Summary of the PhD Thesis

CELL INTERACTIONS IN PREMALIGNANT CUTANEOUS LESIONS

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# Chapter 7

## Results and discussion

### 7.1 Statistical study

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- Discussions

### 7.2 Morphological aspects of tissular micro-medium specific to premalignant lesions

- Results
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### 7.3 Defining the expression TNFα, IL-6, Cox-2 and MMP-2

- Results
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### 7.4 Ezrin immuno-histochemical study in premalignant lesions

- Results
- Discussions

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### Conclusions

### Original ideas

### Open perspectives of the PhD thesis

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### Appendix 1

List of published works on the PhD thesis topic

*The thesis is illustrated with 73 figures and 13 tables and contains 230 reference indexes. This summary selectively illustrates the textual iconography and references and it complies with the numbering and the table of contents of the thesis in extensor.*
**Keywords:** premalignant lesions, squamous cell carcinoma, biomarker, Ezrin.

**Study rationale**
A premalignant lesion, term coined in 1875 by the Romanian doctor Victor Babeș, is that lesion which, without treatment, presents an increased risk of malignant transformation (1,2).

On a global level it has been noticed an increased incidence of lesions that precede squamous cell carcinomas both at epidermis and oral mucous levels. The carcinomas are considered to be extremely aggressive because of local and distant complications. In terms of statistics, this can be translated into increased morbidity and mortality with over 5% each year (17,137).

At the cutaneous level, premalignant lesions such as actinic keratoses, Bowen's disease and actinic keratoses are among the most important risk factors for developing squamous cell carcinoma, being the most commonly considered metastatic cutaneous cancer (5).

At the oral mucosa level, the concept according to which the development of the oral squamous cell cancer requires two initial phases indicates the initial presence of a precursor, a premalignant lesion (3).

Defining changes at molecular level and outlining signalling pathways in the early stages of the development of premalignant lesions, before their clinical manifestation, are important steps for understanding how the cellular response is modulated during carcinogenesis at this stage.
This study brings to the fore a topical pathology relevant to the worldwide medical system and aims the characterization of molecular markers responsible for the modification of cellular interactions / signalling pathways specific to these lesions and their subsequent evolution. This research underlines the existing interrelations both between the markers themselves and between their clinical and therapeutic aspects and manifestation.

**Research objectives**
- Pointing out the severity of premalignant cutaneous lesions as compared to oral ones.
- Emphasizing the risk of malignant transformation of cutaneous and oral premalignant lesions.
- Evaluating the morphological context specific to these premalignant lesions at an ultrastructural level.
- Investigating correlations between the expressions of molecules specific to inflammation and the malignant transformation process via including the modification of a number of signalling pathways.
- Identifying possible correlations between the expression of envisaged molecules and the efficiency of therapies specific to such lesions.
- Investigating the Ezrin expression in the investigation of the considered premalignant lesions
- Correlating the results of the Ezrin expression with ultrastructural aspects and with the expression of proinflammatory molecules.

**Materials and methods**
For this study fragments of cutaneous and oral mucosa were collected through incisional / excisional biopsy with surgical knife, from 63 female patients, non-smoking, without a chronic alcohol consumption, aged 45-60 years, clinically and histopathologically diagnosed with actinic keratosis, Bowen disease, keratoacanthoma, leukoplakia, erosive actinic cheilitis and erosive oral lichen planus. Fragments of normal skin and oral mucosa were obtained by the same harvesting method from perilesional areas, to constitute the control group.

**Reagents:**
- “BCA Protein Concentration Determination Kit” for dosing the protein;
- “TNF alpha ELISA Kit, Human”, to determine TNFα by ELISA technique;
- “IL-6 ELISA Kit Human”, to determine IL-6 by ELISA technique;
- “Fluor-metrical Kit” “MMP - 2 Fluorescence Assay Kit”, to determine MMP-2 by the FRET technique;
- “COX-2 ELISA kit” to determine COX-2 by ELISA;
- “NovocastraTM Kit - NovoLinkTMMax Polymer Detection System, produced by Leica Biosystems Newcastle Ltd Balliol Business Parkwest Benton Lane, for immunohistochemistry.
- Ezrin Polyclonal Antibody (Cell Signaling TechnologyR - BioZyme)

**Methods:**
- Statistical processing of a sample of 1514 patients diagnosed with cutaneous and oral premalignant lesions and squamous cell carcimomas, from 2010 till 2015.
Using the *paraffin inclusion* technique, where some tissular samples were sectioned and stained with hematoxyline for pathologoanatomic diagnosis, others were dewaxed for use *in ELISA* and *FRET*, and another series was processed by the *immuno-histochemical technique*:

- Standard technique for *electron microscopy of transmission*

### Results and Discussions

1. **Statistical study**

#### Results

Out of the 1514 patients, a number of 1,008 patients were diagnosed with squamous cell carcinoma developed de novo or on premalignant lesions, and 506 patients with premalignant lesions.

