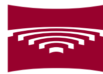


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# CHANGES OF FUNCTIONAL UNIT OF THE NAIL FOR PATIENTS OF ONYHOMYCOSIS

Summary of Doctoral Thesis
for obtaining the degree of a Doctor of Medicine

Subfield – Morphology

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

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# ABBREVIATIONS

| | |
|---|---|
| AI | Apoptotic index |
| AIF | Apoptosis inducing factor |
| AMP | Antimicrobial peptides |
| DNS | Deoxyribonucleic acid |
| FasL | Fas ligand |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| H/E | Hematoxylin and eosin |
| hBD-2 | Human beta defensin-2 |
| hBD-3 | Human beta defensin-3 |
| hBD-4 | Human beta defensin-4 |
| IFN | Interferon |
| IL-1 | Interleukin-1 |
| IL-2 | Interleukin-2 |
| IL-3 | Interleukin-3 |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL-10 | Interleukin-10 |
| IL-22 | Interleukin-22 |
| KOH | KOH test |
| MMP-2 | Matrix metalloproteinase-2 |
| PAMPs | Pathogen associated molecular pattern |
| PAS | Periodic acid-Schiff |
| PCR | Polymerase chain reaction |
| PGP9.5 | Protein gene product 9.5 |
| SCIO | Scoring clinical index for onychomycosis |
| TLRs | Toll Like Receptors |
| TGF | Transforming growth factor |
| $T_h$ | T helpers |
| TNF | Tumor Necrosis Factor |
| TNF-R | Tumor Necrosis Factor Receptor |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| VEGF | Vascular endothelial growth factor |

# 1. INTRODUCTION

Onychomycosis is a chronic, wide-spread fungal infection that is difficult to treat and that causes a gradual destruction of the nail plate. The disease is related to different cosmetic and psychological complications, as well as often recrudescence and not always successful therapy.

Onychomycosis of feet occurs 7 times more often than onychomycosis of hands. Frequency of onychomycosis may increase along with ageing that is explained by changes in the speed of nail growth and blood circulation in the distal parts of extremities [*Szepietowski,* 2007]. Development of onychomycosis is determined by several predisposing factors. The German guidelines for onychomycosis point out the following key factors: genetic predisposition, angiopathy, peripheral neuropathy, wearing tight footwear, deformation of feet, recurring injuries, *diabetes mellitus* and other metabolic changes [*Seebacher,* 2007*, Nenoff,* 2014]. R. Nowicki together with other authors refer also to weakening of immune condition and psoriasis [*Faergemann,* 2006].

It should be noted that Pub Med data base provides large amount of information sources on spread of pathogenic fungi in different regions of the world, on division of range of causative pathogens and the most frequent causative pathogens. Unfortunately, the number of articles on contemporary studies of morphopathology in nails affected by pathogenic fungi is not large. Possibly, it is related to complexity of obtaining relative samples (skills of a doctor, patient's approval of invasive procedure). However, nail biopsy is proposed in situation when clinically it is not possible to differ between nails affected by onychomycosis and other diseases affecting nails (psoriasis, *lichen planus*, longitudinal melanonichia, trachyonychia, tumors) [*Grover,* 2005*; Abdel,* 2006].

In the past years, scientists have actively studied natural antimicrobial protection of the human body. The body produces molecules that are toxic to pathogens of microbes - antimicrobial peptides. Antimicrobial peptides from the groups of cathelicidins and defensins are found in human bodies [*Rodrigues,* 2009*; Lai,* 2010]. Nail unit has been researched from the point of view of the natural innate immunity, proving the antimicrobial action of nail extract against *Candida albicans* [*Dorschner,* 2004].

In case of *Tinea corporis* infection, significant expression of hBD-2 protein was discovered. Histological cuts showed involvement of all epidermis in the reaction of skin against dermatophytes that was expressed as thickening of epidermis on behalf of hyperkeratosis and acanthosis. Infiltration of neutrophils and T cells could be observed in the papillary layer of the skin. Action of hBD-2 antifungicide against *Trichophyton rubrum* has been proven [*Jensen,* 2007].

Therefore it is possible that similar processes of innate immunity occur also in nails affected by onychomycosis. Keratinocytes might produce antimicrobial peptides to fight pathogenic fungi.

All in all, it has to be noted that the natural immunity of nails includes also thickening of the nail plate in an unfavourable conditions, these changes are characteristic of onychomycosis as well. The nail unit affected by onychomycosis morphologically differs from a healthy nail plate, when observing disorders of normal keratinization, inclusions of fungal elements, in certain cases infiltration of lymphocyte can be observed [*Stewart,* 2012]. However, the intensive changes caused by the pathogenic fungi in ill nails might be accompanied with excretion of inflammation cytokines and dilation of blood vessels, possibly, neoangiogenesis. Along with such intensive changes in tissues, restructuring processes of tissues can be observed, a massive focus

in tissues causes the programmed death of cells and this is accompanied with changes in the antimicrobial resistance system of the functional nail unit. The above prescribes the necessity to perform a complex study of various factors impacting / regulating the health of nail.

The **goal** of the present paper was to study functional unit of the nail in case of onychomycosis, assessing processes of inflammation, vascularization, innervation, degeneration, apoptosis, as well as natural anti-microbial resistance processes.

The **hypotheses** of the study are the following:

1. in the nails affected by onychomycosis, excretion of human beta-defensins occurs;
2. in the nails affected by onychomycosis, restructuring processes of tissues occur characterized by excretion of cytokines and destruction of cells.

The following **objectives** were set for the study:

1. to obtain samples of the nail tissues from patients of onychomycosis, as well as to obtain control material and research the histological report on the functional unit;
2. to determine -1, -6 and -10 interleukins and human beta-defensins 2, 3, 4 in the nail tissues affected by onychomycosis and in control nail tissues to characterise the inflammation processes in the tissues and assess the local antimicrobial protection;
3. to determine PGP9,5 and VEGF in the nail tissues affected by onychomycosis and in control nail tissues to analyse the presence of innervation containing neuropeptides and tissue ischemia;

4. to determine MMP-2 and apoptosis in the nail tissues affected by onychomycosis and in control nail tissues to analyse degeneration processes in the tissues and the programmed process of cell death;
5. to perform statistical analysis and to detect the mutual correlation of data.

**Novelty of the study**

For the first time, a comparison between nails affected by onychomycosis and healthy nails is carried out applying immunohistochemical methods. For the first time, human beta-defensins are determined in nails affected by onychomycosis.

**Significance of the study**

The study provides an insight into the process of morphopathology of onychomycosis, allowing to obtain an in-depth understanding of the factors regulating health of nails.

**Structure of the Doctoral thesis**

The Doctoral thesis consists of five chapters: review of literature, materials and methods, results, discussions and conclusions. The review of literature summarizes information on prevalence and topicality of onychomycosis, anatomy and histology of the nail functional unit, pathophysiology of different fungi, diagnostics and nail biopsy method.

The Doctoral thesis is based on 163 literary sources. There are 49 images and 23 tables in the Doctoral thesis.

# 2. MATERIALS AND METHODS

## 2.1. Tissues under study

Material of nail biopsies from 30 onychomycosis patients was used in the study. *Punch* biopsies ("two *punch"* method – 5 and 3 mm biopsies) were performed within a standard procedure for purposes of diagnostics of SIA "J. Ķīsis":

1) when performing differential diagnosis of psoriasis / onychomycosis, i.e., in cases when only clinically changed nails were present without typical symptoms of psoriasis on the skin;
2) in cases when in the clinical conditions of typical onychomycosis, negative results of microscopy / subculture occurred, for purposes of verifying the diagnosis.

Nail biopsy was carried out to 40 patients, 10 of them were diagnosed with *psoriasis* rather than *onychomycosis*.

The material was obtained from 2012 to 2014.

The study was carried out in line with permission given by the RSU Ethics Committee (26.01.2012) and as a result of the study no harm was done to the health of patients.

The following factors were excluded in the patients' anamnesis: systemic diseases, use of antimycotic preparations, use of systemic antibacterial preparations during the last month, continuous subjection to sunlight during the last month.

