International reports suggest a worldwide increase in the incidence of non-melanoma skin cancers (NMSC) [1]. The highest rates were observed in Australia, with a 4.4-fold increase in the reported NMSC in 2011 compared to 1985 [2]: a higher incidence and more rapidly increasing rates were observed in the diagnosis of basal cell carcinomas (BCCs) compared to squamous cell carcinomas (SCCs) [2, 3]. In Europe, alarming epidemiological data came from the United Kingdom, with the highest increase in NMSC [3, 4]. Interestingly, the two populations differ in their sun-exposure behavior, an important risk factor for the development of BCC [3]; in Australia the population is long-term exposed to ultraviolet radiation, while in the United Kingdom intermittent exposure is more frequent [3]. Other factors might explain the epidemiological differences: the skin type and genetic background [3], the variation in sun protection use [3], the aging of the population, the improved diagnosis and better registration procedures [3].

In Romania, there are poor epidemiological data regarding the prevalence of BCCs and their histological subtypes or the epidemiology of NMSC in general. According to the Cancer Report in North Western Region of Romania 2010-2011, the incidence of NMSC was of 116 cases in male patients and 117 in females in 2010 [5]. During the year 2011, 100 cases were reported in men and 82 in women [5].

Although BCCs rarely lead to severe outcomes and have low mortality rates, they can have an important individual impact, being frequently localized on visible areas (face, head, neck), as well as a significant economic burden due to the increasing costs of hospitalization and surgical treatment [3].

Recent advances in the diagnosis of BCCs by histochemical, immunohistochemical and ultrastructural techniques brought new insights in the understanding of tumor etiopathogenesis, revealing complex molecular interactions and tumorigenic pathways. Latest research found evidence that all BCCs derive from primitive basaloid stem cells that subsequently develop in distinct clinical and histological subtypes, probably through different pathophysiological pathways [6, 7]. The phenotypic and genotypic characterization of the enzymatic profile of normal and malignant epithelial cells, as well as the expression of different oncogenic genes improved the diagnostic of BCC by differentiation from other epithelial tumors such as basaloid SCC [8, 9], seborrhiec keratosis [10] or trichoepitheliomas [11].

In the present study, we aim to describe a population of patients diagnosed with BCC in a dermatology clinic, Romania and to characterize the morphology, immunohistochemistry and ultrastructural features of the tumors in order to discover predictive markers of invasive growth. The results could provide insight in the understanding of BCC etiopathogenesis and also to contribute to the discovery of new therapeutic targets.

**Experimental part**

**Materials and methods**

We performed an observational study of 352 patients diagnosed with BCC, hospitalized in the Elias Emergency University Hospital, Bucharest, Romania, over a 5-year
observation period, between January 2006 and December 2010. A cohort of 100 patients was recruited for the experimental study; the histopathological analysis was performed for 100 surgically excised tumors; the histochemical and immunohistochemical profiles were assessed for 20, respectively 10 samples; the ultrastructural features of 4 tumors were analyzed by electron microscopy. An informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in approval by the institution’s human research review committee.

Clinical data was collected by anamnesis, full body examination, tumor assessment by clinical and dermatoscopic criteria. The following information was gathered: age, gender, living area, tumor location.

Histopathological examination

We performed a thorough examination of the biotic tissues obtained by complete surgical excision of the tumors. 100 samples were assessed by standard histopathological techniques, using the standard Hematoxylin and Eosin stain. Tumors were divided into 3 major histologic groups: common BCCs including superficial, nodular, micronodular and morpheaform subtypes, metatypical or basaloid BCCs, and pigmented BCCs.

Histochemical assessment

In order to perform the enzymatic histochemical studies 20 biotomic specimens were frozen with dry ice and sectioned in a cryostat into 5-µm thick fragments [12]. The samples were assessed by histochemical methods for the following enzymes: the aerobic enzyme diaphorase/NADH2 cytochrome c reductase, the glycolic anaerobic enzyme lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), and ATP-ase at pH 9.4 [12-14]. The enzymatic activity was semi-quantitatively assessed, in the following manner: 0 = negative reaction, 1 = mild reaction, 2 = moderate reaction, 3 = intense reaction, 4 = highly intense reaction.