![Fig.7.1.6](image) **Fig.7.1.6** Distribution of carcinomas developed on premalignant cutaneous and oral lesions
Fig. 7.1.5. Histogram of the age variable distribution

The comparison of de novo (oral and cutaneous) carcinomas with those developed on premalignant (oral and cutaneous) lesions was achieved by means of tables of contingency $\chi^2$.

**Table 7.1.X** Frequency of carcinomas developed on premalignant lesions

**Analyse of a 2x2 contingency table**

<table>
<thead>
<tr>
<th></th>
<th>cutaneous</th>
<th>On mucosa</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma de novo</td>
<td>256</td>
<td>422</td>
<td>678</td>
</tr>
<tr>
<td>Carcinoma on lesions</td>
<td>31</td>
<td>229</td>
<td>260</td>
</tr>
<tr>
<td>Total</td>
<td>287</td>
<td>651</td>
<td>938</td>
</tr>
</tbody>
</table>

**Chi-square with Yates correction**

Chi squared equals 57.859 with 1 degree of freedom. The two-tailed P value is less than 0.0001 and statistically considered to be extremely significant.
The result is highly significant with \( p \leq 0.0001 \)
The comparison of oral carcinomas with the cutaneous ones is highly significant, with \( p \leq 0.0001 \)
The comparison of de novo (oral and cutaneous) carcinomas with those developed on premalignant (oral and cutaneous) lesions was achieved by means of tables of contingency \( \chi^2 \)

**Table 7.1. XI** Frequency of oral squamous cell carcinomas

*Analyse of a 2x2 contingency table*

<table>
<thead>
<tr>
<th>Carcinoma</th>
<th>De novo</th>
<th>Premalignant</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>On mucosa</td>
<td>422</td>
<td>300</td>
<td>722</td>
</tr>
<tr>
<td>Cutaneous</td>
<td>257</td>
<td>32</td>
<td>289</td>
</tr>
<tr>
<td>Total</td>
<td>679</td>
<td>332</td>
<td>1011</td>
</tr>
</tbody>
</table>

*Chi-square with Yates correction*
Chi squared equals 85.553 with 1 degrees of freedom.
The two-tailed P value is less than 0.0001 and statistically considered to be extremely significant.

The result is highly significant with \( p \leq 0.0001 \)

**Discussions**
This statistical panel shows that the frequency of oral squamous cell carcinoma is significantly higher than the cutaneous one, regardless of its evolution, de novo or on premalignant lesions. Also, the degree of aggressiveness is greater at the oral level (table7.1.X - 7.1.XI).
The distribution of all cases diagnosed with cancer, oral and cutaneous, indicates a preponderance in males with a M/F ratio=3.27/ 1 (772/236), confirming thus numerous studies that highlight a higher frequency among men, especially in developing countries (fig. 7.1.2) (177).
The obtained results justify once more the highlight on the importance of the doctor-patient relationship in the identifying and early treatment of oral and cutaneous premalignant lesions, an important goal in improving the survival and life quality of these patients.
2. Morphological aspects of the tissular microenvironment specific to premalignant lesions

Results
The tissular fragments collected after processing by means of the above mentioned techniques were examined under photonic microscope *Olympus BX40* with an attached *Olympus E330 camera*. A number of issues significant for the diagnosis of the studied lesions were identified.

By use of *electron microscopy* we identified in the studied premalignant cutaneous lesions the following aspects:
- Enhancement of intercellular spaces associated with partial disruption of linking cell and cell-matrix areas, desmosomes, hemidesmosomes;
- Keratinocyte with an approximately normal architecture and cytoplasmic vacuolation;
- Presence of nuclei increased in volume and in undulating nuclear membrane with rare sinusoidal dilation and increased number of nucleoli;

Among the ultrastructural features highlighted by the data *electron microscopy of transmission* in oral premalignant lesions the following data was found:
- Discontinuity and thickness variations of the basic membrane which may be caused by the presence of an inflammatory process associated with such lesions;
- Presence of desmosomes and HD with disorganized structure at the basal layer;
- Cytoplasm vacuoliations, discontinuities and invaginations in the plasmalemma; in some cases
caveolae and pinocytosis vesicles of different sizes were remarked.

