„CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY

BUCHAREST

DOCTORAL SCHOOL

MEDICINE

SUMMARY OF Ph.D. THESIS

Ph.D. Coordinator: Professor Costache Mariana Ph.D.

Ph.D. Student: Naie Alexandru

2023

Contents of the Ph.D. thesis

INTRODUCTION

Basal cell carcinoma (BCC) is the most common malignant skin tumour worldwide. It is much more common in the Caucasian population, so that the incidence of this condition is inversely proportional to the latitude of the area in which the patient lives, and is closely related to skin pigment status. Thus, similar incidence rates have been reported in Europe, Canada and Asia, with Australia having the highest incidence worldwide. The prevalence of BCC is increasing, partly due to improved methods of diagnosis and partly due to an ageing population, its increasing trend in numbers and exposure to ultraviolet (UV) radiation. The incidence of BCC increases significantly after the age of 40, but in recent years there has been an increased incidence in the younger population, particularly women, due to increased exposure to solar or artificial UV radiation. The likelihood of developing BCC is, therefore, the result of a complex interaction between environmental, phenotypic and genetic factors [1].

The risk factors consist of UV radiation, followed by other factors such as burns, arsenic exposure, chronic injuries, human immunodeficiency virus (HIV) infection or acquired human immunodeficiency syndrome (AIDS), organ transplantation, ionising radiation and other immunosuppressive conditions. Some rare genetic diseases may be responsible for BCC during childhood and adolescence, such as Gorlin syndrome, albinism and xeroderma pigmentosum [2-3].

Several subtypes of BCC with prognostically relevant morphology are recognized in the classification performed by the World Health Organization (WHO). Thus, low-risk BCCs (nodular, superficial, pigmented, fibroepithelial, adnexal/infundibulocystic differentiation) and high-risk BCCs (micronodular, infiltrating, sclerosing/morpheaform, basal-squamous, sarcomatoid) are described. Some subtypes have particular histopathological (keratotic, nodulo-cystic, adenoid) and cytological variants (clear cell, monstrous, pitted ring cell) [4].

Immunohistochemically (IHC), BCC is positive for Ber-EP4/Ep-CAM, CD 10, p 63 and BCL 2; it is negative for EMA (except for transitional areas in basal-squamous and sebaceous/ductal areas of the BCC with adnexal differentiation). Variable expression was reported for CK 5, CK 7, CK 8, CK 15, CK 18 and CK 19. CBC stroma is, usually, negative or non-uniformly positive for CD 10 and CD 34 [5].

The main differential diagnoses are: trichoblastoma, trichoepithelioma and basaloid squamous cell carcinoma for nodular BCC, actinic keratosis for superficial BCC, desmoplastic trichoepithelioma for sclerosing/morpheaform BCC, squamous cell carcinoma for basosquamous BCC, melanocytic lesions for pigmented BCC and several adnexal neoplasms

for BCC with adnexal differentiation. Less common mimics in medical practice are Merkel cell carcinoma, benign fibrous histiocytoma and microcystic adnexal carcinoma [6].

The standard therapy of BCC is surgery. In the head and neck region, Mohs surgery or micrographically controlled surgery provides lower recurrence rates compared to wide excision. In stage I and II progression, a number of other treatments are used, but with lower cure rates and frequent recurrences. Radiotherapy is effective for patients who do not qualify for surgery, but with a lower remission rate than surgery. Side effects include skin reactions such as atrophy, pigmentary changes, hair loss and telangiectasias. The negative impact on quality of life, however, appears to be temporary [7-9].

Advanced BCCs are defined as stage III and IV tumours. Often, these tumours develop over several years but are neglected by patients and caregivers. There is an overlap of high-risk BCC and advanced BCC. High-risk BCC is defined as a tumour with longstanding progression, located in the midface or on the ears, greater than 2 cm in diameter, having an aggressive histopathologic subtype, with perivascular or perineural infiltration, and the patient has a history of radiation exposure or previous treatment failure [10-11].

The aim of the present work is to provide a comprehensive overview of the existing knowledge on BCC, focusing on the clinical, histopathological features, the importance of the tumour microenvironment and in particular the tumour-associated vasculature on tumour invasiveness and the impact of radiotherapy on disease progression.

The study is an observational, retrospective one, of cases of BCC diagnosed in the Pathology Laboratory of the University Emergency Hospital of Bucharest, some of which underwent radiotherapy treatment at the Institute of Oncology Prof. Dr. Alexandru Trestioreanu in Bucharest, between 2013 and 2021, over a period of 8 years. The batch totalled a number of 754 cases. The paper is structured in two parts, divided into 10 chapters. To carry out this study and to compare the results obtained, we consulted a number of 168 bibliographical references. The doctoral study also includes an original iconography using macroscopy and optical microscopy images, numerous graphs and tables.

In the present doctoral research the proposed aim and objectives were achieved, which focused on clinical, microscopic, immunohistochemical characteristics, microvascular density and therapeutic peculiarities of BCC.

The analysis was conducted on a cohort comprising 754 cases of BCC. The age of the patients ranged from 19 to 93 years, with an average of 67.5 years. The sex ratio in the study cohort was in favour of females, with the latter having a lower age at diagnosis.

As regards tumour topography, the vast majority of tumours were located on photoexposed areas, most of them being located on the head and neck, especially on the face

(in order of frequency: nose, cheeks, forehead and periorbital area), followed by thoracic location.

Macroscopically, most of the tumours had a nodular appearance, either with telangiectasias on the surface or ulcerated, pigmented, traumatised, superinfected, calcified, pseudocystic or inflammatory. Microscopically, the histopathological subtypes identified, in order of frequency, are as follows: nodular type - predominant, pigmented, superficial, basal-squamous, infiltrative, infundibulocystic, morpheaform and fibroepithelial.

Localization and histopathological subtype were statistically significantly associated, with fibro-epithelial BCC found exclusively in the chest; in the lower limbs the superficial type predominated, in the head and neck, chest, upper limbs and abdomen, respectively, most tumours belonged to the nodular subtype.