Immunohistochemical assessment

The expression of 4 markers Bcl-2, Ki-67, p53, and PCNA was determined by immunohistochemical nuclear staining in 10 tumors using the protocol of Mateoiu et al. (2010) [15]. The samples were initially formalin-fixed and paraffin-embedded [15, 16]. Serially cut sections of 3µm thickness were stained by standard methods (Avidin-Biotin Peroxidase Complex technique) [15, 16]. The immunohistochemical investigation was performed using the following primary antibodies: monoclonal mouse anti-PCNA (DAKO PCNA, clone PC-10, 1:400 dilution), monoclonal mouse anti-human anti-p53 (DAKO p53, clone DO-7, 1:100 dilution), monoclonal mouse anti-human anti-Bcl-2 (DAKO Bcl-2, clone 124, 1:100 dilution) and monoclonal mouse anti-human anti-Ki-67 (DAKO Ki-67, clone MIB-1, 1:200 dilution) [12, 15, 17]. Each sample was evaluated by two investigators who assessed qualitatively and semi-quantitatively the entire tumor under high-power microscopic magnification (×400) [15]. The staining extent of the molecular reaction was expressed as a percentage of Bcl-2, PCNA, Ki-67, and p53 positive cells [15], scored as follows: 0-5% = 0 (negative reaction), 6-25% = 1+ (mild reaction), 26-50% = 2+ (moderate reaction), 51-75% = 3+ (intense reaction), 76-100% = 4+ (highly intense reaction) [15]. The color intensity was also determined by assessing the major pattern of nuclear staining: grade 0 (no color), 1 (low), 2 (moderate), and 3 (intense) [15, 17].

Ultrastructural evaluation

After histopathological diagnosis, specimens taken from similar regions of the tumors were prepared for scanning electron microscopy. They were fixed for 30 minutes at 4°C with glutaraldehyde 2.5% fixative buffered with 0.1 M sodium cacodylate (pH 7.4), and 1% CaCl2 solution, after the specimens were cut into less than 1 µm with the ultramicrotome [18]. The specimens were then dehydrated by graded ethanol and embedded in EPON A and B [18]. The sections were contrasted with lead citrate and uranyl acetate, using the counterstaining procedure of Venable and Coggeshall [19] and examined with Philips 30 electron microscope. In order to identify the nature of the mucoid material, the specimens were stained with toluidine blue with graded pH values and it was assessed the presence or absence of metachromasia.

The experimental results were correlated with the clinical and paraclinical data.

Results and discussions

Clinical data

The gender distribution revealed a slightly higher prevalence in males (M:F=181:171), consistent with the epidemiologic observations provided in other international reports. The age distribution ranged from the lowest rates in the fourth decade (8 cases, 2%), with equal proportions between genders, followed by a steady increase observed with the aging of the patients in the fifth (11 cases, 3%) and sixth decades (56 cases, 16%). A sharp increase was noted after the age of 60 (277 cases, 79%) (fig. 1).

The majority of patients came from the rural area (75%), where occupational behaviours involve long-term sun exposure. For 55 patients UVR-exposure was considered the main oncogenic factor. The anamnesis revealed a history of X-ray therapy for tinea capitis during childhood or professional exposure to X-ray radiation in approximately 11% of patients (38 cases). The remaining 7 patients had other unidentified triggering factors.

Repeated X-ray exposure represents an important carcinogenic factor involved in the etiopathogenesis of BCC. NMSC are included in the category of occupational cancers, and, surprisingly, the Romanian legislation does not consider UVR exposure and X-ray radiation as carcinogenic factors [20].

A recent study performed in a tertiary referral centre in Romania included data from 321 patients diagnosed with BCC and compared the sun exposure patterns in an attempt to establish significant etiological correlations [21]. Facial
lesions were the most frequent, independent of the history of UVR-exposure. Significant differences were noted in the prevalence of BCCs located on the scalp or the cheek, diagnosed more frequently in exposed and, respectively, non-exposed patients [21]. Histopathological assessment revealed that the nodular subtype was the most prevalent [21]. Statistical analysis showed significant variations of tumor invasiveness depending on UVR-exposure; highly aggressive tumors were diagnosed more frequently in patients with occupational long-term sun exposure [21].

In our study, the relapses were rare, predominantly in female patients, in the fifth decade of life, and were mostly localized on the facial area. These results were probably due to an abusive use of aggressive facial cosmetic treatments.

**Histopathological findings**

The highest incidence of BCCs was noted on the face, followed by the scalp and neck, the truncal area, upper limbs, and lower limbs. The average vertical depth of tumor invasion was of 2.04 mm, higher compared to other recently reported values. A large observational study performed in Hamburg, Germany, included 7,116 patients with 9,467 histologically proven BCCs [22]. The research group analyzed the impact of socio-demographic factors on the morphology of the tumors, by the assessment of tumoral depth, a specific marker of invasion [22]. The highest median tumoral depth, of 1.45 mm, was detected in members of agricultural health-insurances [22].

