2% lidocaine, maintaining an aseptic technique. After acquisition of the skin tissue sample, haemostasis, application of 2% fusidic acid ointment and dressing was provided.

1.2.2. Tissue processing by routine histology method

The obtained skin tissue material was fixed in Stefanini solution (Stefanini *et al.*, 1967). The fixed tissue material was prepared for pouring in paraffin blocks. For dehydration and degreasing of the material alcohol was used. As an intermediate step between the alcohol and paraffin two xylene conversions were used. Following the mixture was homogenized and tissue was infiltrated by two paraffin conversions with the help of paraffin dispenser – paraffin was poured in special cassettes. Next 3–4 μm (micrometers) thick sections were prepared with the assistance of semi-automatic rotary microtome (Leica RM2245, Leica Biosystems Richmond Inc., USA). The cut material

from the microtome razor was ported to 48–50°C water and then on clean and degreased slide glass – we used HistoBond®+ (Paul Marienfeld GmbH & Co. KG, Germany). Afterwards histological sections were dried in thermostat at 56°C for 20–60 minutes. For the tissue staining deparaffinization was carried out with xylene and then the tissue was dehydrated with alcohol 96º. Tissue sections were stained with hematoxylin and eosin (Mayer 1896; Lillie, 1965; Fischer *et al*., 2008). Staining was followed by dehydration with alcohol 70º–96º, clarification with carboxylic acid and xylene, coverslip (code D102450, Deltalab, Spain) attaching and gluing. We attached the coverslip using special bonding glue Pertex (Lot 1710013, Histolab Products AB, Sweden). As a result, we obtained the overview sections in which the basophilic structures of the cell stained blue-violet, but the acidophilic – pink. Slides were examined in Leica light microscope.

1.2.3. Immunohistochemistry by biotin – streptavidin method

Skin tissue samples were fixed in Stefanini solution, dehydrated and poured in paraffin blocks following the steps described above. 3–4 μm thick sections were prepared. Consequently tissue deparaffinization with xylene and washing with alcohol 96º was performed.

Tissue sections were stained by immunohistochemistry using biotin – streptavidin method (Hsu *et al*., 1981) to identify in tissue samples:

- **human beta defensin-2** (hBD-2, code AF2758, obtained from goat, dilution 1:100, R&D Systems, Germany),
- **protein gene product 9.5** (PGP 9.5, code Z5116, obtained from rabbit, dilution 1:600, DakoCytomation, Denmark),
- **calcitonin gene related peptide** (CGRP, code 281328, obtained from rabbit, dilution 1:30, Quartett, Germany),

- **substance P** (code ab14184, obtained from mouse, dilution 1:1000, Abcam, United Kingdom),
- **tumor necrosis factor alpha** (TNF-α, code ab6671, obtained from rabbit, dilution 1:100, Abcam, United Kingdom),
- **interleukin-1 alpha** (IL-1α, code sc-9983, obtained from mouse, dilution 1:50, Santa Cruz Biotechnology, Inc., USA),
- **interleukin-6** (IL-6, code sc-73319, obtained from mouse, dilution 1:50, Santa Cruz Biotechnology, Inc., USA),
- **interleukin-8** (IL-8, code sc-1269, obtained from goat, dilution 1:50, Santa Cruz Biotechnology, Inc., USA),
- **matrix metalloproteinase-2** (MMP-2, code AF902, obtained from goat, dilution 1:100, R&D Systems, Germany),
- **tissue inhibitor of matrix metalloproteinase-2** (TIMP-2, code sc-21735, obtained from mouse, dilution 1:50, Santa Cruz Biotechnology, Inc., USA),
- **tissue inhibitor of matrix metalloproteinase-4** (TIMP-4, code orb106543, obtained from rabbit, dilution 1:50, Biorbyt Limited, United Kingdom).

Tissue samples were rinsed with TRIS buffer (Lot 0713513, Diapath S.p.A., Italy) – 2 times for 5 minutes and placed in boiling EDTA buffer (Lot 0713311, Diapath S.p.A, Italy) in a microwave oven for up to 20 minutes. Following, the container was removed and cooled with the samples in the buffer for up to 20 minutes, until reached 65ºC. The specimen was placed in a TRIS wash buffer and blocking with peroxidase block (code K400611, DakoCytomation, Denmark) was performed for 10 minutes. Next rinsing 2 times for 5 minutes and rinsing in TRIS wash buffer for 5 minutes was performed.

For **antibodies** obtained from **goat LSAB staining system** (code sc-2053, Santa Cruz Biotechnology, Inc., USA) was used. First incubation with the primary antibody for 2 hours, then washing in TRIS wash buffer for 5 minutes and then incubation for 30 minutes with biotin (secondary antibody) was performed. Further it was followed by washing in TRIS wash buffer for 5 minutes, incubation for 30 minutes with HRP-streptavidin complex, then again washing in TRIS wash buffer for 5 minutes. Tissue coating with liquid DAB+ chromogenic substrate system and incubation at room temperature, resulting in a positive structure coloring brown, was done for up to 10 minutes. Then we washed the sample in distilled water for 5 minutes and did the counterstaining with hematoxylin (code 05-M06002, Mayer's hematoxylin, Bio Optica Milano S.p.A., Italy) for 2 minutes.

For **antibodies** obtained from **mouse or rabbit EnVision staining system** (code K400611, DakoCytomation, Denmark) was used. Incubation with the primary antibody for 2 hours was followed by washing in TRIS washing buffer 2 times for 5 minutes and EnVision+/binding phase for 30 minutes. Then again washing in TRIS washing buffer 2 times for 5 minutes was done. Tissue coating with liquid DAB+ substrate chromogenic system and incubation at room temperature was performed, resulting in a positive structure brown coloration, took from 1 to 10 minutes, after which we washed the sample in distilled water for 5 minutes and did the counterstaining with hematoxylin (code 05-M06002, Mayer's hematoxylin, Bio Optica Milano S.p.A., Italy) for 2 minutes.

For **TIMP-2 and TIMP-4 antibodies** we used **HiDef Detection™ HRP Polymer** (code 954D-30, Cell Marque Corporation, USA). First incubation with the primary antibody for 2 hours was performed; afterwards we washed in TRIS wash buffer 3 times and added and incubated with reagent Link or Amplifier at room temperature for 10 minutes. Samples

were rinsed in TRIS wash buffer 3 times and incubated with Label reagent or Polymer Detector at room temperature for 10 minutes. Again rinsing with TRIS wash buffer was performed 3 times. Tissue coating liquid DAB+ chromogenic substrate system was added and incubation at room temperature, resulting in a positive structure coloring brown – 3 to 5 minutes – was performed. For the counterstaining hematoxylin (code 05-M06002, Mayer's hematoxylin, Bio Optica Milano S.p.A., Italy) for 2 minutes was added.

Regardless of the staining system, all samples in the end were dehydrated with 70º–96º alcohol and clarified with carboxylic acid and xylene, as well as the coverslip was glued with the Pertex glue (Lot 1710013, Histolab Products AB, Sweden). Slides were examined in Leica light microscope.