Single tumours were most commonly reported, but there were also cases with multiple tumours, most of them located in the head and neck. In 4.4% of cases the tumours were secondary (recurrent), located in the head and neck. The histopathological subtype was associated with the secondary nature of the tumour, with most recurrences being of the infiltrative and nodular type, followed by those of the basal-squamous type.

Immunohistochemically, the cytokeratins AE1/AE3 and 34BetaE12 were positive in all cases tested, diffusely and intensely, consistent with the epithelial nature of tumour proliferation. BerEp4 was also positive in all cases tested and weakly positive expression was more common in tumour recurrences, unlike in primary tumours, where intensely positive expression predominated. Weak positive expression was also associated with infiltrative, basal squamous and morpheaform forms. EMA expression was associated with the basal squamous histopathological subtype. Most P 63 positive cases were in females. Increased P 53 expression was associated with morpheaform, infiltrative and basal-squamous histopathological subtypes and, also, with tumour recurrence and traumatic tumours. Low BCL 2 expression was associated with superficial and nodular histological subtypes. CD 10 positivity in tumour cells was associated with head and neck localization and that in stromal cells with infiltrative and morpheaform histopathological forms. Increased EGFR values were associated with younger patient age and secondary tumour character. Ki 67 proliferation index had higher median values in head and neck compared to tumours located in the chest. Of the histopathological subtypes, the basal-squamous subtype had the highest Ki 67 values and also tumour recurrence compared to primary tumours.

A relatively new topic regarding tumour structure and pathogenesis determinants is the tumour microenvironment and from this category, we chose to study intratumoural vascularization. Therefore, we found that peritumoural microvascular density is higher than intratumoural and intratumoural low microvascular density predominates. In the thoracic and upper limbs tumours were exclusively characterised by low vascular density, this was also

associated with superficial, infundibulocystic, fibroepithelial and pigmented histopathological types. Recurrences, on the other hand, were characterised by increased vascular density.

Closely related to tumour vascularisation is the expression of VEGF, which is present not only in endothelial cells in the structure of the vascular walls but, also, in tumour cells, predominantly on the invasive tumour front. VEGF in tumour cells correlated with microvascular density, and increased expression was associated with superficial and pigmented histopathological subtypes.

Radiotherapy is a therapeutic option in BCC, either as adjuvant therapy (when resection margins are positive, in case of perineural invasion, multiple recurrences and bone invasion), or as sole therapy (when a surgical procedure may result in a significant cosmetic defect or in unresectable BCC). Thirty-three cases of radiotherapy treated BCCs were evaluated, the dominant histopathological type being the nodular one. After radiotherapy, the recurrence rate was 39%. Median time to recurrence was 39.5 months. 85% of relapsed cases were located in the head and neck - usually periocular, with frequent nodular and ulcerated histopathological subtypes. The total radiation dose was variable for BCCs located on the head and neck (from 10 to 48 Gy), but always above 30 Gy for BCCs located on the trunk.

The doctoral research I will present below faced a number of limitations. Some were of a material nature, not having the possibility to perform molecular biology tests to complete the picture of the tumours evaluated. Also, immunohistochemical testing was performed on a limited number of cases. Information on the history and course of the patients was partly limited. The retrospective nature of the study inevitably made it impossible to obtain a range of information and the observational nature did not allow causal relationships between the variables analysed to be revealed, only trends of association.

The results obtained, particularly in terms of tumour microvascularisation, VEGF and EGFR expression, call for further research, which could lead to the discovery of an alternative treatment, with monoclonal antibody therapy as a possible solution in advanced BCC. However, studies on this topic are still in their infancy and are few in number, and further investigations in larger cohorts and clinical trials are needed to complement the data.

II. WORKING HYPOTHESIS AND GENERAL OBJECTIVES OF THE RESEARCH

BCC is the most common type of malignant tumour and its incidence is increasing. BCC has a low mortality, but can cause significant morbidity mainly due to its local destructiveness. Pathogenesis is related to the interaction between the environment and patient-derived characteristics. There are several therapeutic modalities and their appropriate selection requires knowledge of complications, cosmetic outcomes and recurrence rates [12].

In this context, the present doctoral research aims at an in-depth analysis of BCC through the study of clinical, morphopathological and immunohistochemical characters in order to better understand the dynamics of the biological tumour behaviour of these variables. The present work also aims to highlight the role of tumour vasculature in the dynamics of the disease and, from a therapeutic point of view, the usefulness of radiotherapy.

The objectives of the study include:

1. ***Analysis of general, demographic and clinical characteristics of patients diagnosed with basal cell carcinomas*** - in which, patient characteristics such as gender, age, tumour location, primary/secondary tumour character and single vs. multiple tumours will be detailed. The existence of associations and correlations between these variables will also be assessed.

2. ***Analysis of morphological features*** - in which the most common macroscopic tumour features will be reported and microscopic features (histopathological subtype, presence of tumour necrosis, ulceration, superinfection, calcifications, signs of trauma, with representative iconography) will be detailed. The existence of associations and correlations with other clinico-pathological variables will also be tested.

3. ***Study the immunohistochemical expression*** of positive diagnostic markers (BerEP4, 34BetaE12, AE1/AE3), EMA, CD 10, CD 34, VEGF, EGFR, P 63, P 53, Bcl 2 and the nuclear proliferation index Ki 67. The correlations of the immunoexpression of these markers with other clinico-pathological and tumour morphological variables will also be evaluated.

4. Study of tumour neovascularisation

5. Role of radiotherapy

III. GENERAL RESEARCH METHODOLOGY

1.1. Case selection

This doctoral study is based on a retrospective analysis of cases of BCC diagnosed and treated in two large university centres in Bucharest: University Emergency Hospital of Bucharest (SUUB) and the Oncological Institute of Bucharest (IOB), over a period of 8 years (2013 - 2021). The patients underwent surgical excision in the Plastic Surgery department of Bucharest University Emergency Hospital (SUUB) and the excision specimens were processed for histopathological diagnosis in the Pathological Anatomy department of the same institution; subsequently, some of the patients were treated oncologically by radiotherapy at IOB. The retrospective study was performed by accessing the histopathological registers of the Pathology Department and the computerized database of the SUUB. The study was carried out with the approval of the Ethics Committee, in accordance with international ethical standards and with the informed consent of patients for the use of tissues for diagnostic and research purposes.

