RĪGA STRADIŅŠ
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Pathogenesis of Basal Carcinoma and Features of Development of Local Recurrences in Head and Neck

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The Doctoral Thesis is available in RSU Library and on RSU website: https://www.rsu.lv/en/dissertations

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Dr. med., Professor **Ilona Hartmane**

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Abbreviations used in the Thesis

AJCC	American Joint Cancer Committee
α-SMA	Alpha-smooth muscle actin
5-ALA	5 - aminolevulinic acid
BM	Basement membrane
BCC	Basal cell carcinoma
CYP	Cytochrome enzymes
3'-DAB	3'-diaminobenzidine
7-DHC	7-dehydrocholesterol
DNS	Deoxyribonucleic acid
EM	Electron microscopy
IHC	Immunohistochemistry
FDA	The United States Food and Drug Administration
GLI	Zinc finger protein or glioma - associated oncogene
MAL	Methyl aminolevulinate
PDPN	Podoplanin
PTCH1	Protein patched homolog 1 gene
ROR α	Retinoic acid receptor-related orphan receptor α
ROR γ	Retinoic acid receptor-related orphan receptor γ
SCC	Cutaneous squamous cell carcinoma
SHh	Sonic Hedgehog protein
SMO	Smoothened gene
SPKC	Center of disease prevention and control
SUFU	Suppressor of fused homolog protein
TME	Tumour microenvironment
TNM	Classification of Malignant Tumours
UICC	Union for International Cancer Control

UVA	Ultraviolet A
UVB	Ultraviolet B
UVR	Ultraviolet radiation
VDP	Vitamin D binding protein
VDR	Vitamin D receptor
VDSP	International Vitamin D Standardization Program
WHO	World Health Organization

Introduction

Topicality of the Thesis

Oncology problems have always been the focus of doctors of all specialties. The incidence of malignancy continues to increase worldwide (Sung et al., 2021; Niculet et al., 2022). In recent years, more and more clinicians and morphologists, both in our country and abroad, are paying attention to basal cell carcinoma (BCC) of the skin (Niculet et al., 2022; Derjabo, 2011). Among human malignant tumours, BCC is the third most common after stomach and lung cancer, and its share among skin tumours ranges from 50 to 90 % (Montagna et al., 2017). In recent years, a significant increase in the incidence of BCC has been registered in many countries, moreover, the tumour is mostly diagnosed in European residents with skin phototypes I and II according to the Fitzpatrick scale, for which the lifetime risk of BCC increases up to ~ 30 % (Verkouteren et al., 2017; Bauer et al., 2020; Muzic et al., 2017). Skin cancer also ranks third among all registered malignant diseases in Latvia, and according to SPKC data, the incidence of BCC and skin squamous cell (SCC) cancer tends to increase every year (SPKC database 2010–2019, last updated 31.10.2023).

The histopathogenesis and degree of malignancy of BCC are still the subject of debate. The majority of researchers believe that BCC with locally destructive growth and low metastatic potential ranks between benign and malignant neoplasms. Nevertheless, cases of metastatic basal cell cancer have been described in the world literature with an incidence of 0.0028–0.5 % (Lau et al., 2018). It should be noted that in Latvia, the modified International histological classification is used, clinical guidelines for the diagnosis and treatment of BCC have been developed, but there is no uniform approach, which in turn makes it difficult for specialists to choose the correct and timely treatment method for the patient.

It should be noted that clarifying the reasons for some of the frequent recurrences of BCC is still an unsolved problem, because despite new treatment methods, a high number of BCC relapses and tumour resistance to the latest generation of chemotherapy are still observed worldwide. Therefore, the study of tumour pathogenesis and the factors causing recurrence is an actual research direction in medicine and oncology.

Considering the above, detailed clinical and morphological research on BCC is still relevant, with the complex application of clinical diagnostic and morphological analysis methods.

Aim of the Thesis

The aim is to clarify the clinical, dermatoscopic, and morphological characteristics of primary and recurrent cutaneous BCC in the head and neck region, to characterize patients' vitamin D status, and to investigate the role of vitamin D in the development of head and neck region BCC.

Tasks of the Thesis

1. To collect and analyse clinical data of recruited patients with primary and recurrent cutaneous BCC in the head and neck region, and to characterise the peculiarities of the clinical course.
2. To perform dermatoscopic examination of cutaneous BCC of the head and neck region using the Dermlite DL3N dermatoscope with PigmentBoost function, to analyse tumour dermatoscopic parameters, and assess their significance in determining high-risk BCC.

3. To examine and evaluate changes at the tissue and cellular level in various BCC subtypes using a comprehensive set of methods, including routine histopathology, immunohistochemistry, and electron microscopy.

4. To investigate the vitamin D status of patients with primary and recurrent BCC in the head and neck region, to determine its level in blood serum, as well as to assess the expression of the vitamin D-binding protein in tumour tissues, to establish a potential link with tumour development.

5. Analyse the clinical, molecular marker expression, and vitamin D level data of the Latvian cohort of BCC patients using the clustering analysis method.

Hypotheses of the Thesis

1. Dermatoscopic and morphological evaluation is necessary for effective treatment of BCC and to reduce the risk of tumour recurrence.

2. The SHh signalling pathway plays a role in the pathogenesis of BCC, by participating in the transformation processes of the tumour and its microenvironment.

3. The prevalence of aggressive forms of BCC is associated with low levels of vitamin D in the population.

Novelty of the Thesis

In this study, for the first time, primary and recurrent cutaneous BCC patient data from the head and neck region were compiled, including clinical, dermoscopic, and morphological data. Additionally, tumour recurrence was monitored in the postoperative period for 2 years. Furthermore,

immunohistochemical evaluation of key BCC morphological types was performed for 1) local aggressiveness, 2) invasiveness, and 3) markers significant for recurrence, including IV type collagen, laminin, alpha-smooth muscle actin (α-SMA), podoplanin (PDPN), and *Sonic Hedgehog* (SHh) protein.

Furthermore, the study included an analysis of the vitamin D levels in the blood serum of the enrolled patients, simultaneously assessing the expression of vitamin D binding protein (VDP) in tumour tissues for the first time. To determine the main subsets of the obtained results, a hierarchical clustering machine learning method was used for the first time.

Target population

From September 1, 2016, to September 1, 2019, seventy-nine patients diagnosed and treated for head and neck cancer at the Rīga Stradiņš University, Institute of Stomatology, Department of Oral, Maxillofacial Surgery, and the Latvian Oncology Centre were included in the study. A total of 46 women and 33 men were registered. The age range of the patients was 32–95 years. After surgery, patients were followed for 24 months for early detection and treatment of recurrence. Clinical data of the patients were obtained and studied, clinical and dermatoscopic analysis of the neoplasms was performed, and their anatomical localisation was determined.

Material and technical support

Material and technical materials were used in the research, using the opportunities provided by the AAI Interdepartmental Electron Microscopy Laboratory of Rīga Stradiņš University, the Doctoral Study of Rīga Stradiņš University and the Roche Latvia grant.

Personal contribution

The author of the doctoral thesis carried out: research planning, collection of literature and its analysis, collection and analysis of clinical and dermatoscopic data of patients, selection of immunohistochemical markers based on the analysis of literature on the research topic, control of patients in the postoperative period, microscopic analysis of the results of immunohistochemical reactions in tissue samples, tissue section analysis in an electron microscope, acquisition of images used in the thesis and their processing, developed images used in the doctoral thesis. Together with the supervisor *Dr. med.*, Assistant Professor Anna Ivanova performed the acquisition of tumour material, assisting the patient during surgical operations.

Ethical aspects

The permission of the Ethics Committee of Rīga Stradiņš University was received for performing the research on September 8, 2016 (see appendix).

1 Literature review

1.1 Epidemiology of basal cell carcinoma

BCC is the most common malignant skin tumour that develops from the cells of the basal layer of the epidermis, and its incidence is increasing every year worldwide (Sung et al., 2021; Rubin et al., 2005; Carruci et al., 2008). BCC accounts for up to 80 % of registered non-melanocytic tumours. In the European population, the tumour mainly affects individuals with skin phototypes I and II, who have a 30 % lifetime risk of developing BCC (Muzic et al., 2017; Abbas et al., 2016; Nolan et al., 2020). Up to 80 % of cases of BCC develop in the scalp and neck area (Ghafouri-Fard et al., 2010; Goh et al., 2010). According to international estimates, the mortality associated with BCC is quite low and amounts to 1 case per 100 thousand (Muzic et al., 2017; Abbas et al., 2016).

1.2 Basal cell carcinoma risk factors

There is no definite aetiology of BCC, but there are significant risk factors for the development of BCC: prolonged exposure to the sun and frequent sunburns, fair skin type, male gender, radiation, family history of skin cancers, certain groups of drugs (antibiotics of the tetracycline group, biological drugs, such as methotrexate) use and suppression of the immune system (McDaniel et al., 2021). Some hereditary syndromes (Gorlin-Goltz, Bazek, Rombo syndrome) lead to the formation of multiple BCCs.

1.3 Clinical and morphological presentation
of basal cell carcinoma

Clinically and morphologically, BCC can be manifested as superficial, nodular, infiltrative, pigmented and mixed, as well as subtypes of these forms (Koyuncuer, 2014). Most often, it manifests clinically as a slowly growing skin-

coloured nodule or erythematous patch with surface erosion or ulceration with or without pigment.

The microscopic structure of BCC is as diverse as the macroscopic structure. The epithelial origin of the tumour was determined by E. Krompecher (1903), identifying solid or nodular, adenoid, cystic, hyalinized, parakeratotic and myxomatous forms of BSK of the skin. On the other hand, V. Lever (1948, 1961) identified undifferentiated and differentiated forms of BCC of the skin.

Considering such large morphological variations of BCC and their importance as a prognostic factor, correct interpretation of the tumour type according to the International Histological Classification (The 2022 edition of ICD-10-CM C44.91) is very important. The current version 8 of the American Joint Committee on Cancer (AJCC)/Union International Against Cancer (UICC) International Classification of Tumours (TNM) should be used for BCC staging (Amin et al., 2017; Keohane et al., 2018; Brierley et al., 2019).