Nail material of corpses was used for control purposes. Five samples of nails were obtained via the standard necropsy technique at the structural unit of RAKUS (Riga East Clinical University Hospital), SIA "Valsts Patoloģijas centrs" in 2013. The deceased were from the age of 36 to 57, males and females,

without any clinical changes in toe or finger nails. The selection criteria of the group were the following: non-infectious cause of death, lack of infectious diseases in anamnesis, before death, the patient had not taken a course of antibacterial preparations, the patient does not have any systemic diseases in anamnesis, the patient is not tanned and there are no clinical symptoms of onychomycosis.

## 2.2. Morphological methods

### 2.2.1. Fixation of tissues and methods of routine staining

Fixation of tissues was carried out for 24 hours at SIA "J.Ķīsis" immediately after the surgical manipulation - nail biopsy. For this purpose, saturated *Stefanini* solution (2% formaldehyde and 0.2% pikrin acid, 0.1 M phosphate buffer pH7.2) was used. After fixation, pieces of tissues were transported to Morphology Department of the Institute of Anatomy and Anthropology of the Rīga Stradiņš University and after processing in spirits in an increasing succession and in xylenes, samples were immersed in paraffin.

3-4 μm thick sections were prepared from the material of tissues obtained from each patient, the samples were stained by hematoxylin and eosin [*Lillie,*1976*; Avwioro,* 2011]. The stained preparations were analysed using light microscopy technique, using *Leica DM RB* (Germany) microscope. Histological condition was analysed using *Leica microsystem AG* (Germany) digital camera.

All preparations in the overview sections were assessed from the following positions:

- whether there is or there is not a hypertrophy of the nail plate (thickening of the nail plate);

- whether there is or there is not acanthosis - hyperplasia of the *stratum spinosum* of the nail bed (increase in number of cells);
- whether there is or there is not paraketarosis - clusters of squamous cells (groups) in the corneum layer (plate);
- whether there is or there is not hypergranulosis - hyperplasia of the cells in *stratum granulosum* (increase in number of cells), with the number of cell rows 4 and more;
- whether there is or there is not vacuolar damage of epitheliocytes - parenchymal damage with changed in cell structure;
- whether there is or there is not pericellular oedema of cells;
- whether there is or there is not inclusions - amorphous, thick structures in the *corneum* layer;
- what are the characteristics of collagen fibers (hypo-, normo- or hypertrophic);
- what is the vascularization of the dermis in comparison with the control group;
- number of fibrocytes in the part of connective tissues in one field of view;

To assess the severity of parakeratosis and acanthosis, number of inclusions, spread of vacuolar damage and pericellular oedema in the preparation and to be able to process the data statistically, the following assessment scheme was applied:

- 1– no indication
- 2 – small focus spread (<10%)
- 3 – large focus spread (<50%, more than 10%)
- 4 – total focus spread (<55%)

For hyperkeratosis, a more simple scheme was applied (1 – yes or 2 – no). For granulosis was applied similar: 1 – hypo-; 2 – normo-; 3 – hypergranulosis.

### 2.2.2. Reaction of periodic acid Shiff

Periodic acid *Shiff* (PAS) reaction allows to determine glycoproteins, polysaccharides , different mucopolysaccharides, glycolipids and fatty acids in the tissues [*McManus*, 1946].

Deparaffinized and dehydrated preparations of tissue sections were immersed in 0.5% iodic acid for 10 minutes, afterwards they were rinsed in distilled water. The remaining water was collected with filter paper and preparations were placed in a *Schiff* reagent cooled to 4ºC, afterwards preparations were rinsed in distilled water. The third reagent (*Potassium methabisulphite*) was added for 2 minutes. It was not washed but dried, then the fourth reagent (fixator) was added. Preparations were rinsed in distilled water. *Mayer's Hemalum* solution was left for 3 minutes to differentiate nuclei, afterwards the sections were rinsed in tap water for 5 minutes. Further on, the preparations were dehydrated through spirits in an increasing succession, afterwards the sections were placed in xylene and locked in histologic glue.

### 2.2.3. Biotin Streptavidin immunohistochemical technique and reagents

For immunochistology we used biotin streptavidin method [*Hsu*, 1981]. Deparaffinized samples were placed in a holder that was placed in a container with EDTA buffer (DIAPATH, LOT 0713311). The container with the samples was put into a microwave, afterwards taken out and cooled, then put into a washing buffer 2 times for 5 minutes. 10 minutes a blocking with peroxidase was performed, then 2 times for 5 minutes a rinsing with TRIS buffer was performed (DIAPATH, LOT 0713513). After 30 minutes, tissues were processed with primary antibodies, afterwards washing in TRIS buffer was performed 2 times for 5 minutes. En Vision (Dako, Denmark) or Immuno Cruz

SC-2053 (USA) staining set was used for 30 minutes. Chromogen diaminobenzidine (DAB) was used that was rinsed and the sections were contrasted for 2 minutes with hematoxylin. The structures that coloured brown due to diaminobenzidine (DAB) were considered immunpositive. Antibodies that were used to detect defensins, cytokines and other factors immunohistochemically, have been described in Table 2.1.

Table 2.1.

**Antibodies used in the study**

| Factor | Code | Obtained from | Working dilution | Producer |
|---|---|---|---|---|
| hBD-2 | AF2758 | goat, polyclonal | 1:100 | R&D systems, Minneapolis, USA |
| hBD-3 | NB200-117 | rabbit, polyclonal | 1:1000 | Novus Biologicals, Littleton, USA |
| hBD-4 | (L13-10-D1): sc-59496 | mouse, monoclonal | 1:100 | Santa Cruz Biotechnology, Dallas, USA |
| IL-1 | (B-7): sc9983 | mouse, monoclonal | 1:50 | Santa Cruz Biotechnology, Dallas, USA |
| IL-6 | NYRhIL6 | mouse, monoclonal | 1:50 | Santa Cruz Biotechnology, Dallas, USA |
| IL-10 | ab34843 | rabbit, polyclonal | 1:400 | Abcam, Cambridge, United Kingdom |
| PGP 9,5 | Z511601 | rabbit, polyclonal | 1:600 | DAKOCytomation, Glostrup, Denmark |
| VEGF | SC-7269 | mouse, monoclonal | 1:50 | Santa Cruz Biotechnology, Dallas, USA |
| MMP-2 | AF902 | goat, polyclonal | 1:100 | R&D Systems, Minneapolis, USA |

Abbreviations: hBD-2 – human beta-defensin - 2; hBD-3 – human beta-defensin -3; hBD4 – human beta-defensin -4; IL-1 – interleukin -1; IL-6 – interleukin -6; IL-10 – interleukin -10; PGP9,5 – product of protein gene 9,5; VEGF – vascular endothelial growth factor ; MMP-2 – matrix metalloproteinase-2.

For each series of preparations a positive controls were prepared (tissues with always positive reaction, for example, lungs, intestines, tonsils, *colon* cancer, ovarian cancer) and samples of negative controls (negative reaction without the primary antibody). The stained preparations were analysed using the technique of light microscopy, *Leica DM RB* (Germany) microscope, afterwards the preparations were processed in Image Pro Plus video analyser system. Histological condition was fixated using *Leica microsystem AG* (Germany) digital camera.

### 2.2.4. TUNEL method

To determine apoptosis, a standardized set of equipment was used: *In Citu Cell Death Detection*, *POD Cat. No. 11684817910 Roche Diagnostics* [*Negoescu et al.,* 1998]. Deparaffinized samples of tissues were held in TRIS buffer solution for 10 minutes. Afterwards, blocking of endogenous peroxidase activity was performed for 30 minutes using 3% hydrogen peroxide and the samples were rinsed in TRIS buffer solution 3 times within 5 minutes. Tissue sections were put into EDTA buffer solution, afterwards in microwave for 10 minutes (to disclose antibody), then the preparations were cooled in room temperature. The cooled preparations were washed in TRIS buffer solution 3 times every 5 minutes.

The control preparation sections were covered with DNasI for 10 minutes and washed in PBS solution.

The preparations were washed in TRIS buffer solution and put into 0.1% bovine serum albumin phosphate buffer solution for 10 minutes.

The tissue sections were incubated with TUNEL reagent for 60 minutes in 37ºC temperature. Afterwards, the sections were rinsed in TRIS buffer solution 2 times for 5 minutes and incubated in 37ºC temperature for 30 minutes using POD (contains horseradish peroxidase). After twofold rinsing for 5

minutes in TRIS buffer solution, the sections were covered with homogenuous diaminobenzidine (DAB) solution to determine peroxidase, than rinsed in distilled water for 5 minutes.

Hematoxylin was used to contrast staining.