1.2.4. TUNEL method

Programmed cell death, or apoptosis, in the obtained tissue samples was evaluated using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling, Gavrieli *et al*., 1992; Negoescu *et al*., 1996) method. *In situ* Cell Death Detection Kit (POD catalog no. 1684817, Roche Diagnostics, Switzerland) was used. Sections were deparaffinized using xylene twice and by three different concentrations of alcohol (70º–96º) in decreasing order, also twice with each of them. Then tissue samples were washed in distilled water and phosphate buffer. The next step was the blocking of endogenous peroxidase. Afterwards tissue samples again were washed in phosphate buffer 3 times, boiled for 10 minutes and once more washed in phosphate buffer. To obtain a positive control one section was stained with DNAse I 1 mg/ml for 10 minutes and washed in phosphate buffer. All sections were blocked with

0.1% cow serum albumin phosphate buffer for 10 minutes and incubated with TUNEL mixture for 1 hour 37ºC in a tank with humidified air. To obtain a negative control one section was placed in phosphate buffer only. The sections again were washed in phosphate buffer, incubated with POD according to the instruction of the kit for 30 minutes 37ºC and washed in phosphate buffer. Tissue samples were covered with diaminobenzidine substrate solution for 7 minutes and washed in distilled water. For counterstaining we added hematoxylin (code 05-M06002, Mayer's hematoxylin, Bio Optica Milano S.p.A., Italy) for 2 minutes. Nuclei of apoptotic cells stained brown. Slides were examined in Leica light microscope.

To obtain microphotographs we used DC300F Leica microscope with Leica DFC420 digital camera (Leica Microsystems GmbH, Germany) and the Leica DM6000 B microscope with Leica DFC450 C digital camera (Leica Microsystems GmbH, Germany), as well as image visualization program Image-Pro Plus (Media Cybernetics, Inc., USA) and Leica image visualization program (Leica Microsystems GmbH, Germany).

1.2.5. Methods for statistical evaluation

For the analysis of the structures detected by immunohistochemistry we used semiquantitative counting method (Pilmane *et al*., 1995). The relative frequency of positive structures was evaluated in three randomly selected visual fields for each of the prepared sections as follows:

− no detectable positive structures in the visual field;

−/+ separate positive structures in the visual field;

+ few positive structures in the visual field;

+/++ few to moderate positive structures in the visual field;

++ moderate positive structures in the visual field;

++/+++ moderate to numerous positive structures in the visual field;

+++ numerous positive structures in the visual field;

+++/++++ numerous to abundance of positive structures in the visual field;

++++ abundance of positive structures in the visual field.

To evaluate the TUNEL method and apoptosis findings apoptotic index was also calculated (Potten, 1996) – in three randomly selected visual fields for each of the prepared sections 100 cells were counted, of which separately apoptotic cells were evaluated, then the average number of cells in three fields of vision was determined and the result was divided by 100.

For data evaluation non-parametric statistics were selected. To assess whether psoriasis patients and control group patients have statistically significant differences Mann – Whitney U test (Mann and Whitney, 1947) was used. To evaluate the cross-compliance of two variables Spearman's rank correlation coefficient (Spearman, 1904) was calculated.

The collected data was interpreted as follows:

- 0.00 to 0.19 – very weak correlation;
- 0.20 to 0.39 – weak correlation;
- 0.40 to 0.59 – moderate correlation;
- 0.60 to 0.79 – strong correlation;
- 0.80 to 1.00 – very strong correlation.

Correlation was considered to be statistically significant only if the resulting p-value was less than 0.05, or 5% confidence level.

Statistical analysis was performed with SPSS (Statistical Package for the Social Sciences) version 20.0 (IBM Corporation, USA).

2. RESULTS

2.1. Morphology characteristics

In all 10 skin samples obtained from control patients we found practically unchanged skin histological picture.

In skin tissue overview sections of all psoriasis patients included in the study pathological changes were found in both epidermis and dermis. In epidermis we observed its irregular thickening, marked proliferation of basal and spinous layers, and loss of granular layer with parakeratosis. In some of our patients' skin samples we observed the presence of the characteristic Munro's microabscesses and spongiform pustules of Kogoj. Dermis was characterized by extensive inflammatory infiltrates containing lymphocytes, polymorpho-nuclear leukocytes and macrophages. Arteriole sclerosis and vacuolization of glandulocytes forming the eccrine sweat glands were also observed.

2.2. Immunohistochemical profile and apoptosis

Control group patients

In epidermis hBD-2-containing keratinocytes were few to moderate (+/++) in the visual field, while in dermis few (+) cells contained hBD-2.

In epidermis and dermis numerous to abundance (+++/++++) of structures in the visual field contained PGP 9.5.

In epidermis numerous to abundance (+++/++++) of structures contained CGRP around keratinocytes, while in dermal connective tissue were moderate to numerous (++/+++) CGRP-containing structures.

Numerous (+++) structures contained substance P in epidermis and moderate to numerous (++/+++) structures contained substance P in dermis.

TNF-α was observed in few to numerous (+/++) epidermal keratinocytes and varied from separate (−/+) to moderate (++) positive cells in dermis.

IL-1α-containing keratinocytes were few (+), while in dermis in only some tissue samples IL-1α was present.

Abundance (++++) of keratinocytes and numerous (+++) dermal connective tissue cells contained IL-6.

IL-8 findings also were demonstrative – we found numerous (+++) IL-8-containing keratinocytes and moderate to numerous (++/+++) IL-8 positive dermal connective tissue cells.

MMP-2 expressed in few to moderate (+/++) keratinocytes and in moderate to numerous (++/+++) dermal connective tissue cells.

Numerous (+++) keratinocytes contained TIMP-2, while only moderate (++) TIMP-2-containing connective tissue cells were found in dermis.

Also numerous (+++) TIMP-4-containing keratinocytes were found in epidermis of healthy skin, while dermis presented with moderate to numerous (++/+++) TIMP-4-containing fibroblasts.

Apoptosis was observed in all tissue samples. Numerous to abundant (+++/++++) apoptotic keratinocytes and numerous (+++) apoptotic dermal connective tissue cells were observed.

Psoriasis patients

hBD-2 was observed in all tissue samples. Moderate to numerous (++/+++) epidermal keratinocytes and dermal fibroblasts, macrophages and lymphocytes contained hBD-2. In some tissue samples of our patients we observed abundance of hBD-2-containing epidermal keratinocytes

with more pronounced antimicrobial response in basal and spinous layers (Figure 2.1.).

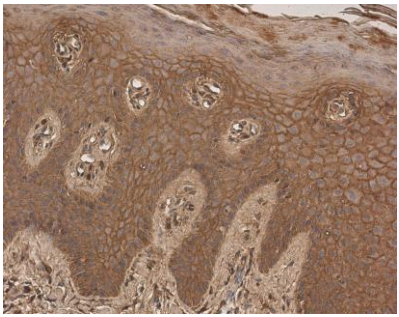

Figure 2.1. **Abundance of hBD-2-containing epidermal and dermal connective tissue and inflammatory cells in 64 years old psoriasis patient. Predominance of hBD-2-containing keratinocytes in basal and spinous layers. hBD-2 IMH, × 250**

PGP 9.5-containing structures in epidermis were few to moderate (+/++) and in dermis moderate (++). We observed fine PGP 9.5-containing nerve fibers in between keratinocytes, in papillary dermis, on epidermis and dermis border (Figure 2.2.), in inflammatory infiltrates, surrounding blood vessels and sweat glands.

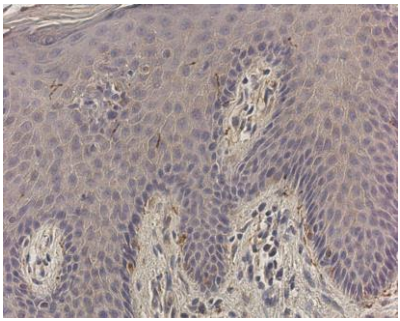

Figure 2.2. **Few (+) fine PGP 9.5-containing nerve fibers running through the spinous layer of epidermis in 51 year old psoriasis patient with long-term disease. At the same time there are moderate (++) PGP 9.5-containing nerve fibers in the tissue on epidermis and dermis border. PGP 9.5 IMH, × 400**

CGRP-containing nerve fibers surrounding keratinocytes in epidermis were moderate (++), while in dermis we found moderate to numerous (++/+++) CGRP-containing nerve fibers. Often we observed more prominent presence of CGRP in basal and spinous layers of epidermis (Figure 2.3.).