1.4   Pathogenesis of basal cell carcinoma

Both the influence of external factors and the characteristics of the patient's organism are important in the development of BCC. The SHh morphogen is an essential regulator of various cellular processes during embryogenesis and adult life. In tumourigenesis, aberrant SHh signalling pathway activation occurs either due to mutation of signalling pathway molecules (i.e., ligand-independent) or increased expression of SHh (ligand-dependent) (Evangelista et al., 2006; Karhadkar et al., 2004; Sanchez et al., 2004; Berman et al., 2003). SHh ligands bind to the transmembrane receptor PTCH1, which regulates the activity of the Hedgehog complex. The most common mutations are PTCH1 inactivating mutations or SMO activating mutations (Kim et al., 2019; Caro et al., 2010). In the case of BCC, mutations in

PTCH1 expression prevent the response to the cell cycle checkpoint cyclin B1 and promotes GLI activation (Hanna et al., 2016; Celebi et al., 2016)

1.5 Role of vitamin D in the pathogenesis of basal cell carcinoma

Today, vitamin D is recognized as a hormonally active lipid-soluble vitamin with many functions related to the complex regulation of physiological processes in the human body (Bijlsma et al., 2006; Lehmann, 2009). In the skin of humans and animals, the synthesis of vitamin D3 (cholecalciferol) takes place from 7-dehydrocholesterol (7-DHC) under the influence of ultraviolet B rays with a wavelength from 290 to 315 nm (Figure 1).
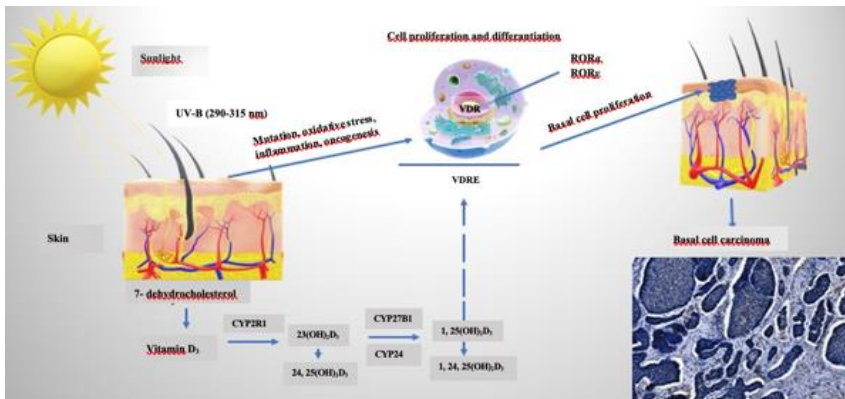
Figure 1.1 **Vitamin D metabolism and cutaneous synthesis**

Under exposure to UVB rays, the process of vitamin D3 synthesis proceeds in keratinocytes, which contain CYP enzymes necessary for the photochemical conversion of the precursor of vitamin D 3, 7-dehydrocholesterol, into its active form (calcitriol 1, 25 (OH) 2D3). The activities of hydroxyderivatives of vitamin D are mediated by the involvement of the ligand-binding domain of the nuclear receptor, VDR. Furthermore, vitamin D3 is implicated in the regulation of biological functions and gene expression of keratinocytes of both healthy and BCC-affected subjects, mediated by the presence of ROR α and γ nuclear receptors. Abbreviations: UVB, ultraviolet B; VDR, vitamin D receptor; RORα and γ, retinoic acid-related orphan receptors α and γ; BCC, basal cell carcinoma.

Accumulating data suggest that the ability of vitamin D to suppress the activation of the SHh signalling pathway explains its protective action in BCC (Asakura et al., 2020; Mahamat-Saleh et al., 2020). One of the described mechanisms of action of vitamin D3 is 1,25(OH)2D-induced VDR transcriptional activation with resulting enhancement of keratinocyte differentiation and reduction of cell proliferation (Moisejenko-Golubovica et al., 2021; Mahamat-Saleh et al., 2020).

1.6 The role of instrumental methods in the basal cell carcinoma investigation

Dermatoscopy (epiluminescent microscopy) is an in vivo method that enables differential diagnosis of skin lesions and early detection of skin tumours – BCC, squamous cell carcinoma and melanoma, as well as differentiating them from other skin diseases (Lallas et al., 2015). The examination is performed based on multi-step algorithms. Dermatoscopic diagnostic criteria for BCC include the presence of arborizing vessels, short fine telangiectasias, maple leaf-like structures, large blue-gray ovoid nests, white streaks, ulceration, multiple small erosions, shiny white areas, focal dots, milky pink to reddish areas, wheel spoke areas, and several blue-gray dots (Popadić, 2014; Puig et al., 2012; Lallas et al., 2013; Wozniak-Rito et al., 2018).

1.7 Treatment of basal cell carcinoma

Today, it is possible to treat BCC with 5-fluorouracil using imiquimod, CO2 laser, curettage, cryotherapy, photodynamic therapy, radiation therapy and surgical techniques including micrographic surgery (Goldenberg et al., 2013; Clark et al., 2014; Villani et al., 2022). In Latvia, surgical treatment is used as a routine method for the radical treatment of skin tumours.

2   Materials and methods

2.1   Patients' characteristics and BCC classification

Seventy-nine patients clinically presented with the suspected BCC of the head and neck treated prospectively in Rīga Stradiņš University, Institute of Stomatology, Department of Maxillofacial Surgery, and the Oncology Centre of Latvia between September 2016 and September 2019 were used in this study. The age range of subjects was 37 – 90 years. Among all BCC patients, 46 were women and 33 were men. Clinical data included information on patients' characteristics, clinical outcomes, complications of BCC, and findings of dermoscopic imaging. Characteristics of a neoplasm included information on the duration and type of the BCC lesion at the time of presentation, anatomical localisation, and the size of the tumour. Patients with vitamin D deficiency were prescribed vitamin D therapy, depending on the severity of its deficiency. The disease relapse was monitored over a 2-year follow-up period.

The study was approved by the Ethical Committee of Rīga Stradiņš University (Decision No. 11/08.09.2016.), and written informed consent was obtained from all patients included in the research. The tumour tissue samples were obtained following the tenets of the Declaration of Helsinki. In all cases, BCC was confirmed, and different types of a tumour were distinguished according to the World Health Organization (WHO) international classification.

2.2   Assays used for the detection of serum vitamin D levels

Blood samples for the assessment of vitamin D levels were prospectively collected from all BCC patients and further transferred to the certified E. Gulbis Laboratory ltd (LATAK accreditation ISO 15189). A conventional chemiluminescence immunoassay using the Cobas 8000 analyser (Roche,

Switzerland) was performed to measure a total vitamin D serum level (Garnett et al., 2019; Vandikas et al., 2022; Enko et al., 2014).

2.3 Dermatoscopic examination used to diagnose BCC and its assessment criteria

The dermatoscopic examination was performed with a handheld dermatoscope (3Gen DermLite DL3N with Pigmentboost; Olympus, USA) using a 30 mm ×10 lens before a tumour mass excision. Both contact and non-contact techniques and a polarized mode were used to visualize BCC lesions. A digital photography of the dermatoscopic presentation of the BCC.

A semi-quantitative assessment of dermatoscopic signs was used for data processing (Argenziano, 2007; Zalaudek, 2010; De Vita et al., 2012; Okuboyejo et al., 2018). Dermatoscopy changes are qualified as follows: weak ≤ 25 %, moderate $26-70$ % and strong visualisation > 70 %.

2.4 Histopathological methods

In this study, the surgically excised BCC masses were further processed as the formalin-fixed, paraffin-embedded, and conventionally sectioned tissue samples. The sections were mounted on adhesive SuperFrost Plus glasses (Gerhard Menzel GmbH, Germany) to better save the tumour tissues exposed to immunohistochemistry. Initially, the sections were routinely stained with haematoxylin and eosin to confirm the diagnosis and detect the type of BCC. The histopathology of the tumour was assessed by two independent observers.

2.5   Immunohistochemical methods

Immunohistochemical reactions were performed using deparaffinized and conventionally prepared paraffin sections. Tissue antigens were detected using a panel of primary antibodies: a monoclonal mouse antibody human α-smooth muscle actin (Abcam, Cambridge, MA, USA, clone 1A4, 1:200), which detect cells expressing the smooth myocyte phenotype (Wu et al ., 2018); monoclonal mouse antibody human podoplanin (Abcam, Cambridge, MA, USA, clone PDPN/1433, 1:200), which is expressed in reactive mesothelial cells and lymphatic endothelial cells and regulates cell migration and tumour invasion in vivo and in vitro (Kimura et al ., 2005; Wicki et al., 2006); a monoclonal mouse antibody human collagen type IV (Dako Denmark A/S, Glostrup, Denmark, clone CIV 22, 1:25), which labels the lamina densa of the basal membrane (Lammers et al., 2011); monoclonal mouse antibody human laminin (Dako Denmark A/S, Glostrup, Denmark, clone 4C7, 1:20), which reacts with epidermal basal membrane laminin family glycoproteins (Wondimu et al., 2013); polyclonal rabbit antibody human VDP (Bioss Antibodies, MA, USA, 1:300), which detects endogenous VDP proteins (Simpson et al., 2015); and a monoclonal rabbit antibody human Sonic Hedgehog (Abcam, Cambridge, MA, USA, clone EP1190Y, 1:200), which recognizes the full-length molecule and the c-product subunit of the human SHh protein (McCann et al., 2011; Kaminagakura et al., 2013). Incubation with primary antibodies was performed by keeping the sections at 4 ◦C for 12 hours and following the manufacturer's recommendations. Amplification of primary antibodies and visualisation of reaction products were performed using the HiDef Detection HRP Polymer system (CellMarque, Rocklin, CA, USA). The substitution of the primary antibody with tris(hydroxymethyl)aminomethane (TRIS) solution was used in negative IHC controls.

The stained slides were scanned with a Glissando Slide Scanner (Objective Imaging Ltd., Cambridge, UK). Bright-field images were generated and analysed by a Leica light microscope (LEICA, LEITZ DMRB, Wetzlar, Germany) using a DFC 450C digital camera at a resolution of 0.5 µm/pixel, using a 20× objective, with a resolution of 0.275 µm/pixel using a 40× objective.

Evaluation of immunostaining was performed semi-quantitatively in 20 randomly selected fields of view for each of the samples (magnification 400×) representing the tumour and stromal features of the region of interest.

2.6   Transmission electron microscopy method

Transmission electron microscopy (TEM) was used to better investigate the ultrastructural features of tumour cells forming an invasive cone in recurrent BCC.