## 2.3. Data processing methods

### 2.3.1. Semi-quantitative method

Semi-quantitative counting method was applied to denote the relative amount of structures determined immunohistochemically [*Tobin et al.,* 1990*; Pilmane et al.,* 1998*; Pilmane et al.* 1999]. The amount of structures was analysed in five fields of view of randomly selected one section. The average amount of structures was chosen for further analysis.

Designations provided in Table 2.2 were used for semi-quantitative method.

Table 2.2.

**Designations of semi-quantitative method**

| **Designation** | **Description** |
|---|---|
| 0 | **none** positive structure is visible in the field of view |
| 0/+ | **rare** positive structures are visible in the field of view |
| + | **few** positive structures are visible in the field of view |
| ++ | **average** number of positive structures are visible in the field of view |
| +++ | **many** positive structures are visible in the field of view |
| ++++ | **a lot** of positive structures are visible in the field of view |

Applying TUNEL method, 5 fields of view were selected randomly, in which positive apoptotic cells (positive staining in nuclei) were counted out of 100 visible cells. The apoptotic index was as well determined [*Itoh et al.,* 2001].

### 2.3.2. Statistical data processing methods

As the number of the tissue samples was not high, non-parametric statistical methods were used to perform statistical processing. Alternative of non-parametric t-test is the Mann-Whitney U test and Kolmogorov-Smirnov two-sample test [*Mitchell,* 1986*; Neely,* 2003]. Mann-Whitney U test was used to check, whether the layout of values in to selections is the same. Observations of both groups were combined and arranged, summarizing ranges in each of the two groups separately and calculating the average range for each group. If division of values in both groups is similar, the higher and lower ranges should be evenly distributed between both groups, therefore the average ranges in groups should be similar.

Results of semi-quantitative method were changed to numbers to place scales of ranges.

Level of credibility was used to analyse data. Results were considered credible, if $p<0.5$. Statistics software package SPSS 17 was used for calculations *(SPSS Inc. USA).*

Correlation coefficient is a quantitative indicator of tightness relation between two or more variables. By the scale of ranges, the so called Spearman correlation coefficient and Kendall tau-b test was calculated [*Christensen,* 1996]. The qualitative relation between variables, on the basis of the value of correlation coefficient, was evaluated as weak, average or tight [*Krastiņš,* 1990].

# 3. RESULTS

## 3.1. General description of clinical data

The average age of onychomycosis patients was 55.8 years with the average SCIO value 22.56.

Clinically, each patient had several changed toenails, on average 2 - 4 of which thickness and colour changes had affected more than 2/3 of the length of the nail plate. The most significant changes had affected the nails of halluxes (fawn, greenish nail, thickening of the plate, onycholysis).

In 27 cases microscopy (KOH test) was positive. In 7 cases, fungi of dermatophytes group grew in a subculture, in 4 cases - yeasts and in 3 cases - mould fungi, in 9 cases the subculturing was not carried out, but in 7 cases the subculture was negative.

## 3.2. Morphology data

### 3.2.1. Routine histology data

Almost in all preparations, all three nail unit structures were well visible: nail plate, bed and connective tissues (see Table in Appendix).

Therefore, assessing the prepared sections of preparation, in 18 cases of onychomycosis, thickening of *corneum* layer or nail plate could be observed, as well as several (14) fragments of hypertrophy in the granular layer of the epidermis up to five to eight cell rows (see Figure 1). In 12 cases normogranulosis was observed and in 4 cases, the granular layer of epithelium was even reduced. In 29 preparations, the upper and deepest layer, foci of parakeratosis of various size and form were found. In general, parakeratosis

could be considered as having small foci in 19 cases, but large foci were detected in 10 cases. Fragments of parakeratosis were mainly found in combination with inclusions of homogeneous, eosinophilic mass (see Figure 2). In 11 preparations, the inclusions could be characterized as having small foci, but in 10 preparations - even having large foci.

Not only eosinophilic inclusions could be observed in the nail plate parakeratosis foci, but also elements of fungi micro-organisms were visible in several preparations as well expressed atrophy of the nail bed was observed in several preparation.

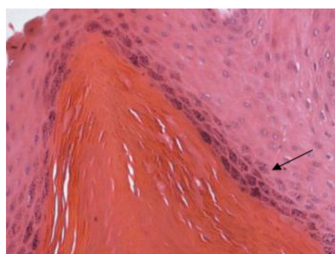

Figure 1. Onychomycosis affected nail bed cell hypertrophy, hyperplasia of *stratum granulosum*. H / E, × 200

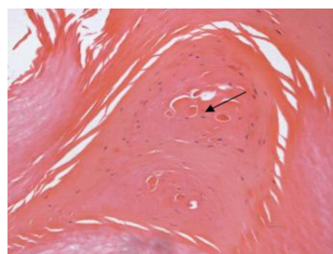

Figure 2. Onychomycosis affected nail plate. Fragment of parakeratosis with multiple roundish forms homogeneous, acidophilous inclusions. H / E, × 200

Acanthosis could be observed only in 8 cases, in both PAS positive and PAS negative preparations. In majority of cases acanthosis had large foci.

In cells of the nail bed affected by onychomycosis a vacuolar or degenerative damage and pericellular oedema was observed. Vacuolar damage was expressed in 16 cases, but pericellular oedema - in 22 cases.

In comparison with the preparations of the control group, the nail connective tissues affected by onychomycosis, hyperplasia of blood vessels, oedema, large foci and in several cases diffuse infiltration of inflammation cells (lymphocytes, plasma cells, macrophages). In several preparations, neoangiogenesis was visible subepidermally. In 12 cases the collagen fibres were hypertrophic, but in 6 cases - atrophic. The number of fibrocytes in one field of view varied from 30 to 120.

In the control group nails, thickening of the nail plate, hypergranulosis or acanthosis was not observed. Inclusions of amorphous substance were not found. The granular layer in one case was hypertrophic, but in other cases - normotrophic. In 3 cases, degenerative damage of the bed cells were visible and in one preparation, pericellural oedema was observed. Rare, diffusively distributed macrophages and rare, small blood vessels could be observed in the dermis. All in all the dermis was without any symptoms of inflammation. The collagen fibres could be characterized as normotrophic. The number of fibrocytes varied from 35 to 50 in the field of view. No blood vessels were detected.

In the nails affected by onychomycosis, in comparison with the control group nails, **parakeratosis is statistically more** expressed (p=0.000), **number of inclusions** (p=0.008), **PAS positivity** (p=0.000), vascularization (p=0.001), as well as significantly higher **number of fibrocytes** (p=0.015).

### 3.2.2. Periodic acid *Shiff* reaction

PAS reaction was positive in 24 cases. Out of those, in 21 preparations, round, homogenous, eosinophilic PAS positive structures could be observed in the nail plate, around several PAS positive aggregates, fragments of the nail plate parakeratosis were visible. In cases of negative PAS reaction, inclusions were not observed.

In all PAS positive preparations, colonies of fungi of elongate form were visible. In several preparations in one field of view, colonies of threadlike fungi and homogenous, round PAS positive elements, most likely spores were visible, moreover, the elements of fungi were in various development stages: spores and hyphae (see Figure 3).

PAS positive elements were not detected in the control nail plate, the bed was as well without a visible morphopathology (see Figure 4).

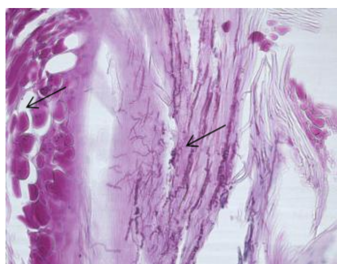

Figure 3. Onychomycosis affected nail plate shows multiple roundish structure of PAS positive homogeneous spores and hyphae. PAS, × 250

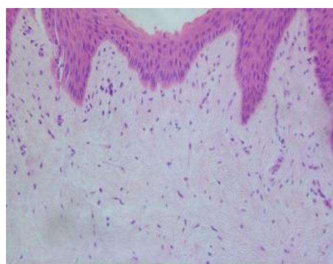

Figure 4. Control nail bed with acanthosis elements. In subepithelial tissue rare lymphocytes and macrophages are seen. H / E, × 100

## 3.3. Data on tissue inflammation and remodelling markers

Intensity of reaction varied in different preparations, however, in the majority of the preparations, expressed intensity of staining was observed.