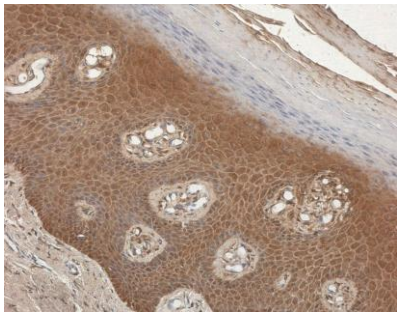

Figure 2.3. **Numerous (++++) CGRP-containing structures especially in basal and spinous layers of epidermis in 68 years old patient with psoriasis and 44 years long history of the disease. CGRP IMH, × 200**

The findings of substance P were limited – only in 24 of our psoriasis patients we found substance P positive structures in epidermis (mostly separate (−/+) positive structures), while all skin tissue samples contained substance P in dermis with also mostly separate (−/+) positive structures.

Few to moderate (+/++) keratinocytes and moderate to numerous (++/+++) fibroblasts and inflammatory cells in dermis contained TNF-α. In dermis of 10 patients we observed abundance (++++) of TNF-α positive cells (Figure 2.4.).

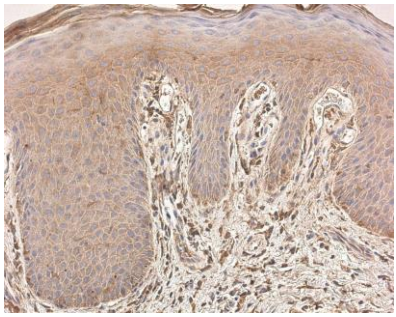

Figure 2.4. **38 years old psoriasis patient with 6 months long history of the disease, but very severe and extensive damage to the skin presented with abundance (++++) of dermal connective tissue and inflammatory cells containing TNF-α. In the middle part of epidermis cytolemma positive for the factor was found. TNF-α IMH, × 250**

Only separate (−/+) keratinocytes contained IL-1α. In dermis few (+) fibroblasts and moderate (++) inflammatory cells expressed IL-1α.

IL-6 findings were rich. Moderate to numerous (++/+++) keratinocytes in epidermis and moderate to numerous (++/+++) fibroblasts and inflammatory cells in dermis contained IL-6. In some of our tissue samples we observed abundance (++++) of IL-6-containing keratinocytes and numerous to abundance (+++/++++) of IL-6-containing fibroblasts and inflammatory cells (Figure 2.5.).

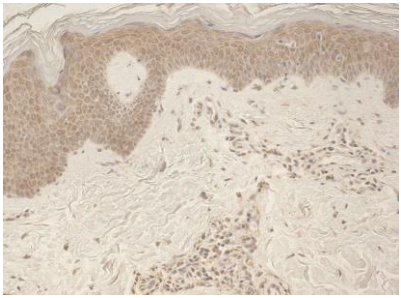

Figure 2.5. **Abundance (++++) of IL-6-containing keratinocytes in epidermis of 65 years old psoriasis patient. Simultaneously, cells of inflammatory infiltrate contain IL-6 in dermal connective tissue. IL-6 IMH, × 200**

IL-8 was found in moderate to numerous (++/+++) keratinocytes and dermal cells (fibroblasts, macrophages, lymphocytes). Simultaneously, IL-8-containing cells were found also perivascularly (Figure 2.6.).

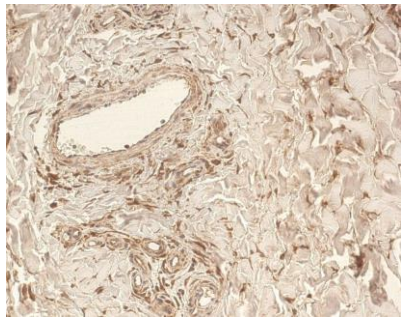

Figure 2.6. **Numerous (+++) IL-8-containing cells perivascularly in the reticular layer of dermis in 40 years old psoriasis patient. IL-8 IMH, × 200**

Moderate to numerous (++/+++) keratinocytes and numerous (+++) dermal cells contained MMP-2. It is important to note that in substantial amount of our skin samples abundance (++++) of keratinocytes (11 patients) and dermal cells (17 patients) contained MMP-2 (Figure 2.7.).

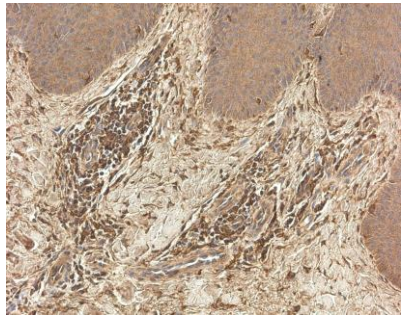

Figure 2.7. **Dermal perivascular inflammatory cell infiltrates rich in MMP-2 in 40 years old psoriasis patient with one year history of the disease. MMP-2 IMH, × 250**

Few (+) keratinocytes and moderate (++) dermal fibroblasts and inflammatory cells contained TIMP-2 (Figure 2.8.). Commonly, we observed more prominent TIMP-2 distribution in basal keratinocytes.

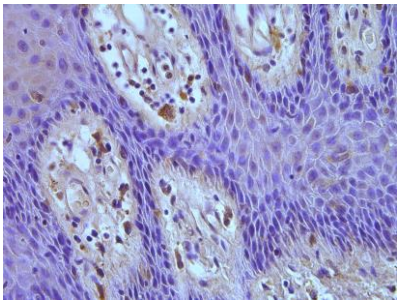

Figure 2.8. **Moderate (++) TIMP-2-containing inflammatory cells in dermal connective tissue in 36 years old psoriasis patient. TIMP-2 IMH, × 400**

Mostly moderate (++) keratinocytes and moderate to numerous (++/+++) dermal fibroblasts, macrophages, lymphocytes contained TIMP-4 (Figure 2.9.).

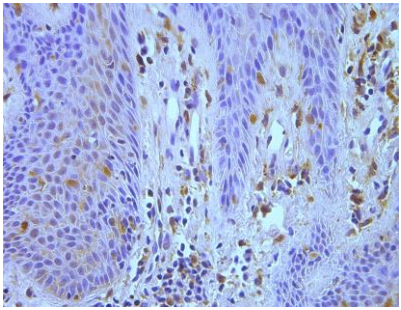

Figure 2.9. **Numerous (+++) TIMP-4-containing cells in the skin of 36 years old psoriasis patient. Cells represent both epidermal keratinocytes and dermal fibroblasts and inflammatory cells. TIMP-4 IMH, × 400**

Apoptosis was observed in keratinocytes (mostly few (+)), dermal fibroblasts and inflammatory cells (mostly moderate (++)), as well as

in glandulocytes forming sebaceous and sweat glands, cells of walls of blood vessels and hair follicles (Figure 2.10.). Apoptotic index was 0.22±0.06 to 0.91±0.16.

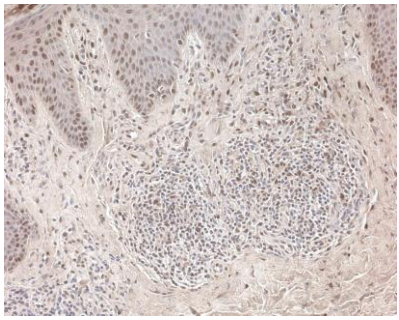

Figure 2.10. **Numerous (+++) apoptotic cells are seen in dermal inflammatory infiltrates in the skin of 19 years old psoriasis patient. Simultaneously, numerous (+++) apoptotic keratinocytes in epidermis. TUNEL, × 200**

2.3. Statistical analysis

Statistically significant difference between patients with psoriasis and control patients was found for hBD-2, PGP 9.5, substance P factors and apoptotic cells in epidermis and dermis, and for CGRP, TIMP-2 and TIMP-4 factors in epidermis.