To specify the region of the tumour and its stroma analysis and follow the BCC Moh's surgery findings (Tehrani H. et al., 2013), 1–2 µm thick sections were made from the epoxy-embedded tissue using an ultramicrotome (LKB, Y2088, Sweden) or semi-thin cuts. After their evaluation and selection under a light microscope, 60–80 nm thick or ultrathin sections were made from the epoxy-embedded tissue an ultramicrotome (LKB, Y2088, Sweden).

BCC tissue analysis was performed in a transmission electron microscope (JEM 1011, JEOL, Japan), changing the magnification from ×1000 to ×10000, and if necessary, the magnification to ×50000. Sections of the valid tumour and its stromal areas for the purposes of the doctoral thesis were only analysed and photographed with Kodak brand photo films (SO-163, Kodak, Rochester, N.Y., USA). After the photographic films were developed, they were scanned using a scanner (Epson Perfection V700 Photo, Seiko Epson Corp., Japan) to obtain electrograms.

2.7   Statistical data analysis

Statistical data analysis was performed to evaluate dermatoscopy and immunohistochemistry results using SPSS versions 24.0, 26.0, Prism 9 software for macOS (GraphPad Software, LLC, USA) and JMP Pro 16 (SAS, USA).

The Kolmogorov-Smirnov normality test was used to check that the collected numerical data were normally distributed. Quantitative data were expressed as mean ± standard deviation, while categorical parameters were expressed as frequency and percentage. Spearman's and Pearson's rank correlation coefficients were used to evaluate the relationships between the immunoexpression results of the antigens analysed in this study, blood serum vitamin D levels, and tumour size, as well as the relationship between gender and tumour type. A two-sample t-test was performed to examine the relation between sex, and different prognostic groups (primary and recurrent, low risk and high risk) of BCC and serum D vitamin level.

Multivariate analysis and a hierarchical clustering method were used to determine the main subsets of the results obtained. Correlation between antigen expression and histopathological type of BCC was investigated by Chi-square test. Fisher's test was used to assess the relationship between dermatoscopic findings and tumour subtype. Kramer's V test was used to assess the association of dermatoscopic structures with BCC types. For pairwise group comparisons, the Wilcoxon signed-rank test with Bonferroni correction was used. For all statistical tests used, a significance level of $p < 0.05$ was chosen. Graphs were generated using Prism 9 macOS (LLC, San Diego, USA) and JMP Pro 16 (SAS, Cary, USA) software.

3 Results

3.1 Results of the clinical data of BCC patients

Collectively, a cohort of seventy-nine patients diagnosed with BCC was used in this study. Among all patients who presented with BCC, 58 % were women and 42 % were men. The mean age of women was 70 years (SD ± 15) and men – 64 years (SD ± 17). Clinical skin phototypes were assessed using the I–III Fitzpatrick classification scale. Of the 79 studied patients, only two patients (2.5 %) were found to have type I, 59 (74.7 %) – type II and 18 (22.8 %) – type III. One male and one female showed the presence of phototype I.

Clinically, in our practice, BCC was most often manifested as a slowly growing skin-coloured nodule or an erythematous plaque with erosion or ulceration on the surface (Figure 3.1 (A), (B)). Primary BCCs were mostly small, but fast-growing aggressive tumours with a large area of destruction were periodically diagnosed (Figure 3.1 (C)). Within the scope of the study, recurrent BCCs were diagnosed as an epidermal ulceration with a thick crust on the surface (Figure 3.1 (D)).
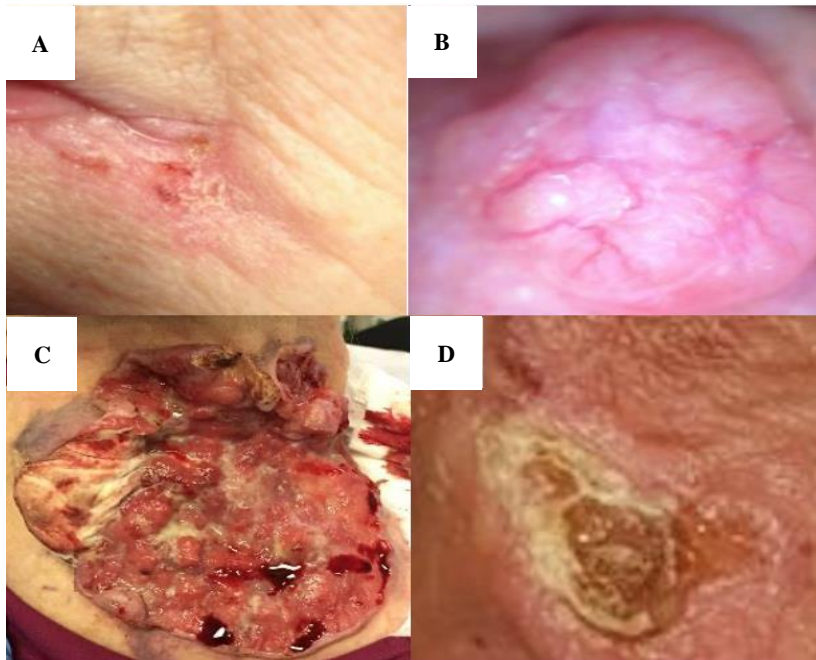
Figure 3.1 **Clinical presentation of basal cell carcinoma**

(A) Erythematous plaque with erosions on the surface in the lower lip region;
(B) A skin-coloured nodule in the nasolabial folds with a vascular pattern on the surface;
(C) Primary aggressive BCC of the neck-shoulder region with a wide ulceration area;
(D) Recurrent BCC of the cheek region with ulceration in the epidermis and a thick crust on the surface.

3.2 Analysis of frequency and anatomical localisation of BCC histopathological types

Out of 79 patients, 15 (19 %) were diagnosed with nodular tumour histopathological subtype, 18 (23 %) with superficial, 10 (12 %) with infiltrative, 7 (9 %) with micronodular subtype and 29 (37 %) with mixed subtype. The most common combinations of mixed BCC included nodular-infiltrative, superficial-nodular, and nodular-micronodular subtypes. No statistical differences in gender distribution were found between histological subtypes (p = 0.102).

BCCs are most often localized in well-blooded areas of the head and neck region, and the most dangerous areas for certain aggressive subtypes of BCCs, such as the nose and nasolabial folds, *a. facialis* in the blood supply zone, the central area of the cheeks in the *a. transversa* and *a. infraorbitalis* blood supply zone, where the tumour was provided with a good blood supply with the supply of the necessary substances for its better development (Figure 3.2) were identified as part of the study.
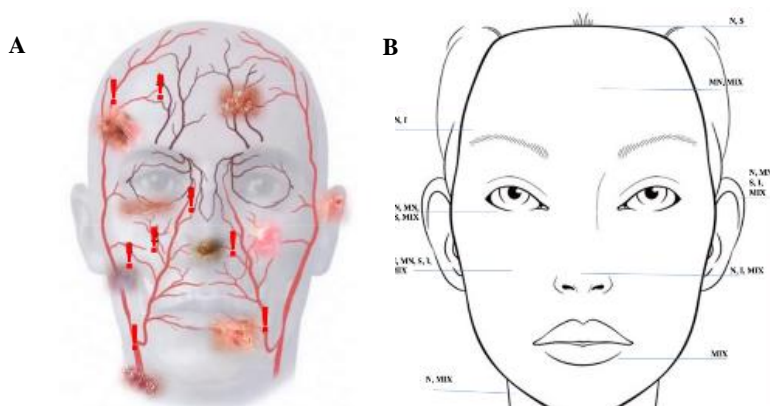

Figure 3.2 **Distribution of basal cell carcinomas in head and neck region**

(**A**) Head and neck blood supply scheme with more frequent BCC localisations shows that the tumour develops more often in the blood supply area of large arteries and their branches. (**B**) The most frequent localisations of BCC in the head and neck region by histological tumour subtype. **Abbreviations:** N – nodular, MN – micronodular, S – superficial, I – infiltrative, MIX – mixed subtype.

Analysis of tumour anatomical location confirmed that the nose and cheek were the predominant regions where both primary and recurrent tumours developed, accounting for 36.7 and 29.1 %, respectively. Nodular (in primary tumours) and mixed BCC (in primary and recurrent tumours) were more frequently located in the skin of the nose compared to other regions of the head and neck, accounting for 42.9 %, 31.6 %, and 70 %, respectively. The anatomical location of the micronodular BCC subtype was nose, 2 (29 %); cheek, 2 (29 %);

eyelid, 3 (17 %); scalp, 1 (5.5 %) and ear, 1 (5.5 %). Recurrent BCCs developed only in the skin of the nose, while localisation of primary tumours was observed in the following anatomical areas: cheek 3 (38 %), nose 2 (25 %), temples 2 (25 %) and ear 1 (12 %). In the second place of recurrent tumours in terms of localisation were five BCCs on the cheek, three on the eyelids and one on the ear. In our study, the third most frequently damaged region was the eyelid (10.1 %).

Mixed-type BCC was anatomically located in the areas presented in Figure 3.3: one (3.45 %) on the temples, two (6.90 %) on the forehead, 6 (20.69 %) on the cheeks, 13 (44.83 %) on the nose and nasolabial folds, one (3.45 %) on the ear, one (3.45 %) on the neck, three (10.34 %) on the eyelids, and two (6.90 %) on the lips.
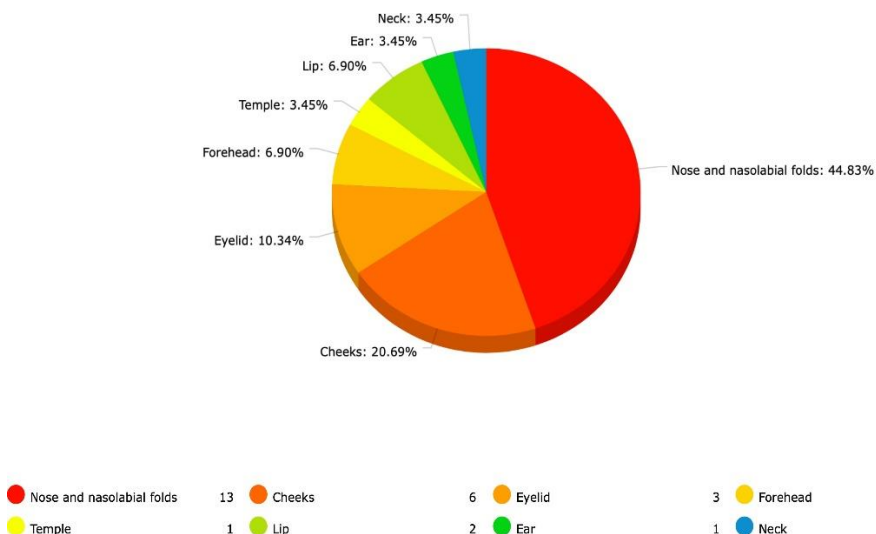

Figure 3.3 **Incidence and localisation of mixed type basal cell carcinoma**

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Histopathologically, aggressive mixed-type BCC was localized on the nose and nasolabial folds as a nodular infiltrative or solid adenoid tumour in most cases. The sizes of the mixed tumours were very different: from 0.2 mm to 2.5 cm, but sometimes up to 4 cm.