The majority of the tumors belonged to the category of common BCCs (83 cases) (51:32), followed by the pigmented subtypes (15 cases) (11:4), almost 3 times more frequently encountered in men. Metatypical BCCs were uncommon compared to other histopathological subtypes: two patients, one female and one male, were diagnosed with this aggressive form of BCC.

We noticed that pigmented BCCs had an uneven distribution of melanocytes that were sometimes observed peritumorally at significant distances, reaching 1.1-1.5 cm. Basosquamous or metatypical carcinomas represent rare and aggressive histopathological subtypes of BCC, characterized by mixed nodular and infiltrative types of growth, showing morphological features of both BCC and SCC [23]. In the experimental study we observed that metatypical BCCs had prominent SCC features, with rare basoloid architectural patterns.

**Histochemical findings**

NADH cytochrome c reductase, also called diaphorase, is an enzyme of the oxidative aerobic metabolism, localized within mitochondria. It was observed an intense and very intense activity in neoplastic cells, independent of the histological subtype (table 1, fig. 2). Also, in all BCC specimens, the diaphorase activity varied greatly in lymphocytes and plasma cells, from a very mild positive reaction towards highly intense histochemical staining. Macrophages presented an intense enzymatic activity. While intratumoral melanocytes showed moderate diaphorase staining, their stromal counterparts presented very intense reactions. Endothelial cells, as well as perivascular fibroblasts and inflammatory cells showed intense and very intense diaphorase activity.

Compared to NADH cytochrome c reductase, lactate dehydrogenase (LDH) had a lower activity, consequently revealing a decreased participation in the anaerobic oxidative metabolism (fig. 2). Both diaphorase as well as LDH did not present activity in highly differentiated cells or during apoptosis. On the other side, malign cells presented...
homogenous LDH staining, being more enzymatically active compared to normal peritumoral cells. We observed significant differences in the enzymatic patterns of inflammatory cells, depending on the type of BCC. Common BCCs presented homogenous and intense LDH activity of the macrophages, lymphocytes and plasma cells, while pigmented and metatypical BCCs had a higher reaction in macrophages compared to other immune cells. A moderate LDH positivity was detected in endothelial cells.

Leucine aminopeptidase (LAP) is an exopeptidase present in the skin, with a critical role in protein degradation. Our experimental study revealed a characteristic activity pattern of LAP, with its unique distribution in areas of positivity rather than individual cellular reactivity. Tumoral cells presented medium, intense and very intense reactions in common BCCs, while pigmented and metatypical subtypes had medium and intense LAP staining. Peritumoral fibroblasts had variable enzymatic expression, decreasing from very intense in the proximity of the neoplastic cells towards milder reactions farther away. It was found an intense LAP activity in endothelial cells. The results revealed the intense participation of lysosomal LAP in the mechanisms of intracellular protection and digestion.

Malignant and inflammatory cells presented lower ATPase activity, mostly of moderate intensity, while endothelial cells were intensely stained, dominating the histological picture (fig. 2). Depending on the presence or absence of an inflammatory infiltrate we noted several histochemical differences. The inflamed tumors showed intense and very intense enzymatic activity in migrating immune cells (lymphocytes, plasma cells, and macrophages), suggesting an active inflammatory reaction and enhanced phagocytosis, while the non-inflamed BCCs presented variable degrees of enzymatic positivity, largely observed in all cellular subtypes. The histochemical results varied greatly depending on different factors: the tested enzyme, the histological subtypes of the tumors, the cellularity of the tumors or of the peritumoral stroma, the inflammatory infiltrate, the membranary and cytosolic structural differences of the cells. Compared to NADH2 cytochrome c reductase, lactate dehydrogenase (LDH) had a lower activity, consequently revealing a decreased participation in the anaerobic oxidative metabolism. Overall, pigmented BCCs showed higher enzymatic activity, both in tumoral cells as well as in some stromal cells such as melanocytes or macrophages, revealing an intense metabolism and phagocytic capacity. Basosquamous carcinoma showed homogenous enzymatic activity, with very intense staining in the majority of squamous cells, revealing a higher number of cells in the active stages of neoplastic differentiation.