Very strong statistically significant positive correlation was found:

- 0.921 (p-value 0.000) – between cells containing hBD-2 in epidermis and dermis,
- 0.915 (p-value 0.000) – between TNF-α-containing cells in epidermis and dermis,
- 0.886 (p-value 0.000) – between hBD-2 finding in dermis and MMP-2 finding in epidermis,

- 0.885 (p-value 0.000) – between cells containing hBD-2 and MMP-2 in dermis,
- 0.878 (p-value 0.000) – between MMP-2-containing cells in epidermis and dermis,
- 0.860 (p-value 0.000) – between IL-8-containing cells in epidermis and dermis,
- 0.824 (p-value 0.000) – between hBD-2 and TNF-α findings in epidermis,
- 0.821 (p-value 0.000) – between hBD-2 and MMP-2 findings in epidermis,
- 0.805 (p-value 0.000) – between apoptotic cells in epidermis and dermis findings,
- 0.803 (p-value 0.000) – between hBD-2 and TNF-α findings in dermis,
- 0.801 (p-value 0.000) – between hBD-2 finding in epidermis and TNF-α finding in dermis.

Strong statistically significant positive correlation was found:

- 0.795 (p-value 0.000) – between TNF-α and MMP-2 findings in dermis,
- 0.786 (p-value 0.000) – between hBD-2 finding in dermis and TNF-α finding in epidermis,
- 0.758 (p-value 0.000) – between TNF-α and MMP-2 findings in epidermis,
- 0.756 (p-value 0.000) – between hBD-2 finding in epidermis and MMP-2 finding in dermis,
- 0.751 (p-value 0.000) – between TNF-α finding in epidermis and MMP-2 finding in dermis,

- 0.745 (p-value 0.000) – between CGRP-containing structures in epidermis and dermis,
- 0.733 (p-value 0.000) – between IL-8 and CGRP findings in epidermis,
- 0.723 (p-value 0.000) – between TNF-α finding in dermis and MMP-2 finding in epidermis,
- 0.639 (p-value 0.000) – between TIMP-2 and TIMP-4 findings in dermis,
- 0.633 (p-value 0.000) – between IL-6-containing cells in epidermis and dermis,
- 0.617 (p-value 0.000) – between IL-8 finding in dermis and epidermis CGRP finding,
- 0.614 (p-value 0.000) – between CGRP-containing structures and apoptotic cells in epidermis,
- 0.606 (p-value 0.000) – between IL-8 and PGP 9.5 findings in dermis.

3. DISCUSSION

In this study, skin biopsy specimens obtained from untreated psoriatic skin inflammatory lesions were examined for hBD-2, PGP 9.5, CGRP, substance P, TNF-α, IL-1α, IL-6, IL-8, MMP-2, TIMP-2 and TIMP-4 immunoreactive structure relative appearance and presence of apoptosis when compared with control normal skin samples.

Psoriasis is a common chronic dermatological disease with substantial variability of findings in different populations and geographic areas. For the most common causes of such marked differences are seen climate, genetic susceptibility and exposure to environmental antigens. On the other hand, no significant gender differences in the prevalence of psoriasis have been identified (Enamandram and Kimball, 2013).

Latvian residents geographically are in a climate zone, which specifically enhances inflammatory processes of psoriasis, as long autumn and winter period decreases and often fluctuates temperature outdoors at the same time with very dry air inside due to the heating, as well as markedly reduces the day time and the received amount of ultraviolet radiation. Therefore, often there is prominent symptom seasonality. However, we did not judge the seasonality, as our study is based on skin samples collected in the autumn and winter period, when psoriasis clinical worsening events are particularly characteristic.

In our study we did not find the relationship between the studied factors and patient's gender. At the same time there were no statistically significant correlations of our studied factors found also with patient's age or duration of the illness, as well as the patient's age and disease duration did not correlate with each other, which means that these parameters in the pathogenesis of the disease are not important.

Skin antimicrobial peptides act as the first line of defense against the external environment pathogens. Both healthy skin and skin affected by inflammation is able to secrete proteins with antimicrobial activity. Most studied so far are hBD and kathelicidins (Borkowski and Gallo, 2011; Gallo and Nakatsuji, 2011; Morizane and Gallo, 2012). The capability of antimicrobial defense in skin affected by psoriasis we assessed by evaluating the presence of hBD-2 in our skin samples. Epidermis and dermis were evaluated separately – in all psoriasis patients' skin biopsy specimens were found hBD-2-containing epidermal keratinocytes and dermal fibroblasts, lymphocytes and macrophages. Between the epidermis and dermis hBD-2-containing cells, we found a statistically significant very strong positive correlation. At the same time, it is important to note that between psoriasis patients and control group a statistically significant difference of hBD-2 factor presence both in epidermis and dermis was found, suggesting that in psoriasis patients it is this skin antimicrobial protein that significantly increases during unmodified (vulgar) psoriasis inflammation.

Up to now, there is a diverse and ambiguous data on the role of hBD-2 in the pathogenesis of psoriasis. hBD-2 has been studied on both protein and gene level and detected in the serum (Metz-Boutigue *et al*., 2010). European residents have a high incidence of psoriasis; therefore, the genetic role of hBD has been studied. Increased copy number of genes encoding hBD has been found in the European population (Hollox *et al*., 2008; Stuart *et al*., 2012).

Similarly to our obtained data, marked hBD-2 findings in the skin of psoriasis patients compared with other inflammatory skin diseases, atopic dermatitis, allergic contact dermatitis and irritant contact dermatitis have been detected (Kamsteeg *et al*., 2010). Also, in blood serum, simultaneously researching psoriasis and atopic dermatitis patients, high hBD-2 concentration and correlation with disease activity or PASI directly in psoriasis patients

has been observed (Jansen *et al*., 2009). Significantly increased presence of hBD-2 was observed in plasma of psoriasis patients and at the same time (within the same study) in keratinocyte cultures (Kanda *et al*., 2011). hBD-2 in combination with other antimicrobial peptides has been found in scales obtained from psoriatic inflammatory lesion, which in turn activates dermal dendritic cells (Lande *et al*., 2015).

The role of hBD-2 in the pathogenesis of psoriatic inflammation and treatment of psoriasis has been proven by immunohistochemical evaluation of 12 skin biopsies of patients with psoriasis before and six weeks after systemic treatment with TNF-α inhibitor Etanercept. A significant reduction in PASI and a decrease of hBD-2 expression in the studied skin samples was observed (Gambichler *et al*., 2011).

Often in the course of psoriatic inflammation directly in connection with antimicrobial protection other factors are emphasized, such as omega 3 and vitamin A effect. However, these studies have been sporadic and mainly the role of vitamin D has been described. Immunohistochemically it has been found that hBD-2 expression in the skin of psoriasis patients is higher in patients with normal vitamin D serum levels as compared to patients with vitamin D deficiency. These local skin immune response changes could be used for psoriasis therapy (Kim *et al*., 2010). Psoriasis patients, included in our study, had skin without a tan, as well as the patients hadn’t received additional vitamin D, in order to exclude the presence of hBD-2 influencing factors. Similarly, in our study psoriasis patients before the skin biopsy specimen acquisition didn’t receive systemic or topical treatment. Thereby, the obtained data shows hBD-2 presence in unmodified inflammation or "output" level.