3.3   Dermatoscopic examination of basal cell carcinoma

Dermatoscopically, the common criteria characteristic of BCC included pink or milky-pink and milky-red colour structureless areas, erosions, ulcerations, short thin telangiectasias, arborizing vessels, blue-gray globules, maple leaf-like structures, white streaks, and translucency as suggested in previous studies (Popadić, 2014; Puig et al., 2012; Lallas et al., 2013; Wozniak-Rito et al., 2018).

Using Fisher's test, it was determined that statistically significant differences existed between certain dermatoscopically diagnosed structures, such as arborized blood vessels, short fine telangiectasias, blue-gray ovoid structures, focal points, ulceration, erosions, white streaks.

The specific dermatoscopic diagnostic criteria for the superficial type of BCC included the presence of short thin telangiectasias and comma vessels, small ulcerations, pink homogeneous areas, and blue-gray ovoid nests (Figure 3.4 (A)). Nodular and micronodular types were presented with the border raised above the central part of the lesion, arborizing vessels, ulceration, blue-gray ovoid nests, and translucency (Figure 3.4 (B), (C)). Infiltrative BCC was commonly presented with arborizing vessels, short thin telangiectasia, shiny white structureless areas, ulceration, and white streaks (Figure 3.4 (D)). Finally, the diagnosis of mixed BCCs was based on histopathological observations and the presence of certain dermatoscopic characteristics that included such standard features of BCC as arborizing vessels, ulcerations, milky-red colour structureless

areas, and less commonly found signs like masses of keratin, superficial scaling, and white streaks (Figure 3.4 (D)).
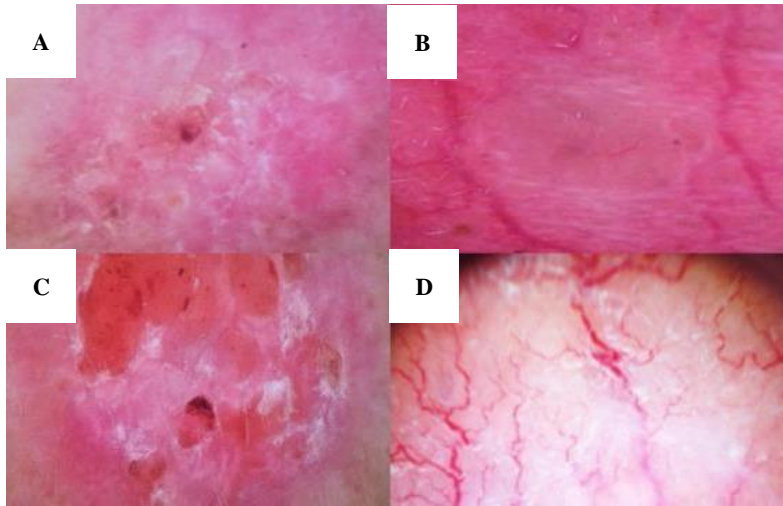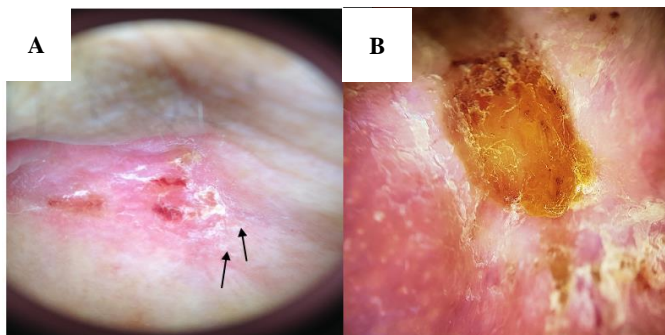

Figure 3.4 **Dermatoscopic findings in different types of BCC**

(A) Milky-pink colour structureless areas, pink homogeneous areas, short thin telangiectasia, and erosion on the central part of the tumour in the case of superficial BCC. **(B)** A translucent nodule raised above the skin and arborizing vessels in nodular BCC. **(C)** The nodular appearance of a tumour with a border raised above the central part of the lesion, arborizing and short thin telangiectasias, milky-pink and milky-red colour structureless areas, ulceration, and erosion in the case of micronodular BCC. **(D)** Arborizing vessels, short thin telangiectasia on shiny white and milky-red structureless areas in the infiltrative and mixed type of BCC.

The diagnosis of mixed BCC was based on histopathological observations and the presence of certain dermatoscopic features, which included the standard features of BCC such as vascularisation, ulceration, milky-red, structureless areas, and less common features such as keratin masses, superficial scaling, and white streaks or bundles. Infiltrative and mixed-type BCC belong to aggressive tumour forms, so it was especially important to recognize them before choosing a treatment method.

The most common vascular pattern in mixed BCC was arborized vessels and short, fine telangiectasias, which were mainly found in small-sized BCC. Shiny white areas (twenty patients, 60.6 %), white streaks (twenty-two patients, 66.7 %), milky pink to red background (twenty-seven patients, 81.8 %), ulcers (thirteen patients, 39.4 %) and several small erosions (eleven patients, 33.3 %) were also frequently detected dermatoscopically. Pigmented structures, on the other hand, were distinguished as blue-gray globules and dots (five patients, 15.1 %), blue-gray ovoid nests (four patients, 12.1 %) and focal points (two patients, 6 %). Pigment-related features of BCC usually presented as maple leaf-like areas (two patients, 6.1 %) and spoke-like structures (one patient, 3.0 %). Statistically significant differences in the case of mixed type of BCC were determined between the frequency of vascularisation, short fine telangiectasias, white streaks and milky pink to red areas ($p < 0.001$), while the dermatoscopic picture of mixed type of BCC is shown in Figure 3.5 (A), (B).


Figure 3.5 **Dermatoscopic findings in mixed type basal cell carcinoma**

(A) Homogeneous shiny structureless areas with a milky pink background; superficial short fine telangiectasia and some fine arborizing vessels. Tumour surface with several small erosions and dermatoscopically indistinct borders in the case of primary mixed-type BCC, which was histopathologically recognized as a combination of superficial and infiltrative type. Filamentous and thread-like thin bands extending beyond the visual borders of the tumour, demonstrating high risk of recurrence and aggressiveness of BCC (black arrows). **(B)** A homogeneous white to pink background and ulceration in the centre of the tumour characterizes recurrent mixed BCC.

3.4 Assessment of Serum Vitamin D levels

The assessment of serum vitamin D levels revealed that women presented with a range of 5.9–40.0 ng/mL, whereas men – with a range of 9–32 ng/mL, respectively (Figure 3.6 (A)). In women, the mean value of vitamin D level was 17.9 ng/mL (SD ± 6.7 ng/ml), whereas, in men, the mean value was 17.7 ng/mL for men (SD ± 7.0 ng/mL), however, these differences were not statistically significant. Only four patients in the study cohort had enough serum vitamin D levels estimated above 30.0 ng/mL. In patients presenting with primary BCCs, statistically significant differences in serum vitamin D levels when assessed in men and women were not found (Figure 3.6 (B)). Simultaneously, in two women diagnosed with recurrent BCC tumours, the mean value of vitamin D level was 13.5 ng/mL (SD ± 5.0 ng / mL), whereas, in 16 men, the mean value was 17.0 ng/mL for men (SD ± 6.7 ng/mL), however, these differences were not statistically significant. Serum vitamin D levels were significantly lower in women presented with mixed BCC tumours when compared to men ($p < 0.05$). Simultaneously, serum vitamin D levels did not differ when patients of both genders presented with different types of BCC were compared (Figure 3.6 (C)).
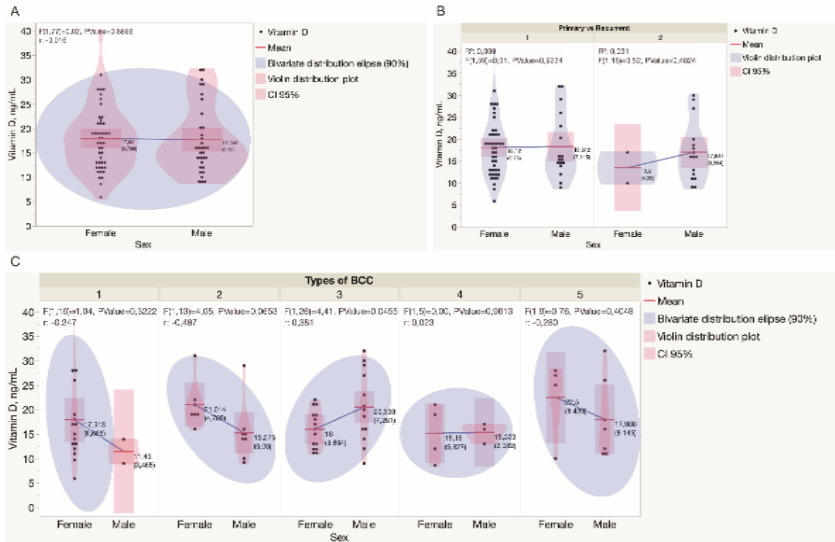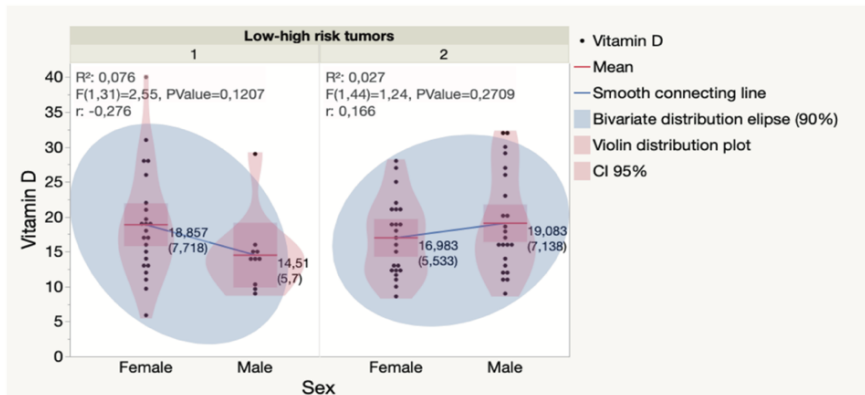
Figure 3.6 **Assessment of serum vitamin D levels in the study cohort**

(A) Assessment of serum vitamin D levels in males and females recruited in the study. Each dot represents a single data point. (B) Assessment of serum vitamin D levels in males and females presented with either primary or recurrent BCC tumours. Each dot represents a single data point. (C) Assessment of serum vitamin D levels in males and females presented with different types of BCC tumours. Each dot represents a single data point. Statistically significant differences in serum vitamin D levels were not observed when different types of BCC diagnosed in both genders were compared, except in mixed BCC tumours, diagnosed in females presented with significantly lower serum vitamin D levels when compared to males (p = 0.0456). **Abbreviations:** (B) 1 – primary BCC tumours; 2 – recurrent BCC tumours; (C) 1 – superficial BCC tumours; 2 – nodular BCC tumours; 3 – mixed BCC tumours; 4 – micronodular BCC tumours; 5 – infiltrative BCC tumours.