**Discussions**

Premalignant cutaneous lesions of the Bowen disease manifested more severe ultrastructural changes (fig. 7.2.15-7.2.16) as compared to those of actinic keratosis (fig. 7.2.17-7.2.18) and of keratoacanthoma
(fig.7.2.19) types, both of the organelles and nucleus and of cytoskeletal elements, especially in the number and distribution of micro-tubules indicating possible disruption of incorporating synthesis and secretion pathways; as with leukoplakia associated with moderate-severe dysplasia it was found the presence of a large number of macrophages presenting a rough endoplasmic reticule and an oversized Golgi complex, indicating an intense activity of cell synthesis and secretion, likely for proinflammatory cytokines.

In the case of oral premalignant lesions, more severe ultrastructural were found in leucoplakias associated with a moderate-severe dysplasia (fig.7.2.30-7.2.35) and in oral erosive lichen planus (fig. 7.2 .23-7.2.27) as compared to the erosive actinic cheilitis (fig.7.2.28-7.2.29) and to leukoplakia with mild to moderate dysplasia (fig.7.2.20-7.2.22). For the erosive lichen planus, these results contradict the theories according to which this type of lesion presents a low risk of malignancy.

3. Determination of the expression of TNFα, IL-6, COX-2 and MMP-2 by ELISA and FRET

Results

Table. 7.3. XII Tissular expression of TNFα, IL-6 and Cox-2(Medium values)

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>TNFα(ng/mg protein)</th>
<th>IL-6(ng/mg protein)</th>
<th>Cox-2(ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinic keratosis</td>
<td>10</td>
<td>0.015</td>
<td>0.013</td>
<td>0.25</td>
</tr>
<tr>
<td>Bowen Disease</td>
<td>5</td>
<td>0.026</td>
<td>0.030</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Table 7.3. XIII Tissular expression of MMP-2 (Medium values)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$n$</th>
<th>MMP-2 (RFU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratoacanthoma</td>
<td>5</td>
<td>0.022 0.024 0.50</td>
</tr>
<tr>
<td>Epidermis witness</td>
<td>20</td>
<td>0.00 0.008 0.19</td>
</tr>
<tr>
<td>Leukoplakia with easily moderated dysplasia</td>
<td>10</td>
<td>0.021 0.016 2.98</td>
</tr>
<tr>
<td>Leukoplakia with severely moderated dysplasia</td>
<td>8</td>
<td>0.024 0.028 3.71</td>
</tr>
<tr>
<td>Erosive cheilitis</td>
<td>10</td>
<td>0.018 0.017 2.37</td>
</tr>
<tr>
<td>oral erosive lichen planus</td>
<td>5</td>
<td>0.020 0.022 2.85</td>
</tr>
<tr>
<td>Oral mucosa witness</td>
<td>43</td>
<td>0.00 0.004 0.54</td>
</tr>
</tbody>
</table>

**Discussions**

The results emphasize the low expression of these molecules for premalignant
cutaneous lesions compared to those of the oral mucosa level (Table 7.3.XII, 7.3.XIII). These results indicate a greater degree of cell phenotype modification in the molecular plan for oral premalignant lesions and are consistent with the survey results that reveal more important ultrastructural modifications in these lesions as compared to the cutaneous ones.

IL-6 facilitates the interaction of epithelial cells with stromal cell and during the epithelium-mesenchymal transition process it synergistically acts with the signalling pathway mediated by TGFβ and induces growth of the vimentin expression by reducing the expression of cadherin-E, on the JAK / STAT3 / Snail (114 170 179) signalling pathway.

By regulating the NF-kB signalling pathway, IL-6 and TNFα intervene equally in both the inflammatory processes of oral lichen planus and in those of the malignant transformation. The large amount of secreted cytokine is associated with the intensity of the inflammatory processes, which are very important for carcinogenicity.

TNFα and IL-6 are also associated with increased levels of cyclooxygenase-2, supporting thus the research of Tsatsanis et al. This indicates that TNFα is a stimulant factor of Cox-2, dependent of the NF-kB activation (200.201).

High levels of TNFα, IL-6 and COX-2 identified in erosive actinic cheilitis lesions can causally be correlated to the exposure to UVB radiations which stimulates both COX-2 and NF-kB (198,199).