The total results disclosed the occurrence of inflammation processes in the nail bed affected by onychomycosis and in the part of the dermis connective tissues, while in the control nail group, positive reactions were observed mainly in the nail bed.

Expressively positive reaction in the preparations of nails affected by onychomycosis were observed in IL-6, IL-10 and MMP-2.

There was no significant difference between the group of nails affected by onychomycosis and the control group of nails in reactions to VEGF and PGP9.5.

A summary of data on all reactions in the nails affected by onychomycosis and control group of nails is provided in Table 3.1.

Table 3.1

**Overview of relative amount of factors characterizing cytokines, degradation enzymes, blood circulation and innervation in the nails affected by onychomycosis and control nails**

| Reaction | Nails affected by onychomycosis (n=30) | | | Nails of control group (n=5) | | |
|---|---|---|---|---|---|---|
| | Bed | Connective tissues | Plate | Bed | Connective tissues | Plate |
| IL-1 | 0 | 0 | 0 | +/++ | 0/+ | 0/+ |
| IL-6 | ++/+++ | +/++ | + | + | 0/+ | 0/+ |
| IL-10 | +/++ | + | + | ++ | + | 0/+ |
| MMP-2 | +/++ | +/++ | + | + | 0 | 0/+ |
| VEGF | | + | | | 0 | |
| PGP9,5 | + | + | 0 | +/++ | + | 0/+ |

Designations: 0 none positive structure is visible in the field of view; 0/+ rare positive structures are visible in the field of view; + few positive structures are visible in the field of view; ++ average number of positive structures are visible in the field of view; +++ many positive structures are visible in the field of view.

Abbreviations: IL-1 –interleukin-1; IL-6 – interleukin-6; IL-10 – interleukin-10; MMP-2 – Matrix metalloproteinase-2; VEGF – vascular endothelial growth factor; PGP9,5 – product of protein gene 9,5

### 3.3.1. Interleukin-1

IL-1 was not detected in the group of nails affected by onychomycosis in none of the parts of the nail unit (see Figure 5). In the control group, there were few or average number of IL-1 positive structures in the nail bed, however, in the connective tissues and nail plate, only several positive structures were detected in the field of view (see Figure 6).

Both tests verified that the difference in number of the existing positive structures in the nail bed (p=0) and connective tissues (p=0.002) affected by onychomycosis, compared to their number in the control nails, is **statistically different.** However, IL-1 positive structures were found only in the control group nails.

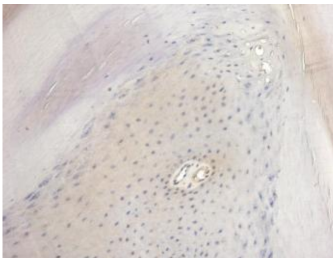

Figure 5. Lack of IL-1-positive structures in onychomycosis affect nail bed and plate tissue. IL-1, IMH, × 200

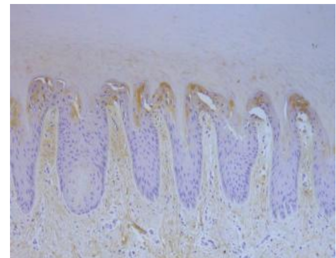

Figure 6. Few to average number of IL-1 positive epitheliocytes in control nail bed tissue. IL-1, IMH, × 100

### 3.3.2. Interleukin-6

Significant amount of cells containing IL-6 were found in the nails affected by onychomycosis. Average or many IL-6 positive structures were detected in the field of view in the nail bed. Few or average amount of positive

structures were visible in connective tissues (explicitly positive structures were visible in the walls of blood vessels of the dermis), few positive structures were detected in the nail plate (see Figure 7).

Few IL-6 positive structures were found in the nail bed of the control group nails, they were mainly found in the form of focal foci, but only separate positive structures were visible in the field of view in the part of connective tissues and plate.

Both statistical tests confirmed that the difference between the amount of positive structures in the nail connective tissues affected by onychomycosis (p=0.010), compared to the amount in the control nails, **is statistically different.**

### 3.3.3. Interleukin-10

In total, small to average amount of IL-10 positive structures were found in the nail bed of the nails affected by onychomycosis, in separate preparations, a large amount of IL-10 positive structures were visible in the field of view. Simultaneously, in several preparations, a total IL-10 positive cytoplasm reaction was observed in the nail bed cells of the nails affected by onychomycosis (see Figure 8). Small amount of IL-10 was detected in the connective tissues and nail plate.

Small to average amount of IL-10 immunoreactive structures were found in the nail bed of the control group nails, small amount was found in connective tissues but in the nail plate only several positive structures were visible.

Statistically significant difference was found only in case of Mann-Whitney test in the nail bed (p=0.021). Therefore, it **cannot be claimed that there is a difference between the control and experiment groups.**

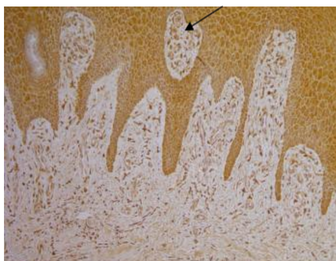

Figure 7. All onychomycosis affected nail bed epithelium, as well as most of the dermal connective tissue cells expressing IL-6. Positive cells form perivascular infiltrates (→). IL-6, IMH, × 100

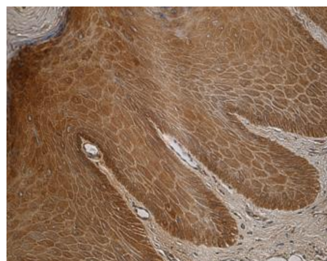

Figure 8. Totally IL-10-positive epithelium of the nail bed. IL-10, IMH, × 200

### 3.3.4. Matrix metalloproteinase-2

MMP-2 was detected in few to average amount of structures in the nails affected by onychomycosis in the nail bed and connective tissues (large amount of MMP-2 was detected in the skin derivatives and walls of blood vessels). In the nail plate such structures were detected in variable amount.

MMP-2 was visible in the control group nails in the nail bed, the enzyme was not detected in the connective tissues, but in the nail plate it was not almost found, except for several positive structures.

Statistically significant difference between the amount of MMP-2 positive structures in the nails affected by onychomycosis and the control group nail (bed, connective tissues, plate) tissues in both tests was found only in the nail connective tissues ($p=0$).

### 3.3.5. Vascular endothelial growth factor

VEGF was viewed only in the part of connective tissues. A lot of VEGF positive structures were found in the nails affected by onychomycosis, in the

control group nails, however, no VEGF positive structures were found (see Figure 9 and 10).

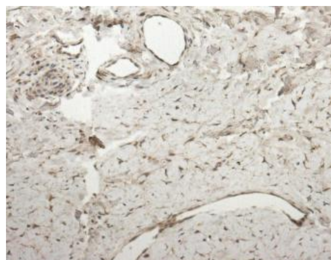

Figure 9. Large amount of VEGF positive cells in the vascular endothelium of onychomycosis affected nail connective tissue. VEGF, IMH, × 200

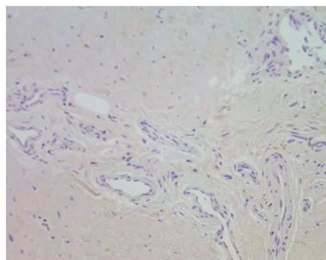

Figure 10. Control nail unit endothelium did not show VEGF positive cells. VEGF, IMH, × 100

Both statistical tests confirmed that the **difference** between the amount of structures containing VEGF in the nails affected by onychomycosis and control group nails **is statistically different** (p=0.010).

### 3.3.6. Innervation marker PGP9.5 containing neuropeptides

Few PGP9.5 positive structures were found in the nail bed and the nail connective tissues affected by onychomycosis. However, average to many PGP9.5 positive structures were observed in several preparations. They were not detected in the nail plate.

Small to average amount of PGP9.5 positive structures were found in the control group nails in the nail bed. Small amount of PGP9.5 was detected in connective tissues but only several positive structures were visible in the nail plate.

From the point of view of PGP9.5 positive structures, in case of the **nail bed**, according to Mann-Whitney test, the difference was statistically significant

at 5% credibility level (p=0.021), however, according to Kolmogorov-Smirnov two-sample test, the difference was **statistically significant only at 10% credibility level.** In cases of **connective tissues and nail plate**, the amount of the observed structures **was not statistically different.** Therefore, in case of PGP9.5 the statistical difference between the nails affected by onychomicosis and the control group nails cannot be found.