Human skin is very well innervated and also in unaltered conditions there can be found a number of neuropeptides. The activation of autonomous nervous system and increase of various neuropeptides in the skin correlates with psoriatic inflammation. It is considered, that not only changes in the skin's

immune system, but also the dysregulation of the peripheral nervous system is important in the pathogenesis of psoriasis. Directly locally in the skin secreted neuropeptides, perhaps, sustain prolonged inflammation of psoriasis. Up to now, increased nerve fiber density in psoriatic skin and an increased secretion of catecholamines and neuropeptides, such as substance P, has been found (Chapman and Moynihan, 2009).

To evaluate the innervation changes in psoriasis affected skin inflammation in our study using immunohistochemistry we detected PGP 9.5, CGRP and substance P. Overall, PGP 9.5-containing nerve fibers in epidermis were few to moderate and in dermis moderate, while CGRP-containing structures in epidermis were an average of moderate and in dermis moderate to numerous. Relatively CGRP findings were slightly more pronounced. A statistically significant strong positive correlation was found between CGRP-containing structures in epidermis and dermis. Particularly surprising were the substance P results – in all patients' dermis and in epidermis of only part of patients (24 of 40) were found mostly few substance P-containing structures. This could be a sign of potentially tachykinin secondary role in psoriasis inflammation. At the same time it is important to note also that there is statistically significant difference between patients with psoriasis and control group patients for PGP 9.5 and substance P in epidermis, in dermis and in epidermis for CGRP factor.

The role of PGP 9.5 directly in the course of psoriasis has been studied little and therefore the available data is also limited. In two trials PGP 9.5-immunoreactive nerve fibers in increased levels have been detected in psoriasis inflammation affected skin (Al'Abadie *et al.*, 1995; Hagforsen *et al.*, 2000). There are available entirely contrary data of two other studies that show prominent decrease of PGP 9.5-immunoreactive nerve fibers in psoriatic inflammation compared with healthy skin or skin of psoriasis patient without inflammation (Johansson *et al.*, 1991; El-Nour *et al.*, 2009).

Reduced expression of PGP 9.5 has also been detected in the experimental mouse model of atopic dermatitis, which was a subject to a long-lasting episode of mild distress. Thus, a constant stress reduces innervation in inflamed skin (Lönndahl *et al*., 2010).

It is not probable to rule out a possible variable PGP 9.5 role in maintaining skin inflammation and formation of neurogenic inflammation. It has been demonstrated that PGP 9.5 has a role in nerve fiber quality evaluation – its' immunohistochemical detection with the help of skin biopsy is recommended as a diagnostic procedure for the diagnosis of peripheral neuropathies. The density of PGP 9.5-containing nerve fibers in dermis directly shows the perception of warmth, heat and pain sensation (Lauria *et al*., 2005). PGP 9.5 is an important neuronal cytoplasmic protein that is found in both central and peripheral nervous system, as well as widely in the cells of diffuse neuroendocrine system (Thomson *et al*., 1983). At the same time PGP 9.5 distinctly marks peripheral nerve fibers of different sizes in various organs (Wilson *et al*., 1988). In light of all the above, assumedly, in the skin of our patients PGP 9.5 non-dominant prevalence reflects the total decrease of nerve fiber quality in psoriasis.

We found a statistically significant moderate positive correlation both between PGP 9.5 findings and apoptotic cells in epidermis and dermis, and also between PGP 9.5 findings in dermis and apoptotic cells in epidermis. Similar to our study, PGP 9.5-immunoreactive nerve fibers and identical disposition of apoptotic keratinocytes in epidermis has been observed in the skin samples of vitiligo patients (Aroni *et al*., 2008). In another study, in the experimental mouse model of stress-induced neurogenic inflammation observed increase of dendritic cells, more rapid maturation and migration and subsequent increased apoptosis of keratinocytes (Joachim *et al*., 2008). Thus, presumably, neural and apoptotic changes are closely related, because neurohormones

and mediators released by inflammatory cells can induce programmed cell death in the course of inflammation (especially neurogenic inflammation).

The influence of CGRP and substance P on psoriatic inflammation has been studied in experimental mouse model. It was observed that by denervating skin or inhibiting these factors significantly reduces the number of lymphocytes and acanthosis. Changes of lymphocyte count were affected both by the activity of CGRP and substance P, while acanthosis was stimulated directly by CGRP (Ostrowski *et al*., 2011). Also, other author study data indicates directly of CGRP effects on enhanced keratinocyte proliferation and subsequent thickening of epidermis both in psoriatic skin, as well as in terms of keloid formation and innervated experimental skin model (Yu *et al*., 2009; Roggenkamp *et al*., 2013; Suarez *et al*., 2014). CGRP promotes not only keratinocyte proliferation, but also the division of epidermal stem cells. This effect of the above mentioned factor activity has been observed *in vitro* (Dong *et al*., 2010). It should be noted that we also at the same time with a strong presence of CGRP observed enhanced keratinocyte proliferation and epidermal thickening, compared with control group tissue samples.

In keratinocyte tissue cultures possible autocrine or paracrine ability to express neuropeptides CGRP and substance P by keratinocytes has been demonstrated – neuropeptide stimulation significantly increased neuropeptide receptor expression in keratinocytes and in result a very large amount of CGRP and substance P was released from keratinocytes. In turn, due to the increase of concentration of these peptides cell proliferation and inflammatory cytokine expression and secretion were stimulated (Shi *et al*., 2013). It is likely that such autocrine or paracrine cell cooperation is possible also in case of other neuropeptides and even other inflammatory mediators.

It is interesting that all three of our studied factors as a complex were studied in hypertrophic burn scars in connection with marked pain and itching

sensation in other authors' works. Findings of all three factors were found increased and they closely correlated with both pain and the sensation of itching. PGP 9.5 correlated with the sensation of itching, substance P correlated with the pain sensation and CGRP correlated with both the sensation of itching and pain (Kwak *et al.*, 2014). These facts could explain certain of our psoriasis patients' subjective complaints of inflammatory skin area itching, while pain was not characteristic (in our studied tissue samples finding of substance P was little). In another study, in skin biopsy samples of patients with psoriasis substance P was found in large amounts and significantly correlated with the intensity of itching (Amatya *et al.*, 2011). In this study the amount of enrolled subjects was slightly smaller or 28, compared to our study of 40 patients. These prominent differences in findings of substance P could be explained just by the fact that the above mentioned study included almost all the patients with complains of a strong concurrent sensation of itching, while only few of our study patients complained of a sensation of itching and it was not continuous.

Overall, traditionally it is believed that substance P plays an important role in the pathogenesis of psoriasis and also other chronic inflammatory skin diseases (Asadi *et al.*, 2012; Lotti *et al.*, 2014). However, our data on the small findings of substance P-containing structures differed sharply from up to now available views in the literature. One study previously also has demonstrated reduced expression of substance P in the skin affected by psoriasis (Pincelli *et al.*, 1992). Presumably, the very role of tachykinins in the pathogenesis of psoriasis is highly variable and perhaps overrated. Although at the same time, perhaps, the different laboratory detection methods of substance P can significantly affect the results.

To evaluate the psoriatic inflammation using immunohistochemistry in skin we detected TNF-α, IL-1α, IL-6 and IL-8. TNF-α-containing cells were found both in epidermis and dermis however; in dermis this finding

was significantly more pronounced. Also, findings of IL-6 and IL-8 were rich – moderate to numerous cells of both epidermis and dermis contained both mentioned factors. We found moderate positive correlation between IL-6 and IL-8 findings in dermis. Separate epidermal keratinocytes and few dermal connective tissue cells contained IL-1α in some of our patients of the study. In another study of skin affected by psoriasis mesenchymal stem cells were derived, which showed increased IL-6 and IL-8 encoding gene expression compared with healthy skin, while IL-1 encoding gene expression was not greater than in the healthy skin (Campanati *et al*., 2014). Thus, we believe that the key cytokines in skin affected by psoriasis are other cytokine release inducers IL-6 and IL-8.