The main clinical landmarks (age, sex, anatomical location of tumours, date of diagnosis), macroscopic aspects of tumours, histopathological diagnosis and, in some cases, immunohistochemical diagnosis were extracted from the accompanying records of surgically excised specimens, histopathological registers and hospital databases. The total number of cases was 754.

For a sublots of cases, IHC tests were performed to highlight tumour features.

Inclusion criteria:

- Cases registered in the Department of Pathology of the SUUB.
- Cases diagnosed histopathologically as BCC.

Exclusion criteria:

- Cases registered outside the 2013-2021 timeframe.
- Cases in which essential clinicopathological information was not accessible
- Cases in which the histopathological diagnosis was immunohistochemically refuted

1.2. Morphological study of basal cell carcinomas

The morphological study of tumours was aimed at the positive diagnosis of BCC and subsequent analysis and recording of prognostically important tumour features.

Surgical resection specimens arrived at the Pathology Laboratory together with the biopsy material accompanying sheet, which contained the patient's identification data and informed consent for histopathological processing of the specimens, type of surgical procedure, clinical diagnosis, date of collection, and the signature and initials of the physician who requested the histopathological examination. Once in the laboratory, the case receives a unique registration number. The excisional specimens were placed in containers of 10% formalin for fixation for approximately 12 hours. The first stage is the macroscopic examination, during which the size of the skin fragment, the tumour, its shape and colour, its distance from the resection margins, the presence of ulceration areas, etc., are determined. Following this examination, the specimen is sectioned and placed in cassettes and processed for histopathological examination. The processing takes place automatically, using the tissue processor, with the aim of stopping vital processes, preserving and preparing the tissue for the finest possible sectioning. The processing steps are as follows:

- dehydration - using ethyl alcohol in various increasing dilutions

- clarification - using toluene

- impregnation with paraffin.

After processing, the tissue fragments are ***embedded*** in paraffin blocks using the embedding apparatus, which melts the paraffin at a temperature of 58-60 °C, which then solidifies by cooling, obtaining rectangular shaped casts that can be fixed in the microtome for sectioning. The paraffin blocks thus obtained will be shaped and numbered with case numbers. ***The sectioning*** is carried out with the aid of a device called a microtome, obtaining sections with a thickness of about 4 μm, which are stretched on the glass blade. These are transparent, thus requiring the next step: staining. The usual staining used is haematoxylin-eosin (H&E) which is also carried out automatically using a specially designed standardised apparatus. ***Staining*** is carried out in several successive steps: deparaffinization (with toluene/xylene), rehydration in three successive ethyl alcohol baths, staining with Mayer haematoxylin (2-5 minutes), washing, differentiation in hydrochloric acid-alcohol, washing, contrast in saturated lithium carbonate solution, washing, staining with Eosin (3 minutes), washing, dehydration in three successive ethyl alcohol baths, clearing.

The final step in obtaining histopathological slides is ***mounting***, which involves applying a slide over the slide using mounting medium (***Entellan or other mounting medium***). The end result involves blue-violet stained nuclei (basophils) and cytoplasm as well as other pink stained connective structures (eosinophils).

Microscopic analysis was performed using the optical microscope (Leica DM 750) and photographs were taken using Leica ICC50HD cameras and Leica Application Suite X software version 3.0.2. This was aimed at a positive diagnosis of BCC and observation of features such as histopathological type, presence of ulceration, superinfection, calcifications, tumour vascularization, etc.

1.3. Immunohistochemical study of basal cell carcinomas

A limited number of cases were tested IHC (Table V.1) in order to assess certain tumour features. IHC is a diagnostic method that combines histological, immunological and biochemical techniques with the aim of identifying cellular and tissue components as specifically as possible through an antigen-antibody reaction. Following the antigen-antibody interaction, the antibody binding site is revealed by an indirect staining reaction. IHC techniques have increased the certainty of the final pathological diagnosis, making differential diagnosis and in some cases the evaluation of prognostic factors possible. Depending on antibody type and technical specifications, the protocol may vary. Tissue sections for IHC testing were 3μm thick being applied on slides treated with an adhesive agent (polylysine). The chromogen used was diaminobenzidine - DAB (manufacturer BIOCARE). The essential working steps were briefly as follows:

- deparaffinisation in toluene and hydration of the tissue section in successive ethyl alcohol baths followed by washing

- pretreatment of the slides according to primary antibody specifications
- inhibition of endogenous peroxidase
- blocking of non-specific antigenicity
- proteolytic digestion and target antigen unmasking
- IHC staining itself, which involves:

    - incubation with the primary antibody
    - application of the labelled enzyme polymer
    - application of the chromogenic substrate (DAB)
    - counterstaining with Mayer haematoxylin.

The final result of the IHC reaction can be assessed using the usual light microscope, the antigens under investigation being stained brown due to DAB chromogen (nuclear, cytoplasmic or membrane labeling, depending on the antigen of interest), on a blue background conferred by Mayer's haematoxylin.

Tabel V.1. Antibodies used in the study

Antibody	Clone	Dilution	Producer
AE1/AE3	AE1/AE3	Ready-to-use	Biocare
EMA	Mc-5	Ready-to-use	Biocare
BerEp4	Ber-Ep4	Ready-to-use	Biocare
34BetaE12	34BetaE12	Ready-to-use	Biocare
CD34	QBEnd/10	Ready-to-use	Zeta
CD10	56C6	Ready-to-use	Biocare
VEGF	EP1176Y	Ready-to-use	Biocare
EGFR	H11	Ready-to-use	Biocare
P63	4A4	Ready-to-use	Zeta
P53	DO-7	Ready-to-use	Biocare
BCL2	100/D5	Ready-to-use	Biocare
Ki67	***MIB-1***	Ready-to-use	Zeta

1.4. Statistical analysis

The variables of the study sample were collected in a Microsoft Excel 2016 database.