### 3.3.7. Apoptosis

Analysing the number of apoptotic cells in the nail bed tissues, it was detected that in the nails affected by onychomycosis, AI on average was 49% but in the control group nails 18% (see Figure 11 and 12). In the group of onychomycosis patients, AI varied from 12 to 79% in the field of view of apoptic cells. In the control group, there were 11 - 27% of apoptotic cells in the field of view.

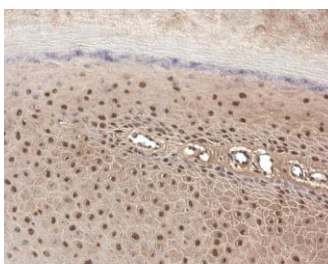

Figure 11. Large number of apoptotic keratinocytes in onychomycosis affected nail bed tissue (AI = 71). TUNEL, × 250

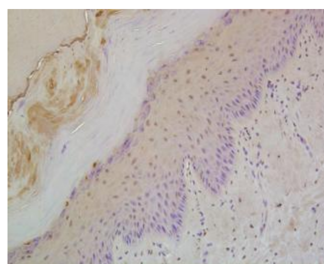

Figure 12. Small amount (<15%) apoptotic cells control the nail bed tissue. TUNEL, × 200

Both statistical tests confirmed that difference between the amount of apoptic cells in the nails affected by onychomycosis and control group nails **is statistically different** (p=0.005). Therefore the processes of apoptosis are more expressed in the nails affected by onychomycosis.

## 3.4. Data on antimicrobial peptides

Intensity of reaction varied in different preparations, however, in the majority of the preparations, expressed intensity of staining was observed.

The total results disclosed a convincing amount of hBD-2 in the nails affected by onychomycosis, compared to the amount of this defensin in the control group nails. The largest amount of hBD-2 positive structures was found in the part of the nail bed and connective tissues in the nails affected by onychomycosis, in the plate, however, it was detected in small number of structures. In the control group nails, hBD-2 was found both in the part of the nail bed and plate, however, in small number of structures.

hBD-3 was found in the nails affected by onychomycosis and in the control group nails, however, in rare positive structures.

Only rare hBD-4 positive structures were found in the nail bed of the nails affected by onychomycosis. hBD-4 was not detected in the control group.

A summary of data on all reactions in the nails affected by onychomycosis and control group of nails is provided in Table 3.2.

Table 3.2

**Presence of beta-defensins in the nails affected by onychomicosis and control group nails.**

| **Defensin** | **Nails affected by onychomycosis (n=30)** | | | **Control group nails (n=5)** | | |
|---|---|---|---|---|---|---|
| | Bed | Connective tissues | Plate | Bed | Connective tissues | Plate |
| hBD-2 | ++/+++ | +/++ | + | 0/+ | 0 | 0/+ |
| hBD-3 | 0 | 0/+ | 0 | 0/+ | 0 | 0 |
| hBD-4 | 0/+ | 0 | 0 | 0 | 0 | 0 |

Designations: 0 none positive structure is visible in the field of view; 0/+ rare positive structures are visible in the field of view; + few positive structures are visible in the field of view; ++ average number of positive structures are visible in the field of view; +++ many positive structures are visible in the field of view. Abbreviations: hBD-2 – human beta-defensin-2; hBD-3 – human beta-defensin-3; hBD-4 – human beta-defensin-4

### 3.4.1. Defensin-2

Defensin-2 was found in many structures of the nails affected by onychomycosis and in the part of connective tissues. Average to large amount was found in the nail bed; few to average amount was found in the part of connective tissues. In the nail plate, it was detected in few structures (see Figure 13).

In the control group nails, only separate positive defensins-2 structures were visible in the field of view in the nail plate and bed. Defensin-2 was not detected in connective tissues (see Figure 14).

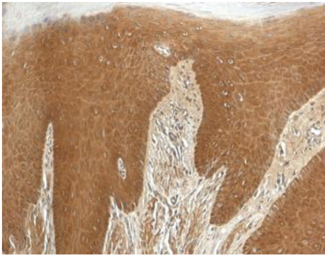

Figure 13. Large amount of HBD-2-positive cells in onychomycosis affected nail bed and average number of HBD-2-positive cells in connective tissue part of the nail. HBD-2, IMH. x 200

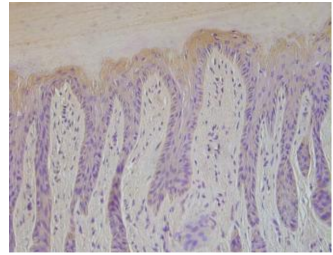

Figure 14. Weak to average intensity dyed HBD-2-containing keratinocytes in control nail bed superficial layers. HBD-2, IMH, x 200

In both statistical tests, **a statistically significant difference** between the amount of positive structures in the **nail bed** (p=0.001) and **connective tissues** (p=0.005) in the nails affected by onychomycosis and control nails **was proven**. Consequently, the amount of hBD-2 found in the nails affected by onychomycosis is significantly larger (in comparison to the control group nails).

### 3.4.2. Defensin-3

Rare defensin-3 positive structures were found in the part of the nail connective tissues affected by onychomycosis, in the nail bed and plate, however, no such structures were found.

Several defenins-3 positive structures were found in the nail bed of the control group nails, in the connective tissues and nail plate, however, no such structures were found.

Both statistical tests show that zero hypothesis as a difference between indices in experiment and control groups can be declined, i.e., it cannot be claimed that the amount of structures observed in the nails affected by onychomycosis and control group nails differs (**statistical difference was not found**).

### 3.4.3. Defensin-4

Defensin-4 was detected neither in the nails affected by onychomycosis, nor in the control group nails.

Both statistical tests show that zero hypothesis as a difference between indices in experiment and control groups can be declined, i.e., it cannot be claimed that the amount of structures observed in the nails affected by onychomycosis and control group nails differs (**statistical difference was not found**).

## 3.5. Statistical correlation of data

The following correlations were found during the study:

1) **Close, positive correlation** between positivity of PAS reaction and detection of inclusions in the nails affected by onychomycosis (p=0.001).
2) **Weak, positive correlation** between the amount of cytokine IL-10 in the nail connective tissues affected by onychomycosis and the age of patients (p=0.032).
3) **Weak up to average close but negative correlation** between severity of onychomycosis and amount of structures containing hBD-2 in the nail unit connective tissues (p=0.002).
4) **Weak, negative correlation** between hypertrophy and acanthosis in the nail affected by onychomycosis (p=0.019).

# 4. DISCUSSION

The author of the paper would like to emphasize that no data were available in the literary sources on similar studies that would describe results of immunohistochemical inspections of various factors impacting the nails affected by onychomycosis. There are several studies on changes in tissues caused by mycoses, mainly studies about mycoses of HIV or immunosupressed patients and studies in veterinary medicine [*Mitchell,* 2007*; Nishikaku,* 2009*; Yuan,* 2009*; Marangon,* 2009*; Villar,* 2012]. There are several studies on defensins in the animal nails and the skin affected by mycoses.

In the present study it was clarified that out of 30 onychomycosis patients, in 7 cases the pathogen was dermatophyte, in 4 cases - yeasts and in 3 cases mould fungi. In total, in 14 cases out of 30 it was possible to clarify a precise pathogen of the disease. In 16 cases, the subculturing for diagnostics of the infection pathogen was either not carried out or it was negative.

*Trychophyton rubrum* was the pathogen in 2 cases, in 5 cases the pathogen was *Trychophyton spp*. These data agree with the Estonian data on *Trichophyton rubrum* as the most often pathogen of dermatophyte - onychomycosis [Järv*,* 2004].

The average age of the onychomycosis patients involved in the study was 55.8 years. In this age, the speed of nail growth is significantly slower than that of younger patients [*Abdullah,* 2011*; Baran,* 2011]. Along with ageing, the size of keratinocytes increases, the nail bed connective tissues get thicker, features of degeneration of blood vessels and flexible fibres appear [*Singh,* 2005].

In the present study, both genders were involved, the number of females was a little bit higher. Other authors view this mater differently. Ones believe that onychomycosis is often prone both to males and females similarly [*Cohen,*

1992], others, on the other hand consider that female patients prevail [*Pires,* 2014] or vice versa - male patients prevail [*Lone,* 2013].