Psoriasis belongs to diseases with predominantly Th1 cytokine profile. TNF-α in psoriatic inflammation is associated with hyperplasia and proliferation of dermal papillae (clinically development of psoriatic inflammatory lesion), capillary dilation and inflammatory infiltrates containing lymphocytes (clinical appearance of erythema) (Tokura *et al*., 2010; Brotas *et al*., 2012; Singh *et al*., 2013). These morphological changes we found also in skin tissue samples obtained during our study.

The role of TNF-α has been shown directly as the leading cytokine in the development of psoriatic inflammation in experimental mouse model. With the help of red bone marrow transplantation it was determined that the production of TNF-α, which promotes the development of skin inflammation, is provided by the permanently residing skin cells, but not those that develop from the red bone marrow and migrate (Nakajima *et al*., 2010). At the same time in another study, in the experimental mouse model of psoriasis TNF-α was released both by keratinocytes and by dendritic cells, macrophages and lymphocytes (Sato *et al*., 2014). Prominent decrease of expression of TNF-α and, subsequently, decrease of inflammation caused by psoriasis has been observed after applied treatment with TNF-α inhibitor

(Menter *et al*., 2007). Keratinocytes express TNF-α, however we believe that TNF-α directly affects psoriasis damaged dermis, suggesting the major inflammatory processes directly in connective tissue.

TNF-α is described to be able to induce apoptosis of keratinocytes in psoriatic inflammation (Zimmermann *et al*., 2011). In our study, we observed a significant increase of TNF-α presence in the epidermis and dermis, as well as apoptotic keratinocytes and apoptosis of dermal cells. Despite the lack of statistically significant correlation between the two above-mentioned factors, however, we believe that in psoriasis patients can be observed in the literature described correlation between the expressions of TNF-α and programmed cell death in keratinocytes and dermal cells, and primary induction is likely to "come" from the TNF-α.

IL-1α finding in our study was small, some of which is partly contrary to the available data in the literature. Thus, in skin biopsies and simultaneously in blood plasma of psoriasis patients without received treatment, reduced quantity of IL-1α has been detected (Tamilselvi *et al*., 2013). Johnston and co-authors of two separate studies have shown increase in expression of IL-1 and direct connection with more pronounced production of antimicrobial peptides, including hBD-2 (Johnston *et al*., 2011). However, it is important to consider that the diagnostic methods differ and these results have been obtained *in vitro*, as well as one of the studies used experimental mouse model of psoriasis, which means that the data that is obtained from one species cannot always be correctly interpreted to another, for example, human. It should therefore be assessed that the role of IL-1 in psoriatic inflammation varies, as well as that at molecular and genetic levels can be significant differences in the expression of the mentioned factor.

Changes in IL-1 coding gene expression and thereby greater risk of developing the disease have been found in psoriatic arthritis patients (Rahman *et al*., 2006; Bowes *et al*., 2012).

In general, the effect of IL-1α activity could be explained by the fact that locally in skin under the influence of foreign antigens dendritic cells and T lymphocytes form cell clusters that promote the proliferation and activation of new T lymphocytes perivascularly in dermis. Such formation of immune cell foci decreases if decreases the amount of macrophages. Treatment with IL-1α in dermal macrophages of contact hypersensitivity experimental mouse model contributes to chemokine production. Thus, IL-1α possibly is more associated directly with acquired immunity of the skin (Natsuaki *et al*., 2014). At the same time IL-1α also actively participates in the provision of the immune function of mucous membranes (Wu *et al*., 2013; Bamias *et al*., 2014).

Interestingly, findings of inflammatory cytokines in our study did not correlate with patient's age, however, in the available literature there is data that shows a small IL-1α and its receptor antagonist decline locally in healthy skin in connection with aging processes (Sato *et al*., 2014).

A significant finding of IL-6 has been discovered in mast cells of experimentally induced psoriasis lesion. Simultaneously, observed also the expression of IL-6 receptor in dermal cells (Suttle *et al*., 2012). Serum IL-6 concentration is described as a good prognostic factor for successful local therapy results, as it protects from immunosuppression (Lo *et al*., 2010). Overall, the IL-6 concentration in the blood serum is described as an effective prognostic factor of systemic inflammation, especially in patients with severe course of the disease (Dowlatshahi *et al*., 2013). The decline of IL-6 concentration was also observed as a response to successful results of systemic therapy in psoriasis patients (Cordiali-Fei *et al*., 2014).

However, it should also be mentioned that there are variable data obtained from genetic research. IL-6 encoding gene polymorphism is associated directly with greater risk of developing psoriasis, but not with the therapy efficacy (Białecka *et al*., 2015). At the same time, in another study

IL-6 genetic variations are seen as the risk of developing psoriasis decreasing (Boca *et al*., 2013).

Concomitant determination of inflammatory cytokines TNF-α, IL-1α and IL-6 may serve as an evaluation indicator of psoriasis diagnosis and therapy (Portugal-Cohen *et al*., 2012).

Both increased findings of IL-6 and IL-8 in our study obtained tissue samples correspond to the data available in the literature. IL-6 and IL-8 encoding gene expression concomitant increase is observed in patients with severe burns (Noronha *et al*., 2014). Thus, we can conclude that the activity of these two inflammatory cytokines in general is coordinated. This is based on in our study observed both IL-6 and IL-8 simultaneous increase in both epidermal keratinocytes and dermal inflammatory cells in psoriatic inflammation and moderate positive correlation between these two factors.

Interestingly, IL-6 and IL-8 also show a subsequent increase in keratinocyte and melanocyte cultures under the influence of ultraviolet radiation. Perhaps, it is the paracrine cell cooperation that ensures high secretion of the two interleukins. Along with these inflammatory cytokines was observed also increase of MMP-9 factor. Thus, presumably, tissue degeneration markers create the conditions for the development of the inflammation and combined with IL-6 and IL-8 affect cellular differentiation and even phenotype (Decean *et al*., 2013).

To evaluate tissue degeneration and remodeling in psoriasis affected skin by immunohistochemistry we detected MMP-2, TIMP-2 and TIMP-4. In general, we found numerous MMP-2-containing cells in dermis of all tissue samples, while also almost all psoriasis patients epidermal keratinocytes contained MMP-2. At the same time few epidermal keratinocytes and moderate dermal connective tissue and inflammatory cells in the majority of patients with psoriasis contained TIMP-2, but moderate epidermal keratinocytes and moderate dermal connective tissue and inflammatory cells in the majority

of patients with psoriasis contained TIMP-4. We often observed patchy expression of TIMP-2 and TIMP-4. It is necessary to stress, that there is a statistically significant difference between patients with psoriasis and control group patients for TIMP-2 and TIMP-4 factors in the epidermis. Strongly with each other correlated TIMP-2 and TIMP-4 findings in dermis of psoriasis patients. So it points to the MMP-2 as the main tissue degradation enzyme in psoriatic skin, which a coordinated several collagenase inhibitor factor – TIMP-2 and TIMP-4, release is trying to suppress. Therefore, we suppose that TIMP-4 in general could be selective inhibitor of MMPs in skin affected by psoriasis.