Statistical analysis was performed using IBM SPSS Statistics 20 software. Descriptive statistics and significance tests were performed. Percentages, frequencies, means, standard error of means, standard deviation, medians and IQR were calculated for descriptive analysis. Chi-Square test and appropriate corrections when criteria were not met (Likelihood ratio, Fisher test) were used to check for intervening associations along with Phi and Cramer's V parameters to

determine effect size; t-independent test with reporting degrees of freedom, difference of means, Levene′s test for testing homogeneity of data. Non-parametric Mann-Whitney U and Kruskal-Wallis tests were used if normality test conditions were not met. The confidence interval was set at 95%. Results were presented numerically and graphically.

IV. SPECIAL PART - SUMMARY OF CHAPTERS

Chapter 6 of the Ph.D. thesis focuses on the clinical and epidemiological study of BCC. The present study included a total of 754 patients diagnosed with BCC over 8 years (2013 - 2021) at SUUB. Their clinical and demographic characteristics were entered into a Microsoft Excel database. Categorical variables (sex, tumour location, etc.) were categorised and for numerical variables (age) ranges were determined and means, medians, standard deviations, IQR (interquartile range), etc. were calculated. Additional statistical tests were performed using IBM SPSS Statistics 20 to check for intervening associations and to test for correlations. Finally, the results were presented numerically, including tables and graphs.

In terms of tumour location, most lesions were located on the head and neck, with the face being the most commonly affected area. In 35% of cases, lesions were located on the nose, while in 24% of cases, lesions were on the cheeks, forehead 9% and periorbital 8%. Over 90% of all BCCs were located on sun-exposed skin areas, revealing a statistically significant association ($p<0.05$). As for non-exposed areas, a high percentage of cases were found on the thorax (11.9%); and on body sites intermittently exposed to sunlight: upper limbs (1.7%) and lower limbs (2.1%) (Fig. 6.4).

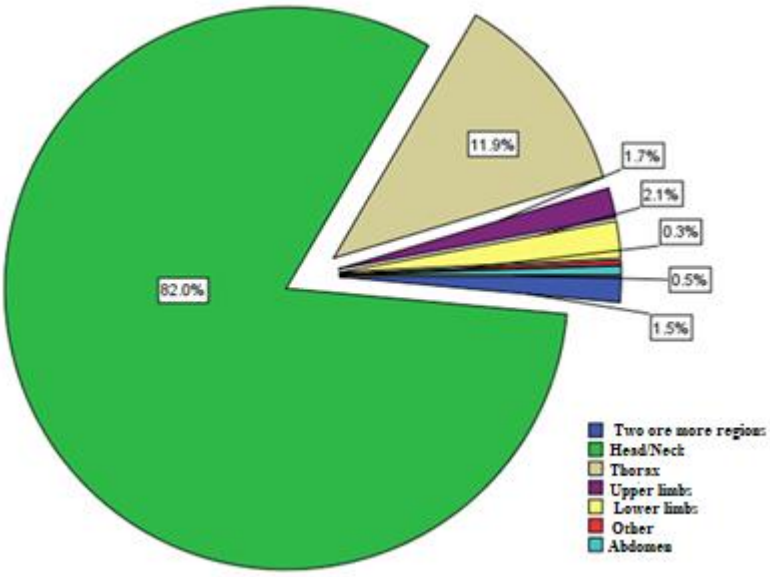

Fig. 6.4 Basal cell carcinoma localization

There was a statistically significant difference between the age means in the group in which borderline excision was performed and in the group in which there were no oncological safety margins, p = 0.47 (df = 752, t = 1.994, Levene's test = 0.941), with younger ages having more frequent borderline excision (Fig. 6.8).

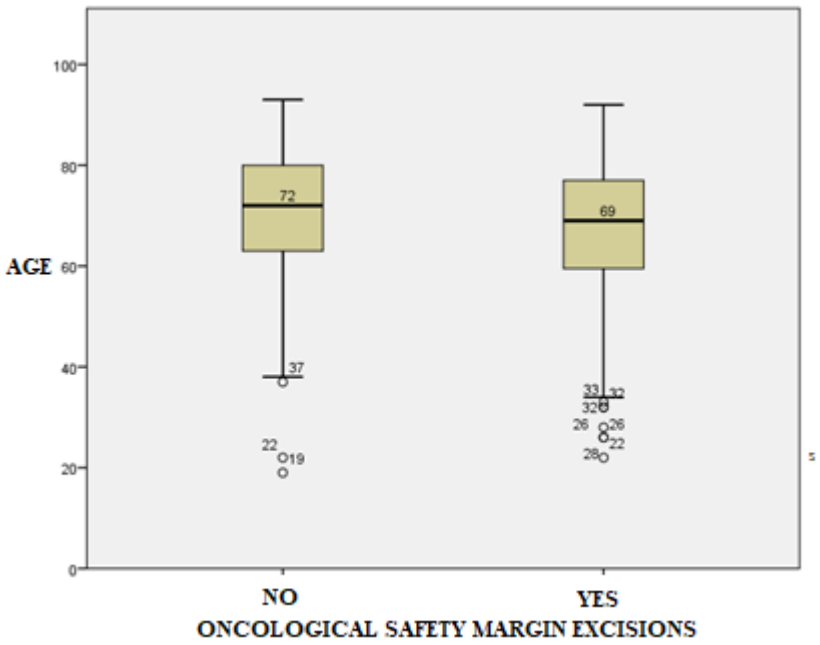

Fig. 6.8. Kruskal-Wallis test accounting for oncological safety margins and patients' age

Chapter 7 of the thesis provides information on the morphological study of CBC. The present study included a total of 754 patients diagnosed with BCC. In the first part, the macroscopic appearance of the tumours was highlighted and in the second part, their microscopic appearance was evaluated based on the usual H&E stained slides using the light microscope. In the first instance, the positive diagnosis of BCC was confirmed and subsequently the tumour features such as histological type, presence of necrosis, ulceration, etc. were inventoried. Photographs were also taken using the camera attached to the microscope, capturing representative images of the features described and complementing the numerical and graphical data. These variables were accounted for in a Microsoft Excel database. Using IBM SPSS Statistics 20 software, statistical tests were carried out to check frequencies and intervariable associations.