The average value of SCIO index of the present study is 22.56 that prescribes a continuous systemic therapy and corresponds with a severe clinical form of onychomycosis. The most frequent form of onychomycosis for the patients has been the distal - lateral. The high SCIO value also reveals that majority of patients have had an expressed subungual hyperkeratosis that requires additional special approach to therapy (keratolytic or regular medical pedicure procedures). According to publications, other authors have also used SCIO in their studies to divide their patients in groups and assess the curing [*Sumikawa,* 2007*; Kawai,* 2014].

Nail biopsy or the so called "*punch*" biopsy was used in the study as the diagnostics verifying onychomycosis that proved itself as accurate and more importantly - safe method to diagnose onychomycosis. Results of the specific PAS staining were analysed for all nail preparations. PAS reaction was positive in 24 cases out of 30 onychomycosis cases, i.e., 80%. It should be noted that in all cases when PAS reaction was positive, as a positive side find inclusions in the nail plate and parakeratosis in staining with hematoxylin / eosin were detected. In all cases the location of nail biopsy had healed well, without any side effects. According to the patients, the felt discomfort only in the first days after the procedure, wounds of several patients were bleeding in the day when the manipulation was performed. These data correspond with several literary sources that justify the necessity of biopsy in case of expressed clinical conditions of onychomycosis but in case of negative results of microscopy and / or culturing [*Karimzadegan-Nia,* 2007*; Barber,* 2009*; Moreno-Coutiño,* 2010].

A positive and close correlation exists between the amount of inclusions in the nail plate affected by onychomycosis and positivity of PAS

reaction. It can be concluded that in cases when PAS reaction was negative, but inclusions were, however, visible, diagnosis of onychomycosis should be considered.

The author of the present paper similarly as other authors [*Stewart,* 2012*; André,* 2013], in case of positive PAS reaction detected elements of fungi in various development stages. Both hyphae and spores were visible. Mainly these elements were visible in the upper part of the nail plate.

Precise pathogen of the disease can be determined only applying culture method, however, according to visual features of elements of fungi, approximate group of pathogens can be detected. Regular, straight, aseptate hyphae with a tendency to place in parallel to the surface of the nail plate are characteristic to dematophytes, but small and round spores, pseudohyphae and short filaments are characteristic of yeasts. Spores without pseudohyphae are considered pollutants and those cannot cause onychomycosis independently. Truncate spores and irregular hyphae with tiny, short filaments are more characteristic of the mould infection [*André,* 2013].

In the present study, the author did not evaluate the visual appearance of spores and accurate data on pathogens in all 30 cases onychomycosis were not available. However, it might be a new and interesting direction of studies in the future.

Assessing morphology of the nails affected by onychomycosis, preparations stained with hematoxylin / eosin and PAS were viewed. In several preparations, it was easier to assess inclusions in PAS reaction. The study statistically credibly proves that the nails affected by onychomycosis compared to the control group nails have significantly more expressed parakeratosis of the nail plate, as well as number of inclusions in the nail plate, pericellular oedema in the cells of the nail bed, and vascularization and number of fibrocytes in the part of dermis. Hyperkeratosis of the nail plate was found in

18 preparations of the nails affected by onychomycosis. Assessing the nail bed of the nails affected by onychomycosis, it can be concluded that in the majority of the preparations a granulosis of epithelium can be detected, in half of the preparations vacuolar damages of epithelium of small foci is visible, in 22 preparations, pericellular oedema is visible. The dermis has an expressed presence of normotrophic and hypertrophic collagen fibres and tiny blood vessels. The number of fibrocytes varied from 30 to 120 cells in the field of view.

The data of the study on disorders of keratinization in the nails affected by pathogenic fungi correspond completely with the data published by Jensen on changes caused by dermatophytes in a smooth skin. Almost in all preparations, foci of parakeratosis with inclusions of fungal elements were visible [*Jensen,* 2007].

Small number of publications is available on morphology of the nails affected by onychomycosis, however, the existing data prove that the other authors have made similar conclusions. Histological changes in the case of onychomycosis are most often characterized by psoriasis forms that include hyperplasia of epithelium of the nail bed, exocytosis of neutrophilic leukocytes, spongiosis of cells and parakeratosis [*André,* 2013*; Martin,* 2013].

In the present study, in almost all cases of onychomycosis thickening of stratum corneum was observed that might be considered as the first and most significant protective reaction of tissues in case of onychomycosis.

The stage of severity and progress of the disease is determined by 2 immune system aspects: quick allergic reaction and delayed hypersensitivity reaction [*Zahur,* 2014]. Factors that determine the outcome of fungal invasion is the antigene presenting by Langerhans cells and accumulation of efector cells. Production of cytokines facilitates the action of neutrophilic leukocytes,

activates the keratinocytes to express HLA-DR and produce even more cytokines, for example IL-1 [*Hay,* 1992].

In case of systemic fungal infection, the significance of IL-1 has been emphasized in creating an immunologic reaction against the fungal microorganisms. It has been proven that in case of systemic fungal infection, IL-1 increases differentiation of T cells to Th17 [*Wüthrich,* 2013]. Campos (2006) and other authors emphasize the increase of TNFα and IL-10 in tissues in case of *Trichophyton rubrum* infection [*Campos,* 2006].

No data could be found in the literary sources on presence of IL-1 in tissues affected by onychomycosis. However, there were data proving that the amount of IL-1 beta and IL-6 was increasing in tissues in case of keratitis [*Zhong,* 2009], as well as explicit production of IL-1, IL-6 and IL-10 and parts of monocytes in the blood in case of *Paracoccidioides brasiliensis* infection [*Kurokawa,* 2007]. In relation to *Candida*, the significance of the complement activation fragment C5a is mentioned. This activation fragment works as a reaction to *Candida* infection and causes cell reaction through IL-6 and IL-1 beta-cytokines [*Zipfel,* 2012].

In the present study, no IL-1 was found in the nails affected by onychomycosis in none of the studied structures of the nail unit. In the control group nails, small or average amount of IL-1 positive structures were found in the nail bed, as well as rare positive structures were visible in the connective tissues of healthy nails and the part of the nail plate. This find shows that IL-1, which is one of the most active pathogens of inflammation [*Gabay,* 2010*; Weber,* 2010], it is not considered a pathogen in case of onychomycosis.

Large amount of IL-6 was found in several structures in the nail bed and the nail connective tissues affected by onychomycosis, in the nail plate, however, only few IL-6 positive structures were visible. In the control nails, small amounts of IL-6 in all parts of the nail unit were detected. Presence of

IL-6 in the nail bed and blood vessels represent an active involvement of immune system in fighting the fungal infection. Damage of tissues caused by pathogenic microorganisms causes immediate synthesis of IL-6 [*Heinrich,* 2003]. Therefore IL-6 can be characterized as one of the most active pro-inflammatory cytokines in the human body. It is synthesized by the activated monocytes, macrophages, T-lymphocytes, endotheliocytes, as well as fibroblasts.

Small or average amount of IL-10 positive structures were found in the nail bed tissues, a large amount of IL-10 positive structures were observed in the field of view of several preparations reaching total cytoplasmic reaction of the bed cells. IL-10 was also found in the nail connective tissues and nail plate but the amount was small in the structures. Small or average amount of IL-10 was found in the nail bed and control group nail connective tissues but only several positive structures were observed in the nail plate. In majority of cases IL-10 is the cytokine that fights against the inflammation. [*Zdanov,*1995*;Ouyang,* 2011]. It is possible that for the studied patients, it is one of the key nail resistance indices.

Therefore, data obtained during our study only partially correspond with results obtained by Zhong (2009) and other authors as regards the cytokines in tissues in case of mycotic infection. In case of the nails affected by onychomycosis, high amount of inflammation cytokines was produced in the nail bed, where IL-6 and IL-10 create a convincing proportion.

One of the most significant components of tissue restructuring are matrix metalloproteinases. MMP-2 together with MMP-9 degrades IV type collagen that is mostly found in the basic element of connective tissues [*McCawley,* 2001].

Matrix metalloproteinases are specific tissue proteolytic ferments that are widely studied in relation to mycotic infections. Unfortunately, no studies

were found that would have researched the matter of restructuring of tissues exactly in onychomycosis, however, the mycotic inflammation of the cornea. Several authors refer to the significance of MMP-2 and MMP-9 in case of damage of the mycotic damages of tissues of cornea [*Mitchell,* 2007; *Boveland,* 2010*; Pärnänen,* 2010].