**Immunohistochemical findings**

The majority of the tumors showed intense and very intense expression of p53 and Bcl-2, the two nuclear markers associated with tumor invasiveness and aggressiveness. The wildtype molecule p53 is an essential tumor suppressor that protects the integrity of the human genome [24]. In response to various stress stimuli it triggers oncogenic cell cycle arrest, apoptosis, and autophagy through transcriptional and biological activities [24-27]. p53 mutations are considered the most frequent genetic alterations in human cancer [28]. In epithelial malignancy sunlight exposure leads to specific UV mutations of p53, PTCH, SMOH, SUFUH and other genes [29]. Mutated p53 inhibits apoptotic pathways, consequently leading to resistance to chemotherapy [26, 30]. Immunohistochemical tests using monoclonal or polyclonal antibodies can detect both wild and mutated types of p53. In the normal skin, UV radiation triggers an increase in wild-type p53 in keratinocytes, while in resting conditions this molecule can rarely be detected, due to its rapid degradation [15, 31-33]. Mutant p53 is more stable and immunohistochemistry usually reflects its presence in the tumor biopsies, rather than the wild-type p53 [15, 26].

In our study, the majority of the tumors (9 out of 10) showed intense staining for p53. Only one superficial BCC was negative for p53 immunostaining. The p53 reaction was more intense in the periphery of the tumors, gradually decreasing in the middle region, becoming pale or even absent in the central, vascular area (fig. 3). While the stromal reaction was weak or absent, metastatic cancer cells, either dermal, or invasive in the subcutaneous tissues, stained intensely.

Mateoiu et al. (2011) compared the expression of several markers of apoptosis and cell proliferation in 21 samples of basal cell carcinoma, either superficial, nodular or morpheaform [15]. It was suggested a potential role of two apoptotic nuclear markers Bcl-2 and p53 in predicting the tumor severity: the majority of invasive, sclerosing BCCs showed intense expression of p53 and low Bcl-2 labeling, while approximately one half of the indolent tumors, nodular or superficial, were positive for p53, but had a much more intense expression of Bcl-2 [15].

Mateoiu et al. (2010) also observed negative results in half of the studied superficial BCCs [15]. A possible explanation is that the antibody used in the immunohistochemical tests might not target the proteins from the mutated genomic areas [15]. Moreover, a negative test does not necessarily equate with the absence of the p53 mutation [15]. Despite a small number of tumors were studied by immunohistochemistry, the results confirm the involvement of the gene p53 in BCC tumorigenesis.

In our study, the intensity of the stromal reaction to Bcl-2 staining was variable and it did not reveal a significant correlation with the Bcl-2 expression in the cancer cells (fig. 3). Metatypical BCC showed intense and very intense immunostaining of the connective tissue and endothelial

![Fig. 3 Immunohistochemical assessment of basal cell carcinoma.](http://www.revistadechimie.ro)
cells, while a moderate stromal reaction was observed in pigmented BCCs. The positive reaction for PCNA and Ki-67 allowed the assessment of the degree of cellular proliferation, both for malignant as well as for stromal cells (fig. 3). PCNA testing showed very intense cytoplasmic positivity of the tumor cells. We observed significant immunohistochemical differences between the common subtypes of BCCs, pigmented tumors and basosquamous BCCs. Negative or mildly positive PCNA results were detected in common and pigmented BCCs, while metatypical tumors had intense and very intense PCNA immunomarking, higher in the peritumoral invading cells. Ki67 positivity varied proportional to the tumoral aggressivity: BCCs with favorable outcomes mildly stained for Ki67, while invasive carcinomas had intensely positive reactions, with an extensive staining of the whole tumor. Stromal Ki67 reaction was low or absent.

Khalesi M et al. (2016) analyzed the expression of PTCH1, COX-2, p53, and Ki-67 proteins from cephalic or truncal BCCs, comparing two histological subtypes, superficial or nodular [6]. The immunohistochemical research pointed out a higher expression of PTCH1 and ki-67 in the superficial tumors [6]. It is assumed that histological variability might appear due to different tumoral etiologies [6].

In 2013, Imiquimod 5% cream was approved by the Food and Drug Administration of the United States of America (FDA) for the topical, noninvasive treatment of superficial BCCs [34]. Imiquimod is an anti-tumoral agent that strongly activates cellular immune response through toll-like receptor (TLR) 7 pathways [34]. It has recently been proven that Imiquimod can directly induce cancer cell apoptosis in BCCs by Bcl-2 and p53 dependent mechanisms. This therapeutic agent upregulates the transcription of p53 gene, stabilizes the protein p53 and activates its nuclear translocation [26]. In the study of Huang SW et al. (2016), mutant p53 skin cancer cells were more resistant to the proapoptotic effects of imiquimod, cells less frequently observed in our research in superficial BCCs.

While the expression of the anti-apoptotic Bcl-2 gene was associated with a favorable outcome [15], an intense expression of proliferating cell nuclear antigen (PCNA) was associated in several reports with aggressive clinical and histological forms of BCC [15].