For a better understanding of the spectrum of serum vitamin D levels observed in BCCs, the indices were analysed separately for primary and recurrent, as well as for low risk (superficial and nodular) and high risk (micronodular, infiltrative, and mixed) tumours. Statistically significant differences in serum vitamin D levels were not observed when low risk and high risk BCCs diagnosed in both genders were compared (Figure 3.7). Similarly, there was not a statistically significant difference found when the results of the

assessment of serum vitamin D levels were compared for patients presented with either different types or primary and recurrent BCC tumours (Figure 3.8).

Figure 3.7. **Assessment of serum vitamin D levels in males and females presented with low and high risk BCCs. Each dot represents a single data point**

Abbreviations: 1 – low risk BCC tumours; 2 – high risk BCC tumours.
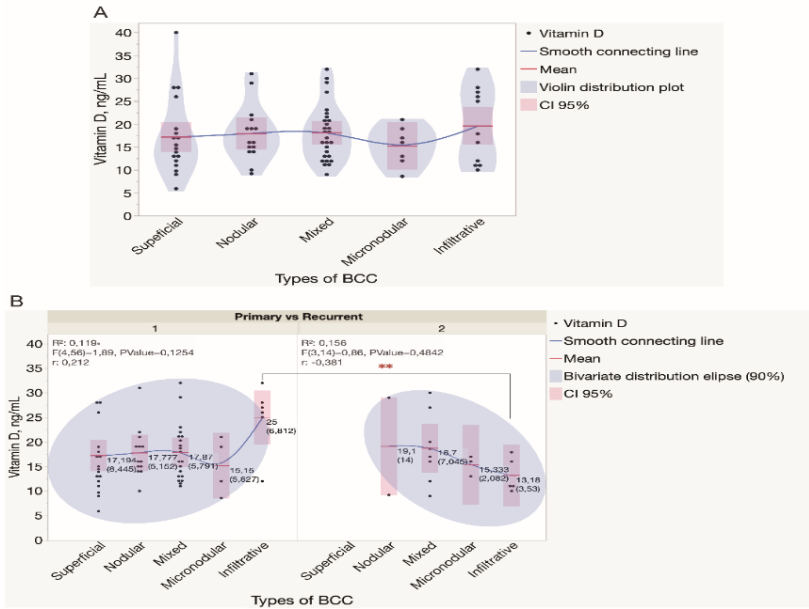
Figure 3.8. **Assessment of serum vitamin D levels
in patients presented with different types of BCC tumours**

(A) Violin distribution plots depict the results of the assessment of serum
vitamin D levels in patients presented with different types of BCC tumours.
Each dot represents a single data point. (B) Bivariate distribution ellipses depict the
results of the assessment of serum vitamin D levels in patients presented with either
primary or recurrent BCC tumours. Each dot represents a single data point.
Abbreviations: (B) 1 – primary BCC tumours; 2 – recurrent BCC tumours.
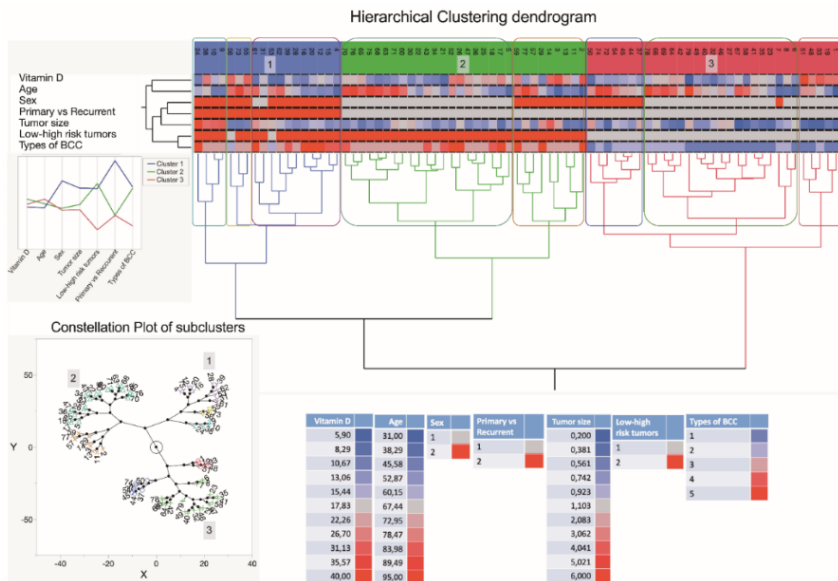
Figure 3.9. **Dendrogram of hierarchical clustering –
relationships between sets of data**

The dendrogram consists of stacked branches (clades) that break down into further
smaller branches. At the lowest level, individual elements appear and then they are
grouped according to attributes into clusters with fewer and fewer clusters on higher
levels. The end of each clade (a leaf) is the data. The sets of data included assessments
of serum vitamin D level patterns of 79 subjects presenting with the different types
of BCC, information about a patient age and sex, a tumour type (primary vs. recurrent),
and a type of BCC, and seen in the right lower part of the Figure. The tumour specimens
were divided into five subtypes based on differences in histopathology and dermoscopic
imaging. The three major data clusters with 2–3 subclusters each are highlighted in the
constellation plot of the dendrogram depicted in the left lower part of the Figure.
Abbreviations: sex: 1 – females; 2 – males; primary vs. recurrent BCC: 1 – primary
BCC; 2 – recurrent BCC; low and high-risk BCC: 1 – low risk BCC; 2 – high risk BCC;
types of BCC: 1 – superficial BCC tumours; 2 – nodular BCC tumours; 3 – mixed BCC
tumours; 4 – micronodular BCC tumours; 5 – infiltrative BCC tumours.

As depicted in the dendrogram presented in Figure 3.9, the data were
separated into three main branches using the hierarchical clustering method. The
blue branch was typically represented by males with low serum vitamin D levels
and large, mostly high risk and recurrent BCC tumours. In turn, the green branch

was represented by both males and females with greatly varying serum vitamin D levels and small-sized primary, however, high risk BCC tumours. Finally, the red branch was represented by males and females with varying serum vitamin D levels and small-sized primary and less aggressive BCC tumours. Furthermore, in general, a negative association between the tumour size and serum vitamin D level was demonstrated by the use of correlation analysis (Figure 3.10).
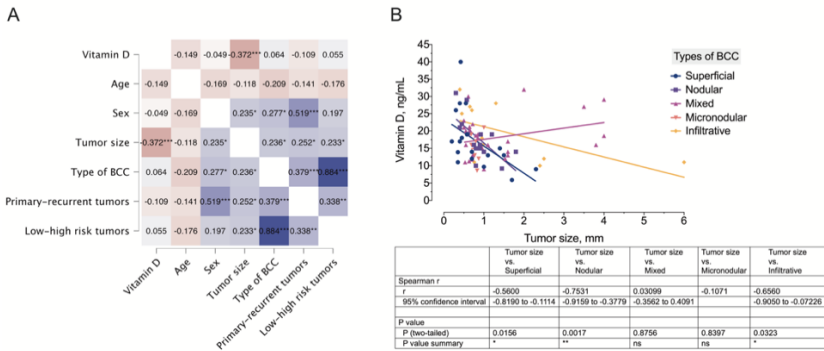

Figure 3.10 **Correlogram of studied variables**

In this plot, correlation coefficients are coloured according to the value. Positive correlations are displayed in blue, whereas negative correlations are in red. Colour intensity is proportional to the correlation coefficients. **(A)** The negative association ($r = -0.372$) between the tumour size and serum vitamin D level is marked by a dark red colour. **(B)** Correlation between the tumour size and serum vitamin D level was observed in different types of BCC.

3.5 Histopathology and immunohistochemistry of the basal cell carcinoma tissue

Type IV collagen and Histopathologically, BCC cells formed nests, cords, and islands. The tumour cells displayed little pleomorphism, and mitotic figures were infrequent. The presence of peripheral palisading, artefactual clefting, and myxoid stroma was characteristic of the BCC tumour.

In superficial BCC, a superficial proliferation of neoplastic cells was demonstrated. A nodular type presented with the nodules displaying the typical nuclear palisading at the periphery of the tumour, whereas a micronodular type demonstrated a cauliflower appearance. In infiltrative BCC, small aggregations or cords of basaloid cells were found penetrating the stroma, interspersing muscular fibres, and revealing perineural invasion. The infiltrative growth often showed heavy stromal collagenisation. Mixed growth BCC commonly revealed an admixture of rounded and irregularly contoured tumour cell nests and cords embedded in a fibrous stroma. In addition, the presence of mitotic figures and apoptotic cellular debris was characteristic of the aggressive growth.