The present study viewed only presence of MMP-2 in tissues, detecting that this proteolytic enzyme is found much more in the nails affected by onychomycosis compared to the control group nails. Small or average amount of MMP-2 was found in the bed of nail affected by onychomycosis and in connective tissues, in the nail plate - small amount of MMP-2 positive structures. Small amount of MMP-2 was found in cells in the control group nails, there was almost none found in the connective tissues and nail plate. Therefore, finding MMP-2 in the nails affected by onychomycosis is similar to the point of view of other authors on intensive restructuring processes in the tissues affected by pathogenic fungi.

If a chronic inflammation occurs in tissues, the changes in the bed of blood vessels can be observed, and this process involves both cytokines (IL-6 and IL-10) and growth factors, as well as MMP of tissues. The role of MMP in the process of angiogenesis has been described as well [*Sariahmetoglu,* 2007].

VEGF has been mentioned as one of the indices of vasculogenesis. Cells produce this growth factor in situations when tissues are suffering from the oxygen deprivation and it is necessary to create new blood vessels [*Karkkainen,* 2000].

There is no data on changes in the bed of blood vessels in case of onychomycosis. In cases of keratitis of mycotic origin, the amount of VEGF-A increases rapidly in tissues, as a result progressive creation of cornea blood vessels develops [*Yuan,* 2009].

VEGF was studied in the part of the nail unit connective tissues in the nail biopsy material taken from the onychomycosis patients involved in the present study. VEGF was not found in healthy nails, in the nails affected by onychomycosis, however, few VEGF positive structures were found but statistical difference between both groups involved in the study has been proven correct. That allows assuming that processes of vasculogenesis occur in connective tissues in case of onychomycosis.

To understand, whether in tissues affected by onychomycosis, the amount of nerve fibres changes, PGP9.5 staining of preparations was carried out. Protein gene product 9.5 is considered the main marker of neurons and neuroendocrine cells [*Day,* 2010].

The data of the present study prove that statistically there is no difference as to the amount of PGP9.5 in the nails affected by onychomycosis and control group nails. No information is available in data bases about determining the amount of PGP9.5 in tissues in cases of onychomycosis and mycosis. It appears that in case of onychomycosis, the innervation of tissues stays the same.

Apoptosis proves restructuring of tissues as well. The key function of apoptosis is to eliminate defective, i.e., changed or infected cells [*Kerr,* 2002*; Diamantis,* 2008]. As opposed to necrosis, during apoptosis, the cells are characteristic of such features as fragmentation of nucleus and degradation of DNS [*Ramsdale,* 2008]. This specific transfer of the signal of cell death as a reaction to impact of microbial organisms, ensures an important role in pathogenesis of different infection diseases.

Unfortunately, there is no data as to the presence of apoptosis process and severity in case of onychomycosis, however, in relation to mycosis the topic has been studied a lot.

When studying expression of *Fusarium oxysporum* infection in the skin of mice, a positive TUNEL reaction was observed in keratinocytes, fibroblasts, endothelial cells, muscles and inflammation infiltrate cells. The authors relate these changes in tissues to impact of fungal toxic metabolytes [*Marangon,* 2009].

It is known that *Candida albicans* induces apoptosis in epithelial cells, including keratinocytes with the help of "Trojan horse" system. Initially, these enzymes connect to the receptors of the cell surface, after wards they are endocyted and only then the release the lyzosomal membranes allowing process of apoptosis in a cell [*Wu,* 2013].

It has been studied that early features of apoptosis after *Candida albicans* infection are observed in more than 50% of cells but late apoptotic alterations are observed only in 15% of cells [*Villar*, 2012].

The number of apoptotic cells was determined in the nail bed of nails affected by onychomycosis during the present study. It was found that AI is on average 49% in the nails affected by onychomycosis, but in the control group nails - 18%. This proves an intensive process of apoptosis in the nails affected by onychomycosis compared to the healthy nails.

Studying impact of *Candida albicans* on macrophages during a fungal infection, early features of apoptosis were observed, after 2 hours - features of nekrosis in cells. An explicit increase in the amount of neutrophilic laukocytes and increase of IL-10 in exudate was observed in the peritoneal cavities of mice 24 hours after the beginning of incubation of macrophages [*Gasparoto,* 2004].

During the present study similar data to the above were obtained, as explicit presence of IL-10 and large amount of apoptotic cells was observed in the nails affected by onychomycosis. These features were mostly present in the nail bed. Visualization of the programmed death of cells in preparations of

bioptates of patients proves an intensive elimination process of defective and inferior cells of the nail bed.

As a reaction to invasion of the strange organisms, human body cells produce not only interleukines that create the inflammation reaction but also antimicrobial peptides, which are the natural antibiotics protecting the body from potential infections.

Such antimicrobial peptides as cathelicidins have been found in the human nails up to now [*Dorschner,* 2004], moreover, only healthy nails were studied. Broader studies on excretion of antimicrobial peptides in case of infection of pathogenic fungi have been carried out only in relation to the skin or mucous membrane [*Jensen,* 2007*; Kawai,* 2006]. It is interesting that the human antimicrobial peptides were mentioned also as regulators of keratinocytes apoptosis [*Chamorro,* 2009].

In the present study, the presence of human defensins 2, 3 and 4 was determined in the nails affected by onychomycosis. Significant difference of hBD-2 was found in comparison of the nails under study and control group nails. This AMP was detected in the nails affected by onychomycosis in many structures both in the nail bed and connective tissues. hBD-2 was not observed in the control group nails.

Similarly as in the present study, hBD-2 was found in the skin affected my dermatophytes rather than nails. A very strong expression of hBD-2 was observed in the granular and spiny layer of the epidermis [*Jensen,* 2007]. There are another data on the excretion of mice defensin-3 (Mdb3), which is considered analogue of human hBD-2, only at the stimulation of lipopolysaccharides. Expression of Mbd3 was detected in the skin and mucous membrane but large amount of Mbd3 was found in tongue and skin of mice feet. Broad range activity was determined for this AMP, including the activity against yeasts and dermatophytes [*Jiang,* 2010].

In hBD-3 and hBD-4 case, statistical difference between the amount of antimicrobial peptides in the nails affected by onychomycosis and control group nails was not found. In both cases, defensins could not be found at all or only small amounts.

Therefore, the study justifies that the nail unit affected by onychomycosis significantly differs from a healthy nail plate. Explicit hyperkeratosis, inclusions and increased thickness of germinative layer and acantosis is detected in the nails affected by the disease; which therefore provide grounds for disorders of keratinization. In the dermis of nails affected by onychomycosis there are more hypertrophied collagen fibres and more explicit vascularization, compared to healthy nails. In general, it can be concluded that onychomycosis infection causes the process of neoangiogenesis in the nail, however, it does not cause any innervation changes. Explicit processes of restructuring and apoptic destruction of defective cells can be observed in tissues. As a reaction to the fungal infection, intensive production of cytokines goes on in tissues, especially production of IL-6 and IL-10, as well as excretion of the natural human antimicrobial peptides - hBD-2.

# 5. CONCLUSIONS

1. Women are more **prone** to onychomycosis, moreover **ageing** is considered a contributing factor. **Dermatophytes** often are the causative pathogens of onychomycosis, followed by yeasts and mould fungi being the last ones.
2. The functional units of nails affected by onychomycosis are most commonly subjected to **disorders of keratinization.** The above is justified by changes in structures of nail bed and nail plate: cellular hyperplasia and hypertrophy, inclusions of nail plate fungi (there is a close correlation between diagnostics of inclusions and histochemical diagnostics – PAS reaction), foci of parakeratosis, explicit degeneration of bed cells, hypertrophied collagen fibers of dermis and neoangiogenesis.
3. Intensive excretion of **antimicrobial peptides beta defensins-2** occurs in the nail bed affected by onychomycosis; the process determines the selective involvement of the protein in the scheme of pathogenesis of the said illness. Human beta defensin-3 and human beta defensin-4, however, cannot be related to invasion of fungi in the human nails.
4. The similar detection of **PGP9.5** fibrils and positive structures in the nails affected by onychomycosis and nails of control group indicate lack of innervation's involvement in the pathogenesis of the illness.
5. The small number of endoteliocytes containing **VEGF** in the nail bed tissues in case of onychomycosis, however, is sufficient to induce a focal neoangiogenesis and verify tissue ischemia that indicates at the blood-vessel component's involvement in the pathogenesis of the illness in the part of the nail connective tissues.