In terms of clinical appearance, more than 60% of the tumours had a nodular appearance, some covered by an intact epithelium, with telangiectasias on the surface, others

ulcerated, traumatised, superinfected or showing calcifications. About 10% of the tumours were pigmented, lending themselves to differential diagnosis with melanocytic tumours. In a smaller proportion, superficial forms mimicking inflammatory conditions or vaguely demarcated, whitish, pseudo-scarred forms were identified.

Localization and histopathological subtype were statistically significantly associated, $p < 0.01$, $df = 48$, Cramer's $V = 0.208$, weak power of association. Thus, in the head and neck, most tumors belonged to the nodular subtype, followed by the pigmented, basal-squamous and infundibular-cystic subtypes. In the chest, the most common histological type was also nodular, followed by superficial and then pigmented. The nodular subtype also predominated in the upper limbs, followed equally by superficial, basal-squamous, infiltrative and pigmented. The superficial type predominated in the lower limbs, followed by nodular and pigmented. In the abdomen, nodular and superficial types had an equal number of representatives. In most histological subtypes, the head and neck predominated, with the exception of the fibroepithelial form, which was found exclusively in the thorax (Table VII.1).

Table VII.1. Distribution of cases depending on localization and histological subtype

General localization	**HP subtype**								
	N	**S**	**B-S**	**I-C**	**F-E**	**I**	**P**	**M**	**C**
Two or more areas	5	1	0	0	0	1	3	0	1
	45,5%	9,1%	0,0%	0,0%	0,0%	9,1%	27,3%	0,0%	9,1%
Head/neck	440	19	39	21	0	32	55	11	1
	71,2%	3,1%	6,3%	3,4%	0,0%	5,2%	8,9%	1,8%	0,2%
Thorax	45	19	5	0	5	0	16	0	0
	50,0%	21,1%	5,6%	0,0%	5,6%	0,0%	17,8%	0,0%	0,0%
Upper limbs	9	1	1	0	0	1	1	0	0
	69,2%	7,7%	7,7%	0,0%	0,0%	7,7%	7,7%	0,0%	0,0%
Lower limbs	5	6	1	0	0	0	4	0	0
	31,2%	37,5%	6,2%	0,0%	0,0%	0,0%	25,0%	0,0%	0,0%
Other	0	1	1	0	0	0	0	0	0
	0,0%	50,0%	50,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
Abdomen	2	2	0	0	0	0	0	0	0
	50,0%	50,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

N = nodular; S = superficial; B-S = basal-squamous; I-C = infundibulocystic; I = infiltrative; P = pigmented; M = morpheaform; C = combined

The vast majority of tumours evaluated were primary (95.6%), the remainder being tumour recurrences. A significant percentage (8.8% of cases) were previously traumatised

tumours, which contributed to the patient's change in appearance and presentation to the doctor. Also, in a significant number of cases (N=434; 43 respectively 78) there were ulcerations, superinfections and microcalcifications (Table VII.2).

Table VII.2. Characteristics of basal cell carcinoma

TUMOUR CHARACTERISTICS	
Single/multiple	unică = 698 (92,6%) multiplă = 54 (7,2%)
Primary/recurrence	primară = 721 (95,6%) recidivă = 33 (4,4%)
Traumatised	nu = 688 (91,2%) da = 66 (8,8%)
Ulcerated	nu = 320 (42,4%) da = 434 (57,6%)
Superinfected	nu = 711 (94,3%) da = 43 (5,7%)
Microcalcifications	nu = 676 (89,7%) da = 78 (10,3%)

Chapter 8 provides information on the CBC IHC study. Immunohistochemical tests were performed on samples with limited numbers of cases. Thus, AE1/AE3, BerEp4, 34BetaE12, EMA were tested in 60 tumours; p 63, p 53, Bcl 2, CD 10 and Ki 67 in 50 tumours; EGFR in 15 tumours.

Testing was performed using paraffin blocks corresponding to tumours with clinical and histopathological BCC criteria. In all cases, lesion diagnosis was confirmed by identification of histopathological features in H&E-stained slides. Final preparations, in the

form of slides with immunolabelled tissue, were obtained using standardised techniques, following a specific protocol according to antibody type and manufacturer.

In 50 cases, **P53** expression was tested, with immunolabeling absent in 24% of cases (N=12) and ranging from 5-25% in 28% of cases (N=14), 26-50% in 26% of cases (N=13), 51-75% in 14% of cases (N=7) and above 75% in 8% of cases (N=4).

P 53 expression was associated with the histopathological form of BCC ($p < 0.001$, df = 28, Cramer's V = 0.579, strong association), with the morpheaform, infiltrative and basal squamous subtypes characterized by increased expression, above 50% and even above 75% for basal squamous carcinoma (Fig 8.11).