3.5.1 Collagen type IV and laminin expression immunohistochemical analysis

Initially, analysis of the immunohistochemical results was consistent with the detection of expression levels of major molecules of the BM – collagen type IV and laminin establishing a barrier restricting the dissemination of tumour cells. Contours of tumour masses labelled by the anti-laminin (Figure 3.11) and anti-collagen (Figure 3.12) antibody for the presence of these molecules displayed a linear but greatly varying staining pattern – both continuous and discontinuous. A discontinuous or absent pattern of immunostaining was often revealed in the infiltrative BCCs. Levels of collagen type IV and laminin expression were similarly distributed varying from low to moderate and high – 60.3, 35.6, 4.1 %, and 71.1, 25.2, 3.6 % for collagen and laminin, respectively.
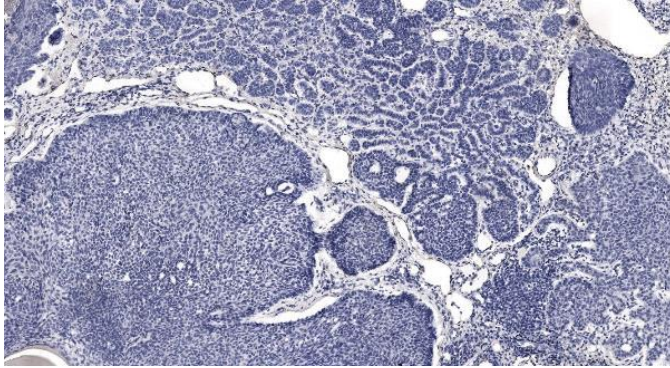
Figure 3.11 **Weak continuous laminin expression appearing along the basal aspect of the surface and follicular epithelium, the expression within the BM delineating differently sized and shaped nodules and micronodules of the mixed recurrent tumour is almost nil**

Laminin immunohistochemistry, ×200.
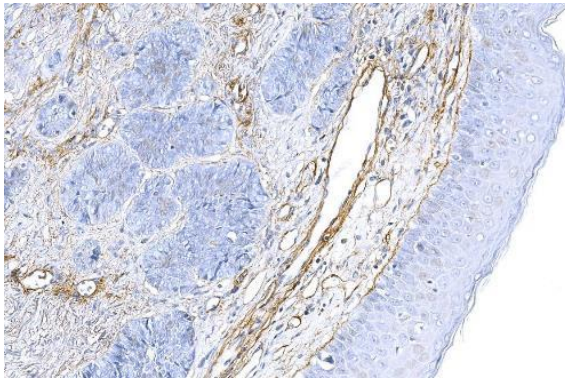
Figure 3.12 **The linear and both continuous and discontinuous immunostaining decorating the base of the tumour nests and vascular beds of the mixed recurrent tumour**

Type IV collagen immunohistochemistry, ×250.

Immunoexpression of type IV collagen in mixed-type BCC was highly variable, demonstrating continuous, discontinuous, and even completely absent expression (Figure 3.13 (A)). It was determined that for

mixed BCC 49.47 % showed absence of type IV collagen expression, 40 % showed discontinuous immunostaining and only 10.53 % showed continuous BM immunostaining. It should be noted that in infiltrative BCC variants collagen expression was absent in up to 96 % of tumours. Collagen-type immunostaining was identified as a linear pattern along the basal aspect of the tumour nest (Figure 3.13 (B)).

Neck: 3.45%
Ear: 3.45%
Lip: 6.90%
Temple: 3.45%
Forehead: 6.90%
Eyelid: 10.34%
Nose and nasolabial folds: 44.83%
Cheeks: 20.69%

🔴 Nose and nasolabial folds 13	🟠 Cheeks 6	🟠 Eyelid 3	🟡 Forehead 1
🟡 Temple 1	🟢 Lip 2	🟢 Ear 1	🔵 Neck 1
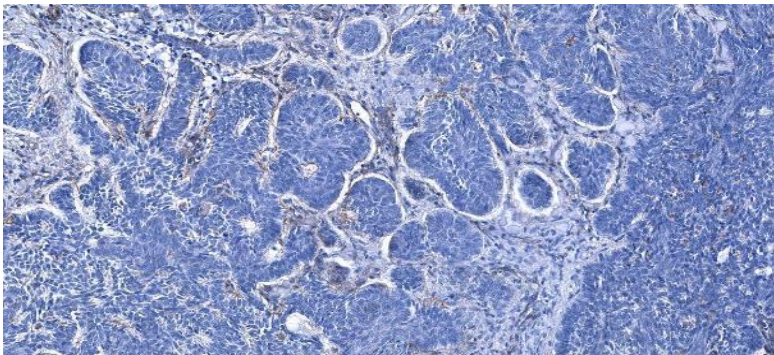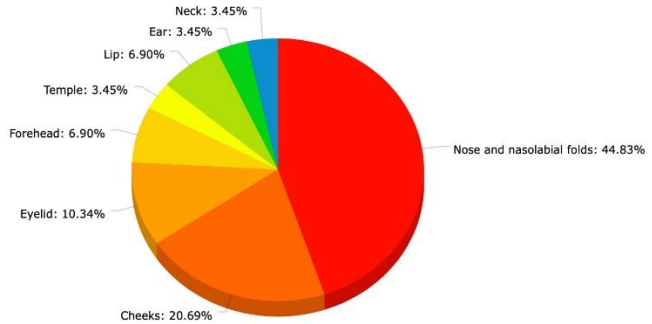
Figure 3.13 **Type IV collagen expression in mixed-type basal cell carcinoma**

Panel (**A**) shows the diversity of collagen expression and the statistical evaluation of its immunohistochemical detection results. Panel (**B**) linear-type immunoreaction reflecting the localisation of type IV collagen in the BM *lamina densa* with continuous but, when surrounding small nodules, with discontinuous or even absent labelling. Immunohistochemistry of type IV collagen. ×200.

3.5.2 Immunohistochemical analysis of *α*-SMA, PDPN and SHh expression

The results of the immunohistochemical expression of α-SMA and SHh estimated for tumoural and stromal compartments of primary and recurrent tumours in different histopathological subtypes of BCCs.

Levels of the tumoural α-SMA expression were distributed varying from weak to moderate and strong. Simultaneously, we confirmed a decrease in weak stromal α-SMA expression levels in recurrent BCC when compared to primary BCC, followed by two times higher strong stromal α-SMA expression levels demonstrated in recurrent BCC. Comparing the levels of α-SMA expression in primary and recurrent BCCs studied, we found statistically significant differences for both tumoural and stromal compartments ($\chi^2 = 16,191$; df = 2; $p < 0.0001$; and $\chi^2 = 26,510$; df = 2; $p < 0.0001$), respectively.

When compared to α-SMA, levels of the tumoural Shh expression were almost equally distributed and varied from weak to moderate and strong for primary and recurrent BCC. Similar to the α-SMA assessment, we found an increase in strong stromal Shh expression levels in recurrent BCC. Comparing the levels of SHh expression in primary and recurrent BCCs studied, we found statistically significant differences for stromal but not tumoural compartment ($\chi^2 = 7.121$; df = 2; $p = 0.028$; and $\chi^2 = 0.915$; df = 2; $p = 0.633$), respectively.

The levels of SHh assessed for both BCC compartments differed when compared to α-SMA levels. Moderate and strong levels of the tumoural SHh expression characterized infiltrative, superficial, the mixed, and micronodular subtypes. Simultaneously, stromal SHh levels were three times lower but two times higher for infiltrative and superficial subtype, respectively, when compared to α-SMA levels. Comparing SHh expression in subtypes of BCC studied, we found that tumoural expression revealed in the nodular subtype significantly differed when compared to all other subtypes except micronodular – infiltrative

(p < 0.0001), superficial (p < 0.0001), and the mixed (p < 0.0001). Stromal expression revealed in the nodular subtype significantly differed when compared to all other subtypes – infiltrative (p < 0.0001), micronodular (p = 0.009), superficial (p < 0.0001), and the mixed (p < 0.0001).

We found that one-fourth of neoplastic cells within the samples demonstrating infiltrative BCC subtype revealed a strong level of α-SMA expression followed by one-fifth in the nodular, and the mixed subtype. Simultaneously, almost one-fifth of the stroma of infiltrative subtype revealed a strong of α-SMA expression followed by one-tenth in the mixed, and around one-tenth in micronodular subtype. Tumour masses and stroma of the superficial subtype revealed a negligible amount of neoplastic epithelial and stromal cells displaying a strong level of α-SMA expression. Furthermore, when assessing the α-SMA expression displayed in different histopathological subtypes of BCCs, we found that nodular BCC demonstrated a weak to moderate and rarely a strong level of α-SMA immunopositivity within tumour nests and cords (Figure 3.14) paralleled by very weak or almost nil stromal reactivity (Figure 3.15).
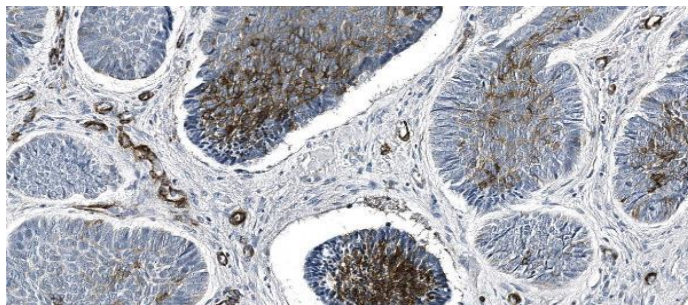
Figure 3.14 **Solid nodules of primary BCC demonstrate rather diffuse, weak to moderate α-SMA positivity, whereas the lower one – stronger and more compact immunopositivity appearing locally**

Vascular α-SMA positivity appears in myxoid stroma interspersing the nodules.
α-SMA immunohistochemistry, ×250.
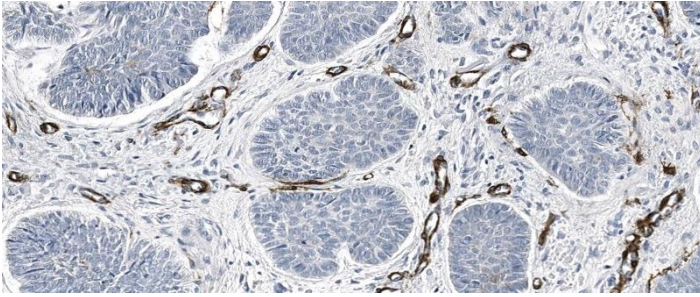
Figure 3.15 **Expression of *α*-SMA, both tumoural and stromal,
is almost nil; *α*-SMA positivity is restricted to vascular appearing
in myxoid stroma interspersing the nodules of this primary BCC**

α-SMA immunohistochemistry, ×250.