6. Detection of small up to average number of structures containing **MMP-2** in the nail bed affected by onychomycosis and in the connective tissues of nail unit affected by onychomycosis supports intensive restructuring processes in the tissues affected by pathogenic fungi.

7. In case onychomycosis, a large **production** of separate specific **inflammation cytokines** takes place in the nail bed where the most active morphopathological processes of the illness occur. As to cytokines, IL-6 is the main pro-inflammatory factor of onychomycosis and IL-10 is the main anti-inflammatory factor, IL-1, however, cannot be related to invasion of pathogen fungi in nails.

8. Clear and statistically credible increase of apoptotic cells in the nail units affected by onychomycosis, especially in the nail bed tissues, justifies intensification of **process of programmed cell death** as a compensating mechanism to eliminate defective cells in the nail functional unit.

9. **Statistically credible positive correlations** between severity of onychomycosis and amount of hBD-2 positive structures in the tissues, as well as between the age of patients and structures containing IL-10 in the nail tissues support the **index for clinical assessment of onychomycosis and age** as modulators / inductors of the immune tissue reaction exactly for this illness.

# PUBLICATIONS

## Articles (4)

1. **Murashko O.,** Kisis J., Kroica J. *Causative agents of onychomycosis in Latvia* // Dermatovenerologie, 2009; 54 (1): 16 – 18.
2. **Zaikovska O.,** Kisis J., Pilmane M. *Defensins and cytokins in nail unit affected by onychomycosis: a pilot study* // Papers on Anthropology XXII, 2013; 225 – 233.
3. **Zaikovska O.,** Pilmane M., Ķīsis J. *Nagu patomorfoloģiskās pārmaiņas onihomikozes gadījumā* // RSU Zinātniskie raksti 2013, 2014: 274 – 281.
4. **Zaikovska O.,** Pilmane M., Kisis J. *Morphopathological aspects of healthy nails and nails affected by onychomycosis* // Mycoses, 2014; 57(9): 531 – 536.

## Guidelines (1)

Practical recommendation of onychomycosis for practical doctors (one of authors). Accepted on 11 February 2014.

## Presentations in Latvia (7)

1. 17 March, 2010 – oral presentation *"Onychomycosis causative agents in Latvia"* Latvian Association of Dermatovenereologist.
2. 18 - 19 March, 2010 – poster *"Onychomycosis and Vitamin D in organism"*, scientific conference of Rīga Stradiņš University.
3. 21 - 22 March, 2013 – poster *"Immunochistological examination of onychomycosis affected nails"* scientific conference of Rīga Stradiņš University.
4. 19 - 21 September, 2013 – poster *"Morphological diagnostic methods of onychomycosis"*, 7 th Congress of Latvian doctors.

5. 7 - 9 November, 2013 – poster *“The presence of antimicrobial peptides in onychomycosis affected nails”* Baltic Morphology VII Scientific conference.
6. 10 - 11 April, 2014 – poster *“Inflammation and remodellation processes in onychomycosis affected nais”*, scientific conference of Rīga Stradiņš University .
7. 26 - 27 March, 2015 – poster *“Histology and innervations and vascularisation in onychomycosis affected nails”*, scientific conference of Rīga Stradiņš University .

**Presentation abroad (4)**

1. 23 – 26 April, 2009 – oral presentation *“Causative agents of onychomycosis in Latvia”* 6th European Academy of Dermatology and Venereology Spring Symposium Bucharest, Rumania.
2. 22 – 23 September, 2011 – oral presentation *“The morphology of nails affected by onychomycosis and antimicrobial peptides”* Baltic Morphology VI, Tartu, Estonia.
3. 27 – 30 September, 2012 – oral presentation *“Mycosis and psoriasis – similar and different”* and co-chair on symposium “Fungal skin and scalp infections” 21 th Congress of European Academy of Dermatology and Venereology, Prague, Czech Republic.
4. 2 – 6 October, 2013 - poster *“Morphology and immunohistochemical examination of nails affected by onychomycosis”* 22 th Congress of European Academy of Dermatology and Venereology, Stambul, Turkey.

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# TESTIMONIALS

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*Appendix.* **Histology data**

| P. | Nail plate | | | | Nail bed | | | | Dermis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Parakeratosis | Hyperkeratosis | Inclusions | PAS | Granulosis | Akanthosis | Vakuol. | Pericel. oedema | Collagen fibers | Vascular. | Fibrocytes |
| 1 | 2 | no | 3 | + | normo- | no | no | 2 | normo- | 2 | 70 |
| 2 | 2 | present | 3 | + | hiper- | no | 2 | no | hiper- | no | 120 |
| 3 | no | no | 2 | + | normo- | no | 2 | 2 | atrof- | 3 | 100 |
| 4 | 2 | present | 3 | + | hiper- | no | no | no | hiper- | no | 75 |
| 5 | 3 | no | 3 | + | hiper- | no | no | 3 | normo- | 2 | 85 |
| 6 | 3 | no | no | + | hipo- | 2 | 2 | 2 | hiper- | 2 | 60 |
| 7 | 2 | no | no | + | hiper- | no | no | 2 | hiper- | 3 | 110 |
| 8 | 3 | present | 2 | + | normo- | no | no | 2 | normo- | 3 | 110 |
| 9 | 2 | no | 2 | + | hiper- | 3 | 3 | 3 | atroph- | 2 | 60 |
| 10 | 2 | present | no | - | hiper- | no | no | 2 | normo- | 2 | 70 |
| 11 | 2 | no | no | + | normo- | 3 | 3 | 3 | atroph- | 2 | 50 |
| 12 | 3 | present | 3 | + | normo | no | no | no | normo- | 2 | 80 |
| 13 | 3 | no | no | - | hiper- | 3 | 3 | 3 | normo- | no | 60 |
| 14 | 2 | no | 2 | + | normo- | no | no | no | atroph- | 3 | 40 |
| 15 | 2 | present | no | - | hiper- | no | no | 3 | normo- | 3 | 70 |
| 16 | 2 | present | no | + | hiper- | no | 2 | no | hiper- | 2 | 50 |
| 17 | 3 | present | 3 | + | normo- | no | 2 | 3 | normo- | 2 | 70 |
| 18 | 3 | present | 2 | + | normo- | no | 2 | 3 | atroph- | 2 | 50 |
| 19 | 2 | present | no | - | hipo- | no | no | 3 | hiper- | 3 | 110 |
| 20 | 2 | present | 3 | + | hiper- | no | 2 | no | normo- | 2 | 60 |
| 21 | 3 | present | 2 | + | normo- | no | no | 3 | hiper- | 2 | 80 |
| 22 | 3 | no | 2 | + | normo- | 2 | no | no | normo- | 3 | 65 |
| 23 | 2 | present | 2 | + | hiper- | no | no | 2 | atroph- | 3 | 30 |
| 24 | 2 | present | 2 | + | hipo- | no | 3 | 2 | normo- | 2 | 30 |
| 25 | 2 | no | no | - | hiper- | 3 | 2 | 2 | normo- | 2 | 80 |
| 26 | 3 | no | 2 | + | normo- | 2 | 2 | no | hiper- | 2 | 40 |
| 27 | 2 | present | 3 | + | hiper- | no | 2 | 3 | hiper- | 3 | 80 |
| 28 | 2 | present | 3 | + | hiper- | no | 2 | 3 | hiper- | 2 | 40 |
| 29 | 2 | present | 2 | + | normo- | no | 2 | 3 | hiper- | 3 | 120 |
| 30 | 2 | present | 3 | + | hipo- | 4 | 2 | 2 | hiper- | 2 | 67 |
| 1K | no | no | no | - | normo- | no | 2 | no | normo- | no | 50 |
| 2K | no | no | no | - | normo- | no | no | no | normo- | no | 45 |
| 3K | no | no | no | - | normo | no | 2 | 2 | normo | no | 50 |
| 4K | no | no | no | - | hipo | no | no | no | normo | no | 40 |
| 5K | no | no | no | - | normo | no | 2 | no | normo | no | 35 |

Abbreviations: P. – patient number, vakuol. - vakuolisation, pericel. – pericelular, vascular. – vascularisation, 2 – small amount of lesions, 3 – large amount of lesions, 4 – total damage.