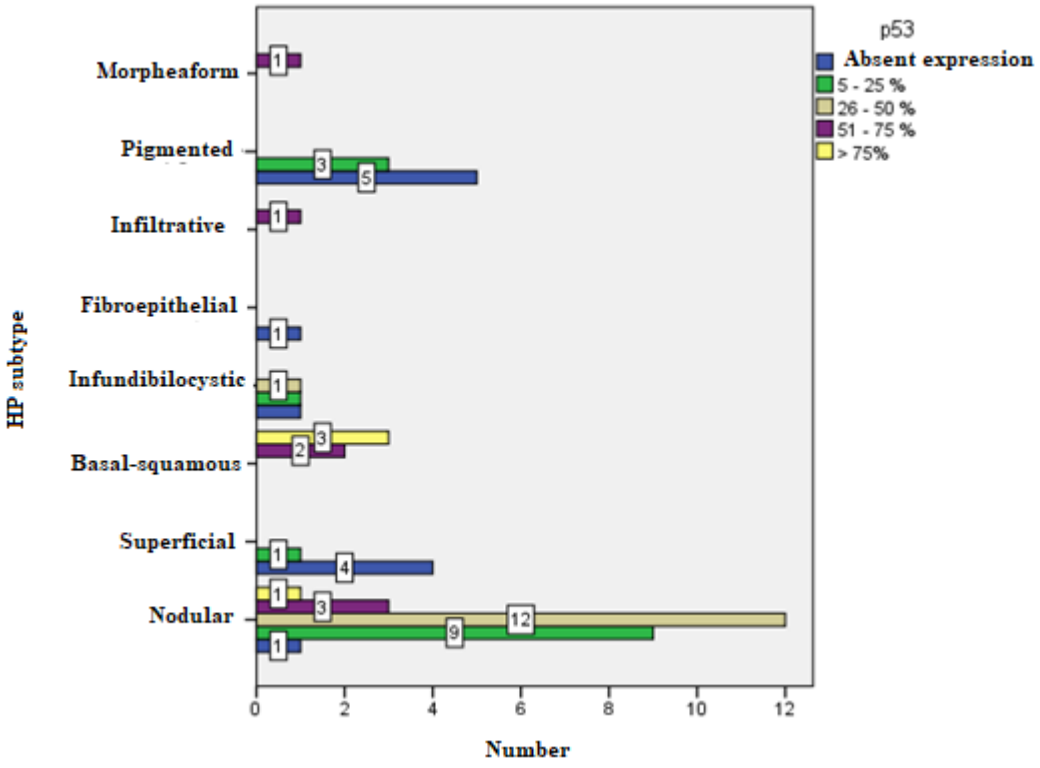

Figure 8.11. Relationship between P 53 expression and histopathological subtype of basal cell carcinomas

Chapter 9 focuses on the role of vasculature in BCC. From the initial batch of patients, 30 cases were selected for evaluation of microvascular density, initially on the usual preparations: H&E stained slides. Subsequently, using the standardised techniques described in the previous chapters, vascular structures were revealed by IHC assays and specifically for CD 34 in all 30 cases. VEGF expression was also assessed in 20 cases, but in addition to vascular structures, it also marked tumour cells.

VEGF expression was analysed in 20 of the cases included in the study, determining two classes of tumours: those with more than 10% positive cells (high expression) and those with less than 10% positive cells (low expression). VEGF was expressed in endothelial cells of blood vessels both in the tumour and peritumour, in keratinocytes of the basal layer of the epidermis, but also in tumour cells, predominantly on the invasive tumour front, in 12/20 cases, VEGF expression being significantly higher than in the adjacent epidermis.

16 of 20 BCCs (80%) showed low VEGF expression and 4 (20%) showed high expression, mostly with diffuse cytoplasmic expression. At the margin of invasion, higher immunolabelling intensity was observed in some cases, without statistical significance. A statistically significant correlation was found between VEGF expression in tumor cells and microvascular density ($p < 0.05$).

VEGF expression did not vary significantly in relation to patient characteristics (age, gender), tumor location, single/multiple, primary/secondary, presence/absence of ulceration, superinfection, microcalcification, or tumor trauma. However, it was associated with histopathological form ($p = 0.016$, $df = 7$, $Phi = 0.919$ - strong association power), with the nodular subtype having exclusively low expression of this marker. The superficial and pigmented subtypes had an increased expression (Fig 9.10).

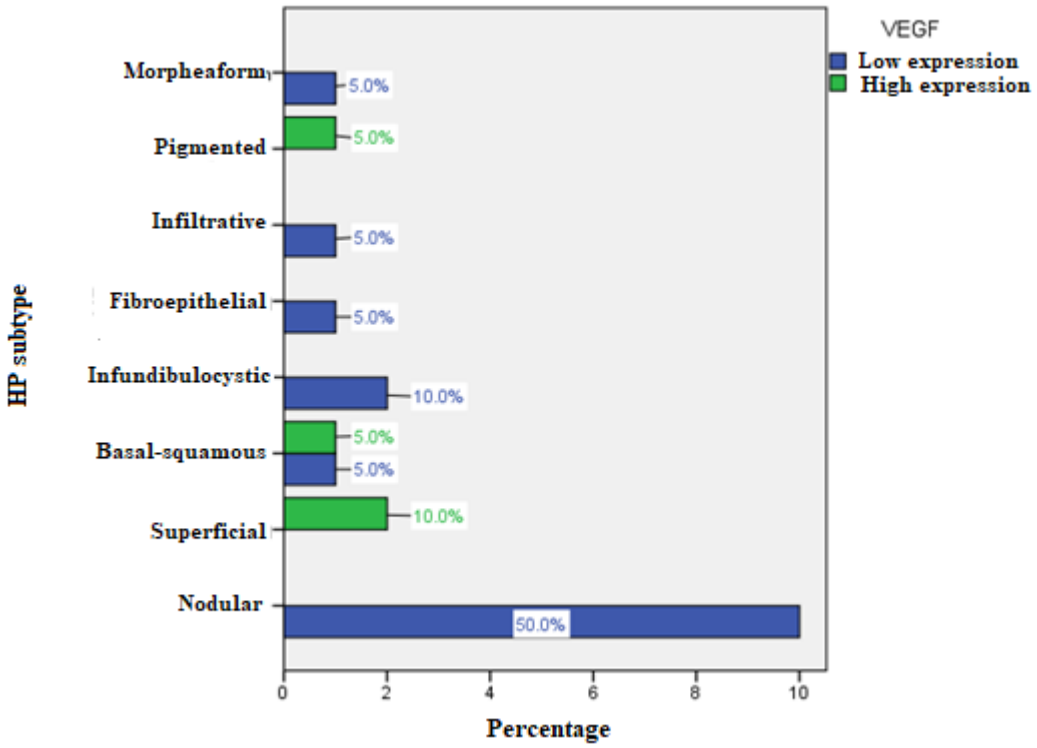

Fig. 9.10. Relationship between histological subtype and VEGF expression

In Chapter 10, we provided an overview of the role of radiotherapy in BCC. The aim of this study was to assess whether histological subtypes and location of BCC alone can be correlated with radiotherapy regimens and disease-free survival rates.