MMPs in serum and synovial fluid have become valuable diagnostic biomarkers for evaluation of psoriatic arthritis and its treatment with TNF-α blocking agents used also in the treatment of psoriasis (Giannelli *et al.*, 2004; Chandran *et al.*, 2013). Currently is available data also that MMPs are important biomarkers for evaluation of psoriasis activity and treatment outcome. It should be noted, however, that more often, the blood plasma is studied and relatively limited information is available on MMPs changes directly in the skin tissue samples. It is described that simultaneous detection of MMP-3 and highly sensitive C-reactive protein in blood plasma could help differentiate patients with psoriasis from patients with psoriasis and psoriatic arthritis (Chandran, 2012). However, it is not noted, that the blood plasma shows the total body, not the selective for each organ-specific factor size.

In psoriasis patients compared with healthy control subjects, MMP-9 increase has been observed in peripheral blood mononuclear cells, blood plasma and skin samples, but at the same time a decrease in all these samples was observed after treatment with TNF-α blockers (Buommino *et al.*, 2012). Prominent changes of MMP-9 are observed directly in patients with large area psoriasis (Lee and Lew, 2009). Also, MMP-1 and TIMP-1 have been studied in blood plasma of patients with psoriasis and, possibly, they could serve

as a biomarker for evaluation of the course of psoriasis (Flisiak *et al*., 2008). Prominent expression of MMP-9 and MMP-12 together with TIMP-1 and TIMP-3 is determined in patients with psoriasis skin samples, mostly perivascularly and in macrophages (Suomela *et al*., 2001).

The MMP-2 has been studied at the level of genes and in individual patient subgroups a relationship with psoriasis, so-called, skin gene locus susceptibility region has been established (Vasku *et al.*, 2009). Also, in our study the increased expression of MMP-2 was observed, but skin samples obtained directly from markedly inflamed area of skin were studied. Our data shows that the increase of MMP-2 expression is specific to psoriatic skin, but, perhaps, in these patients also genetic changes are characteristic.

We found a reduced expression of TIMP-2 in psoriatic epidermis, while, presumably, in compensation moderate number of dermal connective tissue cells and inflammatory cells contained TIMP-2. Our study findings are partly consistent with the findings of other authors of increased presence of TIMP-2 in skin affected by psoriasis, although again, in the study of other authors, special attention was paid to the changes in epidermis. Both in this study and in our study epidermis affected by psoriatic inflammation presented with expression of TIMP-2 particularly dominant in the basal keratinocytes (Fleischmajer *et al*., 2000). It is also proven that TIMP-2 encoding gene expression is increased in psoriasis patients (Zhang *et al*., 2013).

Currently available data on TIMP-4 generally is limited. TIMP-2 and TIMP-4 as a complex have been studied in connection with wound healing. A significant difference of TIMP-2 has been observed in smooth healing process and chronic ulcers. The presence of TIMP-2 markedly decreased in chronic non-healing ulcers. At the same time the expression of TIMP-4 in chronic ulcers was detected perivascularly (Vaalamo *et al*., 1999). Thus, presumably, the changes of TIMP expression and mutual balance directly affect both wound healing and tissue regeneration

in general. TIMP-4 concentration in blood serum is significantly increased in systemic sclerosis patients (Elias *et al*., 2008). In psoriatic skin TIMP-4 has not been studied. We found TIMP-4-containing cells in both epidermis and dermis and simultaneously a positive correlation between it and TIMP-2. Thus, presumably, TIMP and, in particular, TIMP-4 has a compensator inhibitory role in tissue degeneration and subsequent remodeling.

To evaluate apoptosis in our study we used the TUNEL kit. We found a statistically significant difference of amount of apoptotic cells in epidermis and dermis between patients with psoriasis and control group patients. We found the presence of apoptotic cells in almost all skin samples of patients with psoriasis. Overall, both epidermis and dermis contained moderate amount of apoptotic cells, and among them we found a statistically significant strong positive correlation, which shows programmed cell death as the compensatory mechanism for the removal of damaged cells from psoriatic skin region without scarring.

Apoptosis as programmed cell death is an integral part of the life cycle of a cell. In psoriatic skin very extensive changes affect, firstly, the epidermis and the life cycle of it composing keratinocytes. Parakeratosis is often observed. In similar to skin experimental psoriasis model using the TUNEL reagents variable apoptosis findings are observed directly in parakeratotic epidermal regions (Yamamoto-Tanaka *et al*., 2014). Also in skin biopsies of psoriasis patients with the same method increased presence of apoptotic cells in the epidermis as compared with healthy skin has been found (Kawashima *et al*., 2004; Gündüz *et al*., 2006; Doger *et al*., 2007).

In several studies apoptosis in psoriasis patients has been examined in relation to the applied treatment. Psoriatic skin that was treated with vitamin D3 analogue calcipotriol ointment – the most widely used psoriasis medication of topical treatment, which is included in public compensation program for Latvian psoriasis patients has been studied. This medication

is believed to reduce keratinocyte proliferation while promoting their differentiation. A significant increase in apoptotic keratinocytes was observed directly in the treated skin areas (El-Domyati *et al*., 2007; Tiberio *et al.*, 2009; Raychaudhuri *et al*., 2014). Also, after combination therapy with tar preparations and ultraviolet radiation more pronounced programmed cell death in the skin is observed (Ranna *et al*., 2014).

There is data on increased apoptosis finding in the mesenchymal stem cells of red bone marrow and at the same time their altered activity in psoriasis patients – these changes are likely to be the reason for abnormal immune response in psoriasis patients altogether (Hou *et al*., 2014). Similarly, in psoriasis patients significantly increased with apoptosis mechanism associated cell membrane particles of endothelial cells, platelets, monocytes and macrophages have been found in blood plasma, which significantly increases the cardiometabolic risk (Takeshita *et al*., 2014).

Thus, it clearly appears that apoptosis does not only have to be assessed in isolation as cell death, but at the same time it directly affects the inflammatory processes of psoriasis, as well as with psoriasis often associated confounding diseases. Currently it is known that psoriasis patients have genetically altered composition of genes encoding the immune system, cell cycle and apoptosis (Tervaniemi *et al*., 2012; Filkor *et al*., 2013).

In the conclusion of the discussion it should be noted that in our study for the first time directly with the immunohistochemistry method a complex analysis of the morphological changes of skin of psoriasis patients with tissue sample at the time of acquisition of untreated inflammation has been performed. Key processes in inflammation of psoriatic skin are related to detectable prominent increase of antimicrobial peptide hBD-2 and tissue degeneration marker MMP-2-containing epidermal and dermal cells, changes of neuropeptides-containing innervation, compensatory findings of apoptotic cells, selective / individual changes of inflammatory cytokines.

In the development of neurogenic inflammation of the skin presence of CGRP structures plays a key role. TNF-α, IL-6 and IL-8 are important inflammatory cytokines, while IL-1α is not the key inflammatory mediator in the pathogenesis of psoriasis. As an absolutely innovative has to be mentioned the finding of TIMP-4 (and with it associated TIMP-2), which indicates compensatory tissue remodeling opportunities, while from the MMPs pleiad also MMP-2 is one of the main psoriatic skin degeneration factors.

4. CONCLUSIONS

1. In the skin of psoriasis patients with different disease duration and progress activation of skin antimicrobial peptides, specific neuropeptides-containing innervation (sensory), inflammatory cytokines, degeneration enzymes and programmed cell death is essential, suggesting their involvement in the morphopathogenesis of psoriasis. At the same time findings of these factors statistically significantly do not correlate with patient age, sex, disease duration and season, excluding these parameters from involvement in the pathogenesis of the disease.
2. A healthy human skin is characterized by presence of antimicrobial proteins, inflammatory mediators and pro-inflammatory cytokines, while overall cytokine-stimulating factor (IL-6 and IL-8) expression remains high. At the same time in healthy skin sensory innervation is dominant with specific presence of CGRP. Apoptosis is the characteristic compensatory mechanism in cell cycle regulation of intact skin keratinocytes and dermal connective tissue cells.
3. In the skin of psoriasis patients of different age statistically significantly increased hBD-2 points to its role in therapy unmodified psoriatic skin local protection.
4. In psoriasis patients comparative predominance of CGRP-containing innervation over PGP 9.5-containing nerve fibers indicates a total skin innervation quality deterioration or damage, which is not related to the sensory nerve fibers. Selective CGRP prominent presence indicates its role, not substance P, as the main neurogenic inflammation factor and broader with other processes in the skin (keratinocyte proliferation, thickening of epidermis) related functions in the skin of psoriasis patient.