In the light of the above data, the increased expression of MMP-2 both in premalignant and oral cutaneous lesions
that was observed in this study also suggests the activation of oncogenes involved in the modulation of the expression of this proteinase in the case of such lesions. This is a molecular characteristic of premalignant oral and cutaneous lesions for which there are no references in the literature.

4. Immune-histochemical study of the Ezrin expression in premalignant lesions

Results
This immune-histochemical research aimed to identify the Ezrin expression, a molecule less studied at the level of premalignant cutaneous lesions, with only one rather irrelevant study for his type of lesions in the literature and ignored until nowadays for premalignant oral lesions. The results of this research highlight a number of changes in the expression of this molecule, regarding both the intercellular bond and the development of important signalling pathways through which cellular interactions take place. These cellular interactions will guide concerned cellular systems towards proliferation and / or migration. Findings can be considered part of the disturbances of the intricate molecular system which causes the acknowledged ultrastructural features: disorganization of the coupling cellular zones, modification and distribution of tonofilaments and partial disruption of the normal structure of the plasmalemma and basal membrane.
Discussions
Ezrin involvement in intercellular communication, with the extracellular environment as well, its role in the complex mechanisms of cell adhesion led us to try in premiere to evaluate in a comparative analysis the expression of this protein at the level of cutaneous and oral structures with premalignant lesions.
To this regard, the disorder of the cell-cell and cell-matrix coupling areas observed in the electron microscopic study with a greater frequency in the case of Bowen disease (fig. 7.2.15, 2.7.16), of erosive lichen planus (Fig. 7.2 .22,7.2.23) and of leukoplakia with moderately severe dysplasia (fig. 07/02/30 to 07/02/35) as compared with actinic keratoses (fig. 7.2.17,7.2.18),
erosive actinic cheilitis (fig. 07/02/28 , 07/02/29) and leukoplakia associated with a mild-moderated grade of dysplasia (fig.7.2.20-7.2.22) is also due to the modification of the expression of the Ezrin normal membrane that seems to be translocated into the cytoplasm in the above mentioned lesions, taking into account the intensely cytoplasmic immune positivity found in the present study (fig. 7.4.38,7.4.43 a, b, c, d, 7.4.44 a, b, c).

The correlation of the MMP-2 expression with that of the Ezrin shows that it can be adjusted by the Ezrin by means of AKT (PKB) and MAPK signalling pathways.

The mechanism and the exact role of the Ezrin in the tumult of the tumour growth and dissemination is not completely understood, and there are several hypotheses: role in adhesion and communication, importance of some signalling pathways: MAPK, CD44, Rho kinase, HGF, identified as proteins associated with the tumour metastasis (224).

The Ezrin overexpression is associated with an aggressive tumour phenotype and for this study it represents a sign that concerns the evolution towards the malignancy of these lesions.

Possibly, the fact that Ezrin maintains the stability of adhesion junctions under conditions of quasi-normality, intense or moderated immune reactivity at a cytoplasmic level and keeping the lower intensity of the membranous one, does not necessarily mean the total breakup of intercellular links, process revealed at the ultrastructural level. It means that the disintegrated material support further enables the preservation of polarity under the influence of a number of signals of the extracellular
environment to the reception of which the Ezrin also participates, still totally undisturbed and unconverted to another kind of proliferation and evolution.

**Conclusion**
The statistical processing highlights that the frequency of oral cell squamous carcinoma developed de novo or on premalignant lesions, is significantly higher than that of squamous cutaneous carcinoma.
In the tumours developed on premalignant lesions there is a much higher frequency in the case of oral mucosa as compared to the cutaneous one.
The comparative electron microscopy study of the two types (cutaneous/oral) of premalignant lesions reveals the increased prevalence of important ultrastructural changes, some of which being very specific to tumour cells, in cases of premalignant oral lesions. This aspect highlights their severity as compared to the cutaneous ones and is consistent with the results of the statistic research.
The intensity of the Ezrin immune positivity also correlates with the increased expression of TNFα and IL-6 cytokines as shown by the biochemical study indicating the Ezrin influence of the NF-kB-mediated signalling pathway, and the association of the Ezrin cytoplasmic overexpression with the increased levels of COX in the dysplastic lesions, suggests the activation of the C protein-kinase.
The results were relevant to what I wanted to highlight on some of the most common premalignant cutaneous and oral lesions, with unpredictable evolution, major malignancy potential, tendency to associate with a series
of development, maturation and specialization disturbances.

**Selective bibliography**


Kamperos G, Nikitakis N, Sfakianou A et al. Expression of NF-κB and IL-6 in oral precancerous and cancerous