There are multiple histopathological types of BCC described, with no single, generally accepted classification. In the present study, BCCs were classified into four categories: nodular (with three histological variants: ulcerated, clear cell and pigmented), micronodular, adenoid and ulcerated. The nodular type was the most common in the study group (26 patients), and the overall distribution of histological subtypes and variants can be seen in the following table.

Table X.1. Distribution of BCC according to histopathological type, with corresponding radiation dose

Histological subtype of BCC	Total number of patients	Patients with recurrence	Minimum Dose (Gy)	Maximum Dose (Gy)
Nodular	26	10	18	50
Micronodular	1	-	30	30
Adenoid	2	1	10	30
Ulcerated	4	2	30	50

V. CONCLUSIONS AND PERSONAL CONTRIBUTIONS

In the present doctoral research, the proposed aims and objectives were achieved, which revolved around the characterization of BCC, the most common type of malignant tumour in the skin, from a clinical, morpho-pathological, immunohistochemical point of view, the relationship with the tumour microenvironment and specifically with the tumour vasculature and the identification of the role of radiotherapy in the evolution of this type of tumour.

The study was conducted on a cohort comprising 754 cases of BCC diagnosed at the University Emergency Hospital in Bucharest. Patients ranged in age from 19 to 93 years, with an average age of 67.5 years. The gender ratio in the study group was in favour of the female gender (female/male: 1.13/1). In the female gender, BCC was diagnosed at younger ages than in the male gender.

1. In terms of tumour topography, the overwhelming majority (over 90%) were located on photo-exposed areas, with most being located on the head and neck, especially the face (in order of frequency: nose, cheeks, forehead and periorbital area). The thoracic location followed by the lower limbs and then the upper limbs.

2. The vast majority of the tumours included in the study were excised within safe oncological limits, but there was a significant percentage of cases (25%) without safe limits in the head and neck; also, excision with safe oncological limits was performed more frequently at younger ages.

3. Macroscopically, most of the tumours had a nodular appearance, some with telangiectasias on the surface, others ulcerated, traumatised, superinfected or with calcifications. There were also pigmented forms, lending themselves to differential diagnosis with melanocytic tumours. In a smaller proportion, pseudoscarring or superficial forms were identified, mimicking inflammatory conditions.

4. Microscopically, the histopathological subtypes identified, in order of frequency, are as follows: nodular type - predominant, pigmentary, superficial, basal-squamous, infiltrative, infundibular-cystic, morpheaform and fibroepithelial.

5. Of the histopathological subtypes, the nodular type was present in the majority of cases (67.1%), followed by the pigmentary (10.5%), superficial (6.5%), basal-squamous (6.2%), infiltrative (4.5%), infundibulo-cystic (2.8%), morpheliform (1.5%) and fibroepithelial subtypes.

6. Patients with nodular BCC were diagnosed at older ages (mean age 67 years), compared with those with morpheaform (65.7 years) and superficial (mean age 61 years) BCC. Morpheliform BCC was predominantly diagnosed in females.

7. Localization and histopathological subtype were statistically significantly associated, with fibroepithelial BCC found exclusively in the thorax; in the head and neck, thorax, upper limbs, abdomen, most tumors belonged to the nodular subtype; in the lower limbs the superficial type predominated.

8. In 7.2% of cases there were multiple tumours, the number of which ranged from 2 to 9, most often multiple tumours were 2. The highest percentage of multiple tumours in relation to location was found in the abdomen, and in absolute number most cases were in the head and neck.

9. The vast majority of tumours evaluated were primary (95.6%), the remainder representing tumour recurrences. There were no cases with metastases or fatal outcome. BCC patients often presented concomitantly with other skin lesions influenced by chronic sun exposure, such as actinic keratoses or solar lentigines. Tumour relapses were located exclusively on the head and neck.

10. 8.8% of the tumours studied were previously traumatised, 57.6% were ulcerated, 5.7% were superinfected and 10.3% showed microcalcifications. Ulcerated lesions were more common in the study group in males and in older age groups. The presence of microcalcifications was statistically significantly associated with tumour location, most commonly found on the upper limbs.

11. Histopathological type was statistically significantly associated with the presence of ulceration, with the basal-squamous subtype having the highest percentage of ulcerated tumours. Histopathological subtype was also associated with tumour trauma, presence of superinfection and microcalcifications, with most tumours with these characteristics being nodular.

12. In the case of morpheaform and infiltrative histopathological types, most often surgical excision was not performed with oncological safety limits.

13. The histopathological subtype was associated with the secondary character of the tumour, with most recurrences being of the infiltrative and nodular type, followed by the basal squamous type.

14. Immunohistochemically, AE1/AE3 and 34BetaE12 were positive in all cases tested, diffusely and intensely, consistent with the epithelial nature of tumor proliferation.

15. BerEp4 was also positive in all cases tested, 86% high intensity, 14% low intensity, with low positive expression more common in tumour relapses than in primary

tumours, where high positive expression predominated. Strong positive expression was also associated with nodular, pigmentary, fibroepithelial and infundibular-cystic histopathological forms, whereas weak positive expression was associated with infiltrative, basal squamous and morpheaform forms.

16. EMA was positive in 11.7% of cases, with EMA expression associated with the basal-squamous histopathological subtype.

17. P 63 was positive in 82% of BCCs tested, with most positive cases in females.

18. P 53 was positive in 76% of cases evaluated, with variable intratumoral extension, associated with histopathological form, with morpheaform, infiltrative and basal-squamous subtypes showing increased values of this marker. Higher values were also recorded in tumour relapses and traumatic tumours.

19. BCL 2 was positive in the cases tested, with variable extent, high values in superficial and nodular histological subtypes and low values in tumour relapses.

20. CD 10 showed positive expression in both tumour cells (80%) and intratumoural stroma (14%). Positivity in tumour cells was associated with head and neck localization and in stromal cells with infiltrative and morpheaform histopathological forms.

21. EGFR was positive in 80% of cases, of variable intensity, with increased expression associated with younger patient age and secondary tumor character.