5. Main cytokines in psoriasis-affected skin are other cytokine release inducers IL-6 and IL-8, which indicates unchanged role of these factors in skin. TNF-α, predominantly, affected psoriasis damaged dermis, suggesting main inflammatory processes directly in connective tissue, instead of epidermis, while the main pro-inflammatory cytokine IL-1α finding in only some patients indicates probably very individual skin response to the progress of the disease.
6. In the skin of psoriasis patient pronounced is the expression of degeneration enzyme MMP-2, while as the main MMP inhibitors significantly increase the mutually correlating TIMP-2 and TIMP-4 (TIMP-4 in particular). Overall, tissue degeneration factor and their inhibitor common (TIMP-2) and selective (TIMP-4) increase indicate intensive remodeling processes of psoriasis-affected area of the skin.
7. Pronounced findings of apoptotic cells of psoriasis-affected skin indicate programmed cell death as a complex mechanism for the removal of damaged cells from the skin without scarring.
8. In the prognostic diagnostic algorithm of psoriasis patient the detection of such molecular markers as hBD-2, CGRP, IL-6, IL-8, MMP-2 and TIMP-4 should be included.

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6. LIST OF PUBLICATIONS

Scientific papers (7)

1. **Mozeika E.**, Pilmane M., Kisis J. Role of Human Skin Antimicrobial Peptides in Psoriasis // Acta Chirurgica Latviensis, 2010 (10/2): 69–75.
2. **Mozeika E.**, Pilmane M., Kisis J. Distribution of human β-defensin 2, TNF-alpha, IL-1 alpha, IL-6 and IL-8 in psoriatic skin // Papers on Anthropology XX, 2011: 289–302.
3. **Mozeika E.**, Jemec G.B., Nürnberg B.M. Hedgehog pathway does not play a role in hidradenitis suppurativa pathogenesis // Exp Dermatol., 2011 Oct; 20(10): 841–842.
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6. **Sidhom E.**, Pilmane M., Kisis J. The Distribution of Matrix Metalloproteinase-2, Tissue Inhibitor of Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-4 in Psoriatic Skin // Br J Med Med Res., 2015; 8(10): 883–890.
7. **Sidhom E.**, Pilmane M., Kisis J. Local antimicrobial, protease and cytokine defense systems in psoriatic skin – accepted for publication in Indian Journal of Dermatology, Venereology and Leprology.

Monograph chapters (1)

1. Ķīsis J., Hartmane I., Žīgure S., Karls R., Karpoviča I., **Sidhoma E.**, Kažociņa Z. – Rīga Stradiņš University, Department of Infectology and Dermatology work group. Psoriasis clinical guidelines. Published in homepage of the National Health Service, registered 23.04.2013. No. KV 03 – 2013, 2012, 109 pp.

Abstracts and presentations in international conferences (8)

1. **Mozeika E.**, Pilmane M., Kisis J. Antimicrobial peptides in psoriatic skin lesions – an immunohistochemical study, 9th Congress of the Baltic Association of Dermatovenerology (BADV) abstract, Tartu 2010, abstract published in homepage of the congress. (Oral presentation)
2. **Mozeika E.**, Pilmane M., Kisis J. Immunohistochemical analysis of inflammatory markers and neuropeptides in psoriasis, 3rd International Congress on Psoriasis abstract, Paris 2010, JEADV (2010) 24 (Suppl. 4), 7. (Poster and Oral presentations)
3. **Mozeika E.**, Pilmane M., Kisis J. Distribution of human β-defensin 2, TNF-alpha, IL-1 alpha, IL-6 and IL-8 in psoriatic skin, Baltic Morphology 6th scientific conference abstract, Tartu 2011, abstract book 28. p. (Oral presentation)
4. **Mozeika E.**, Pilmane M., Kisis J. Distribution and appearance of antimicrobial peptides, neuropeptides and inflammatory markers in psoriatic lesions, 6th International Congress Psoriasis From Gene to Clinic abstract, London 2011, abstract book 66. p. and British Journal of Dermatology 12.2011. supplement. (Poster presentation)

5. **Mozeika E.**, Pilmane M. Local immune response in the skin of psoriasis patients, Oral presentation in the Annual Meeting of Finnish Anatomists, Tampere 2012.
6. **Sidhoma E.**, Pilmane M., Ķīsis J. Role of Local Antimicrobial Response and Apoptosis in Psoriatic Skin, Baltic Morphology 7th scientific conference abstract, Riga 2013, abstract book 59. p. (Oral presentation)
7. **Sidhoma E.**, Pilmane M., Kisis J. Role of antimicrobial peptides, proteases, neuropeptides and apoptosis in the inflammatory events of psoriasis, European Workshop on "Skin Immune Mediated Inflammatory Diseases (SIMID)" abstract, Verona 2014, abstract book and Clinical Dermatology 2014; 2 (Suppl 1): 35. (Poster presentation)
8. **Sidhom E.**, Pilmane M., Kisis J. The role of antimicrobial defense and tissue inhibitors of matrix metalloproteinases in psoriatic inflammation, European Academy of Dermatology and Venereology (EADV) 12th Spring Symposium abstract, Valencia 2015. (Poster presentation)

Abstracts and presentations in local conferences in Latvia (7)

1. **Možeika E.**, Pilmane M., Ķīsis J. Cilvēka ādas dabīgie antimikrobie peptīdi pacientiem ar psoriāzi, annual Rīga Stradiņš University Scientific Conference 2010 abstract, abstract book 111. p. (Poster presentation)
2. **Možeika E.**, Pilmane M., Ķīsis J. Psoriāzes slimnieku ādas dabīgā imūnā atbilde, annual Rīga Stradiņš University Scientific Conference 2011 abstract, abstract book 134. p. (Poster presentation)
3. **Možeika E.**, Pilmane M., Ķīsis J. Human antimicrobial peptides in skin of patients with psoriasis in ontogenetic aspects, annual Rīga Stradiņš University Scientific Conference 2012 abstract, abstract book 134. p. (Poster presentation)

4. **Sidhoma E.**, Pilmane M., Ķīsis J., Sidhom T. Antimikrobo peptīdu un iekaisuma citokīnu izvērtējums psoriāzes pacienta ādā, annual Rīga Stradiņš University Scientific Conference 2013 abstract, abstract book 159. p. (Poster presentation)
5. **Sidhoma E.**, Pilmane M., Ķīsis J., Sidhom T. Dermatožu neskartas ādas imūnā atbilde, annual Rīga Stradiņš University Scientific Conference 2014 abstract, abstract book 69. p. (Poster presentation)
6. **Sidhoma E.**, Pilmane M., Ķīsis J. Dermatožu neskartas ādas audu remodelācijas raksturojums, annual Rīga Stradiņš University Scientific Conference 2015 abstract, abstract book 59. p. (Poster presentation)
7. **Sidhoma E.**, Pilmane M., Ķīsis J. Neiropeptīdu sadalījums psoriāzes pacienta ādā, 7th Congress of Latvian Physicians, abstract published in homepage of the congress:
   http://arstukongress.lv/?&s=1361881321&fu=read&id=28
   (Poster presentation)