By contrast, micronodular, the mixed and infiltrative subtype of BCC often demonstrated a marked, strong stromal expression of *α*-SMA presented either as a diffuse or a peritumoural (Figure 3.16), heavily decorating the base of tumour nests (Figure 3.17). In the case of diffuse expression, some mixed tumours presented with actin-rich stroma enveloping *α*-SMA negative tumours nests and strands (Figure 3.18).
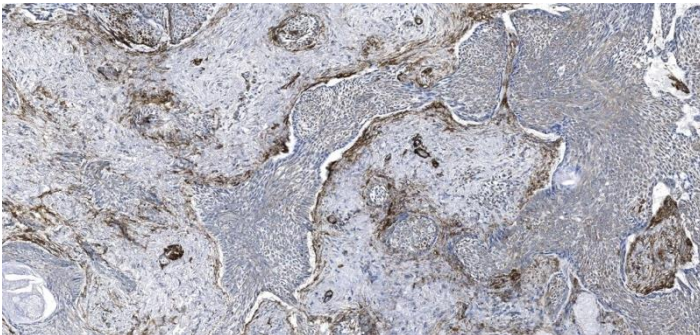
Figure 3.16 **Low power view of mixed recurrent BCC demonstrating
stromal peritumoural and vascular *α*-SMA expression appearing
in the myxoid stroma**

α-SMA immunohistochemistry, ×100.
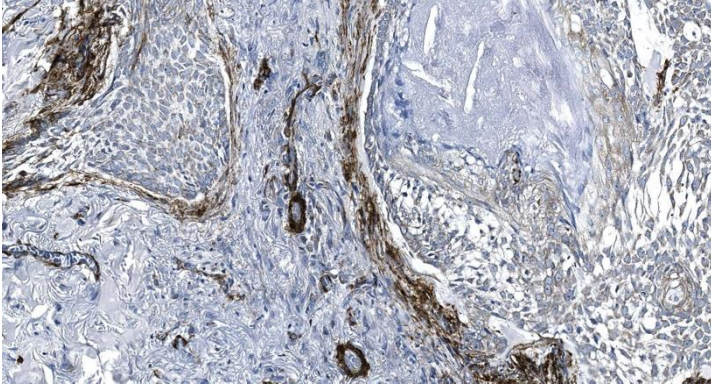
Figure 3.17 **Strong stromal peritumoural expression of *α*-SMA displayed as a heavy decoration at the base of tumour nests and cords observed in mixed recurrent BCC; *α*-SMA positivity within vascular channels**

α-SMA immunohistochemistry, ×200.
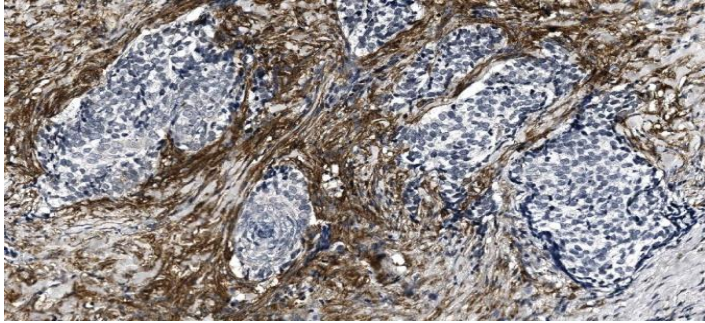
Figure 3.18 ***α*-SMA negative tumour nests and strands of mixed recurrent BCC are surrounded by actin-rich stroma**

α-SMA immunohistochemistry, ×200.

The superficial subtype presented with a weak *α*-SMA expression, both tumoural and stromal, furthermore, the last one often diminished in areas with inflammatory infiltration (Figure 3.19).

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Figure 3.19 **Weak tumoural expression of *α*-SMA observed in primary superficial BCC; stroma is heavily infiltrated with inflammatory cells, and *α*-SMA positivity is restricted to vascular beds**

α-SMA immunohistochemistry, ×100.

Comparing *α*-SMA expression in subtypes of BCC, we found that tumoural expression revealed in the superficial subtype significantly differed when compared to all other subtypes – nodular ($p < 0.0001$), micronodular ($p = 0.003$), infiltrative ($p < 0.0001$), and the mixed ($p < 0.0001$). Similarly, stromal expression revealed in the infiltrative subty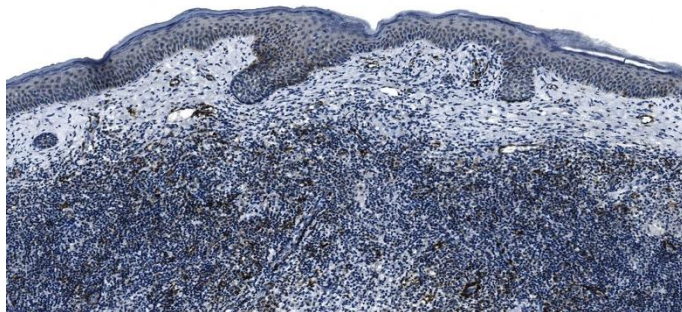pe significantly differed when compared to all other subtypes – nodular ($p < 0.0001$), micronodular ($p = 0.036$), superficial ($p < 0.0001$), and the mixed ($p < 0.001$).

Similar to the *α*-SMA expression, nodular tumoural masses demonstrated mostly weak SHh immunopositivity accompanied by extremely weak stromal reactivity (Figure 3.20). Of note, that almost one half of infiltrative BBCs, followed by more than one-third of superficial and mixed tumours, and about one-third of micronodular displayed a strong SHh expression within neoplastic buds and strands (Figure 3.21), whereas the others – moderate to strong SHh expression (Figures 3.22 and 3.23).
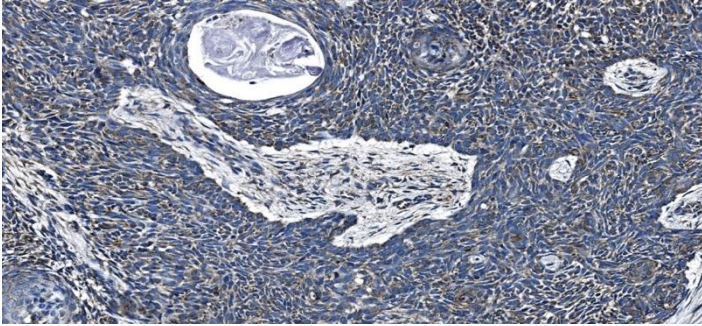
Figure 3.20 **Nodular BCC masses displaying a weak expression of SHh; stromal expression is almost nil**

Shh immunohistochemistry, ×250.
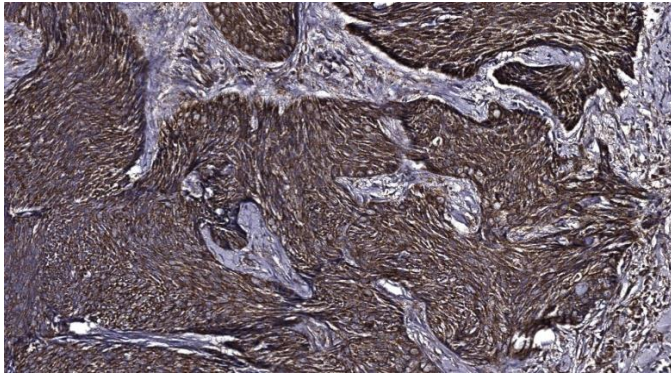
Figure 3.21 **Mixed primary BCC demonstrates tumour strands revealing partial stromal invasion and heavily decorated with the anti-SHh antibody; stromal component exhibits Shh immunopositivity as well**

SHh immunohistochemistry, ×200.
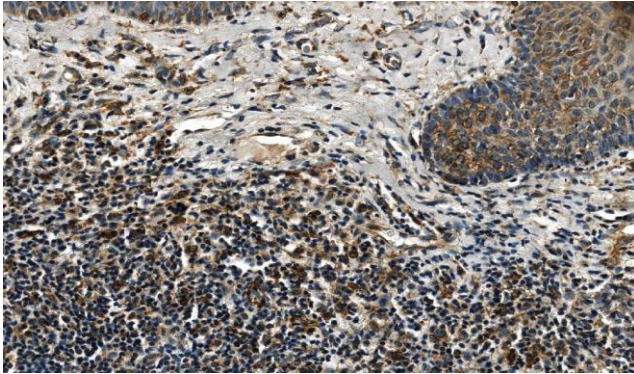
Figure 3.22 **Moderate to strong tumoural expression
of SHh accompanied by weak to moderate stromal expression observed
in primary superficial BCC; marked stromal infiltration**

SHh immunohistochemistry, ×250.
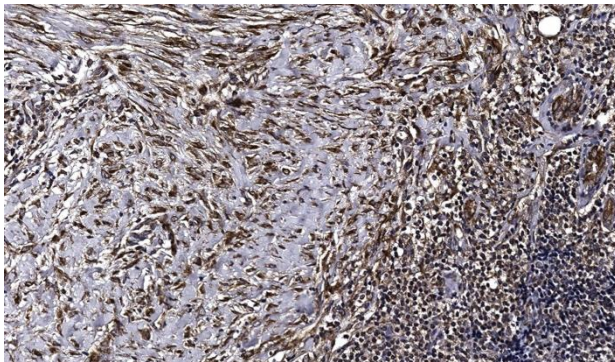
Figure 3.23 **Diffuse, rather regular moderate stromal SHh expression
demonstrated in primary superficial BCC**

SHh immunohistochemistry, ×200.