22. Ki 67 proliferation index ranged from 3 to 90% of tumour cell nuclei, with a mean of 19% and a median of 15%. In the head and neck, median Ki 67 was higher than in the chest. The basosquamous subtype had the highest median Ki 67 values. At the same time, relapses had higher values than primary tumours and tumours without oncological safety limits had higher values than those with safety limits.

23. Peritumoral microvascular density is higher than intratumoral.

24. Regarding intratumoral vascularity, 70% of tumors were characterized by low vascular density and 30% by high vascular density.

25. Patients with low vascular density had older median age than those with high vascular density. In the chest and upper limbs, tumours were characterised exclusively by low vascular density.

26. Microvascular density was associated with the histopathological type of the tumour, with superficial, infundibulocystic, fibroepithelial and pigmentary subtypes showing exclusively low vascular density, whereas infiltrative, basal-squamous and morpheaform subtypes had predominantly high density. Likewise, relapses were characterized by increased

vascular density, whereas primary tumors had representatives in both classes. Tumors excised within oncological safety limits were characterized by a most frequently low vascular density.

27. VEGF was expressed in endothelial cells of blood vessels both in the tumor and peritumor, in keratinocytes of the basal layer of the epidermis, and in tumor cells, predominantly on the invasive tumor front.

28. 80% of tumours tested showed low VEGF expression and 20% showed high expression.

29. VEGF in tumor cells correlated with microvascular density.

30. VEGF was associated with the histopathological subtype of the tumour, the nodular type having exclusively low expression of this marker and the superficial and pigmentary types having high expression.

31. Of the 33 cases treated with radiotherapy, the dominant histopathological type was nodular. The vast majority was located in the head and neck and only a small percentage in the trunk.

32. 61% of cases presented remission, 39% of cases relapsed after radiotherapy.

33. Median time to relapse in cases where radiotherapy was not curative was 39.5 months.

34. 85% of relapsed cases were located in the head and neck, usually periocular, and the most common histopathological subtype was nodular and ulcerated.

35. The total RT dose is highly variable for BCCs located on the head and neck (from 10 to 48 Gy), but is always above 30 Gy for BCCs located on the trunk.

Selective bibliography

1. Christenson L.J. Incidence of Basal Cell and Squamous Cell Carcinomas in a Population Younger Than 40 Years. JAMA. 2005; 294:681. doi: 10.1001/jama.294.6.681.
2. Göppner D, Leverkus M. Basal cell carcinoma: from the molecular understanding of the pathogenesis to targeted therapy of progressive disease. J Skin Cancer. 2011; 2011:650258.
3. Parren LJ, Frank J. Hereditary tumour syndromes featuring basal cell carcinomas. Br J Dermatol 2011;165:30
4. Messina J, Epstein E H Jr, Kossard S. Basal cell carcinoma. In World Health Organization Classification of Skin Tumours; Elder D E, Massi D, Scolyer R A, Willemze R, Eds.; IARC Press: Lyon, France, 2018; pp. 26–34.
5. Vega Memije ME, Luna EM, de Almeida OP, Taylor AM, Cuevas González JC. Immunohistochemistry panel for differential diagnosis of Basal cell carcinoma and trichoblastoma. Int J Trichology. 2014 Apr; 6(2):40-4. doi: 10.4103/0974-7753.138583..
6. Stanoszek LM, Wang G Y, Harms P W. Histologic Mimics of Basal Cell Carcinoma. Arch. Pathol. Lab. Med. 2017, 141, 1490–1502.
7. Narayanan K, Hadid OH, Barnes EA. Mohs micrographic surgery versus surgical excision for periocular basal cell carcinoma. Cochrane Database Syst Rev. 2012;2: CD007041.
8. Mendenhall WM, Amdur RJ, Hinerman RW, Cognetta AB, Mendenhall NP. Radiotherapy for cutaneous squamous and basal cell carcinomas of the head and neck. Laryngoscope. 2009; 119:1994–9.
9. Skiveren J, Mikkelsen MR, Daugberg H, Wulf HC. Skin reactions and quality of life after X-ray therapy of basal cell carcinoma. J Skin Cancer. 2012:825095.
10. Varga E, Korom I, Raskó E, Kis E, Varga J, Oláh J, et al. Neglected basal cell carcinomas in the twenty first century. J Skin Cancer. 2011; 2011:392151.
11. Wollina U, Pabst F, Krönert C, Schorcht J, Haroske G, Klemm E, et al. High-risk basal cell carcinoma: an update. Expert Rev Dermatol. 2010;5:357–68
12. Kim DP, Kus KJB, Ruiz E. Basal Cell Carcinoma Review. Hematol Oncol Clin North Am. 2019 Feb; 33(1):13-24. doi: 10.1016/j.hoc.2018.09.004.

List of published scientific papers

Articles published in extenso in journals indexed in international databases with CNCSIS B(+) citation

1. **Naie A**, Dumitru A, Costache S, Tampa M, Matei C, Georgescu S..R, Anghel R, Costache M. Impact of histological subtypes and anatomic location of basal cell carcinomas on the outcome and the regimen of radiotherapy as a treatment modality. Medicine in evolution, Vol. XXV, No. 3, 2019, 233-238,. ISSN 2065-376X (see **Annex 1, pg. 155-162**).

2. Naie A., 2, Dumitru A. Costache S., Tampa M., Matei C., Georgescu S.-R., Anghel R., Costache M. Microscopic and macroscopic features of 91 basal cell carcinoma patients, observed throughout a 2 year-period. Medicine in evolution, Vol. XXVI, No. 1, 2020, 1-8, ISSN 2065-376X (see **Annex 2, pg. 163-171**).

3. Mariana Costache, Tiberiu Augustin Georgescu, Ana Maria Oproiu, Diana Costache, Alexandru Naie, Maria Sajin, Adriana Elena Nica. Emerging concepts and latest advances regarding the etiopathogenesis, morphology and immunophenotype of basal cell carcinoma. Rom J Morphol Embryol 2018, 59(2):427-433 (see **Annex 3, pg. 172-178**).