In addition to the above-described potential roles of p53 and Bcl-2 as predictive biomarkers of severity and as targets for different therapeutic agents, Ramezani et al. (2016) recently suggested that Bcl-2, along with CD10, CEA, and EMA might be useful in the differential diagnosis of BCCs and SCCs, due to their characteristic immunostaining profile [8].

**Ultrastructural findings**

The ultrastructural study by electron microscopy revealed that neoplastic cells presented various changes in each cellular component. In all tumors, the stromal reaction was intense showing fibroblast proliferation, dense inflammatory infiltrates and active angiogenesis. This pattern could explain the general good prognosis of BCCs.

The nuclei had an increased volume, were rich in euchromatin, and presented 2-3 large nucleoli (fig. 4). Moreover, the cytoplasm was rich in cellular organelles, useful in the synthesis of vital molecules or involved in important defense mechanisms. Mitochondria were large, with many cristae. The rough endoplasmic reticulum had numerous ribosomes attached to its surface, while the Golgi apparatus was also well developed. The ultrastructural examination also revealed a rich number of organelles involved in oxidative damage protection or intracellular digestion. Lysosomes, rich in acidic hydrolases, were observed in different stages of activity. Cellular membranes presented numerous extensions of various sizes and shapes, with rare or even absent intercellular junctions (e.g. desmosomes).

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**Fig. 3 Immunohistochemical assessment of basal cell carcinoma.**

a. Ki67 nuclear immunostaining of the tumor cells, marking the degree of neoplastic proliferation. Ki-67 Index relatively increased in the tumor, indicating a favorable prognosis.

b. PCNA: intense and very intense tumoral PCNA staining, with a weak stromal reaction. Positivity is higher within the desmoplastic differentiation islands in comparison with the rest of the tumor. 300X.

c. d. PCNA: intense and very intense tumoral PCNA staining, with a weak stromal reaction. Positivity is higher within the desmoplastic differentiation islands in comparison with the rest of the tumor. 300X.

**Fig. 4 Ultrastructural features of basal cell carcinoma.**

Tumoral cells with euchromatic nuclei and large nucleoli. E.M. 11000X.

The tumoral stroma was also intensely represented. Frequent fibroblasts with large euchromatic nuclei and 2-3 nucleoli, had a cytoplasm rich in lysosomes and organelles involved in protein synthesis (mitochondria, rough endoplasmic reticulum, Golgi apparatus).

Peritumoral stroma included frequent, active fibroblasts and rare melanocytes with normal ultrastructural features. We observed abundant evolving capillaries, suggesting intense blood vessel neoformation. We analyzed the
ultrastructural features of various types of inflammatory cells. The macrophages had an intense phagocytic activity, with the presence of dense cytoplasmic lysosomes. The lymphocytes were increased in size, with euchromatic nuclei. Plasma cells had equal amounts of nuclear euchromatin and heterochromatin.

The ultrastructural study by electron microscopy also revealed the presence of various cellular alterations, characteristic for each BCC subtype.

Pigmented BCCs presented large and unevenly distributed melanocytes, sometimes detected peritumorally at very large distances of 1-1.5 cm. Different from their distribution in nevocytic nevi, melanocytes did not form aggregates. The cells had nuclei rich in euchromatin, with 1-2 nucleoli and an increased nucleocytoplasmic ratio. We observed two ultrastructural types of melanocytes: the first was characterized by long, thick, well-developed dendrites, had numerous cellular organelles and dense cytoplasmic melanomas; the second type was smaller in size and presented a lower degree of differentiation, had short and rare dendrites, few cellular organelles and isolated granules of melanin in the cytoplasm. The stromal reaction was similar to the one observed in other BCC subtypes, characterized by fibroblast proliferation, dense inflammatory infiltrates and active angiogenesis.

In basosquamous carcinomas, tumoral cells also had large, hyperchromatic nuclei, with 2-3 nucleoli, and active cytoplasmic organelles. Multipolar mitotic figures, tri or tetra-polar, were frequently noted. Ultrastructural changes were more evident in the areas of squamous cellular infiltration rather than regions of BCC. Basaloid cells had darker nuclei compared to squamous cells, mainly due to a higher content of euchromatin.

Conclusions

In conclusion, our study revealed a great morphologic diversity of the tumors showing unique characteristics for each tumoral subtype (common, pigmented and metatypical BCC). Histochemical, immunohistochemical and ultrastructural investigations provided a detailed description of the tumors, critical in the understanding of tumor progression, and highlighted potential biomarkers of severity (Bcl-2, p53, ki-67). In order to establish statistical relevant correlations, larger studies are needed.

References