Analysing BCC, it was determined that some tumours did not express or weakly expressed podoplanin (Figure 3.24 (B), (C), (D)), whereas others expressed podoplanin exclusively in the invading front. Finally, some tumours expressed podoplanin within the basal cell layer with frequent cytoplasmic staining (Figure 3.24 (A)).
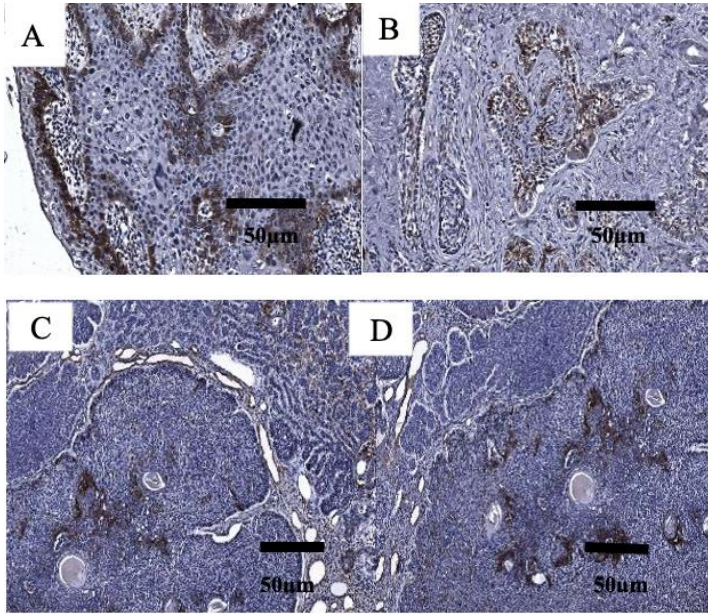
Figure 3.24 **Podoplanin expression in tumour and stroma**

(**A**) Irregular tumour nodules growing into the stroma in primary BCC. Several tumour cells in the basal part express PDPN, ×200. (**B**) Tumour cell filaments in mixed recurrent BCC show PDPN immunopositivity, while some lack it, ×200. (**C, D**) Mixed type BCC is characterized by both large nodules and micronodular structure. Expressed focal PDPN expression was determined, as well as less expressed along the tumour basement membrane; micronodular structures express PDPN very weakly, ×100.

3.5.3 Correlative immunohistochemical analysis of VDP and SHh in low- and high-risk BCC

To better understand the potential protective action of vitamin D in the development of BCC, VDP expression was determined immunohistochemically and correlated with SHh expression in low- and high-risk tumours. A pattern of DBP and SHh immunostaining varied when different types of BCC were compared. To better assess the possible associations between the expressions of cutaneous tissue markers, a correlation analysis was performed separately for different BCC types. Assessments of cutaneous DBP and SHh indices observed

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in low and high risk BCCs using IHC assays and correlation analysis are depicted and summarized in Figures 3.25 and 3.26. In superficial BCC, mostly moderate expression of both tissue markers was revealed in superficial BCC (Figure 3.25 (A) and (B)). Furthermore, in superficial BCC, a strong negative correlation between SHh and DBP expression in both tumoural $(r = -0.77)$ and stromal $(r = -0.49)$ compartments was found (Figure 3.25 (C)). Worth noting, weak DBP expression was commonly demonstrated in aggregations of basaloid cells and peripheral cells of the tumour nodules with scanty cytoplasm that displayed the typical nuclear palisading. In contrast, moderate to strong expression of DBP was found in differentiated tumour cells (Figure 3.25 (D)). Simultaneously, a weak expression of SHh was demonstrated in nodular BCC masses (Figure 3.25 (E)). The only natural intrinsic correlation was found for the DBP marker in nodular BCC (Figure 3.25 (F)).

Overall, a more pronounced expression of SHh was observed in tumour cells with low expression of DBP, and vice versa (Figure 3.26 (A) and (B), (D) and (E), (G) and (H)). In both infiltrative and mixed BCCs, high SHh expression was found in the tumour tongues presented as admixtures of rounded nodules and nodules with irregular contours, and small irregular tongues of tumour cells embedded in the fibrous stroma (Figure 3.26 (B) and (H)). Characteristically, high risk infiltrative BCCs, often with scanty cytoplasm, poorly expressed DBP (Figure 3.26 (G)). Intrinsic correlations were found for the DBP and SHh markers presented in the tumoural and stromal compartment of a neoplasm when assessing high risk BCCs by the use of statistical data analysis (Figure 3.26 (C) and (F)). Simultaneously, in infiltrative BCC, a negative correlation between DBP and SHh stromal expression $(r = -0.56)$ was found (Figure 3.26 (I)).
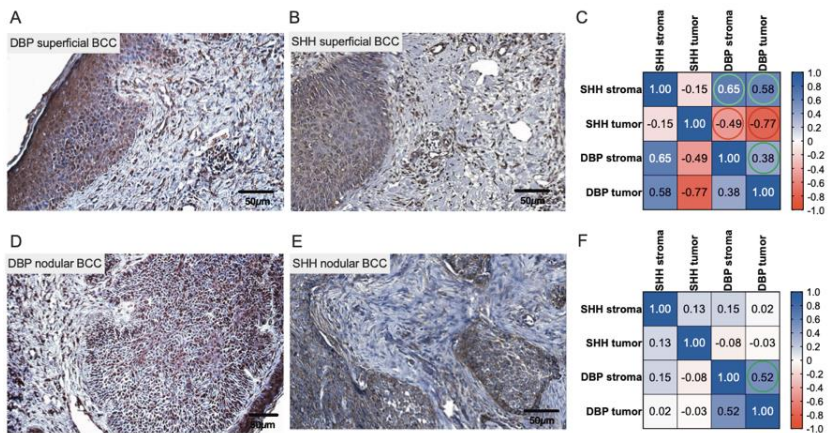
Figure 3.25 **Assessment of cutaneous DBP and SHh expression in low risk BCCs by the use of IHC**

Representative images demonstrating cutaneous structures (tumoural (∗) and stromal (□)) decorated by the anti-DBP (**A** and **D**) and anti-SHh (**B** and **E**) antibodies and recognized by the presence of brown reaction products found in the BCC samples. Scale bars: 50 μm. Correlograms that highlight associations between the expression of DBP and SHh in superficial (C) and nodular (F) BCCs. A strong negative correlation between SHh and DBP expressions is marked by red colour (C).
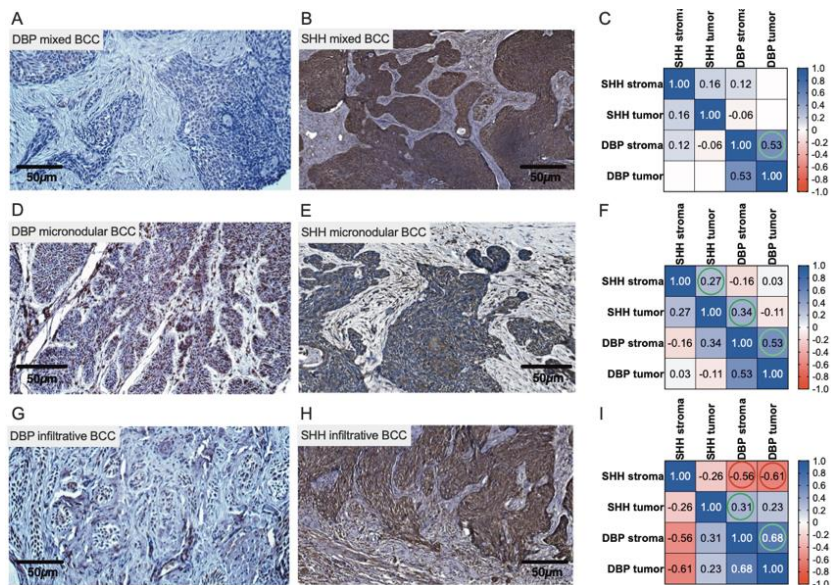
Figure 3.26 **Assessment of cutaneous DBP and SHh expression
in high risk BCCs by the use of IHC**

Representative images demonstrating cutaneous structures (tumoural (∗)
and stromal (□)) decorated by the anti-DBP **(A, D, G)** and anti-SHh **(B, E, H)** antibodies
and recognized by the presence of brown reaction products found in the BCC samples.
Scale bars: 50 μm. Correlograms that highlight associations between the expression
of DBP and SHh in mixed **(C)**, micronodular **(F)**, and infiltrative **(I)** BCCs. A negative
correlation between SHh and DBP expressions is marked by red colour **(I)**.

Finally, to better assess the complex associations between serum vitamin
D level patterns detected in the Latvian cohort of patients presenting with the
different types of BCC and the cutaneous tissue expression of DBP and SHh
confirmed using IHC in these individuals, a Spearman's rank correlation
analysis was performed (Figure 3.27 (A)–(C)). Similar to the results obtained
and interpreted when describing a dendrogram (Figure 3.9), in this final
assessment, males recruited in the given study characteristically presented with
lower than females serum vitamin D levels, high risk (r = −0.87), and recurrent
(r = −0.87) BCC tumours.
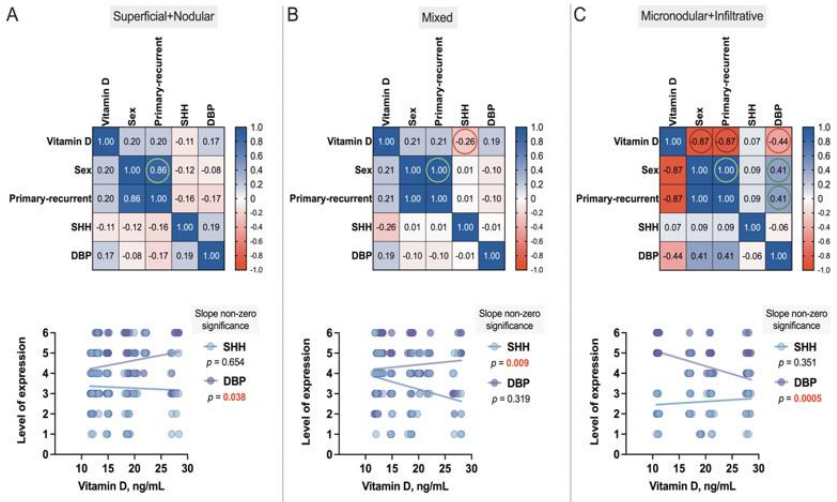
Figure 3.27 **Correlograms of the studied variables**

The correlograms depict the complex associations between serum vitamin D level
patterns of 79 subjects presenting with the different types of BCC and the cutaneous
tissue expression of DBP and SHh confirmed using IHC in these individuals. Each dot
represents a single data point. The levels of expression are assessed as a sum of the
tumoural and stromal BCC tissue indices. In these plots, correlation coefficients are
coloured according to the value. Positive correlations are displayed in blue,
whereas negative correlations are in red. Colour intensity is proportional
to the correlation coefficients.

3.6   Ultrastructural analysis of basal cell carcinoma

Finally, for the better assessment of a tumour architecture in the front, the
cellular morphology was explored using TEM. The cell displayed an irregular
cell shape, and the intercellular spaces were dilated. Almost all cellular cords
presented with loss of cell-to-cell junctions; only primitive junctions were
preserved (Figure 29A, B, C). There were only occasional tonofilaments
observed in the cytoplasm. The changes of the BM included the presence of
multi lamination, splitting, and development of a discontinuous course
(Figure 3.28 (D)).
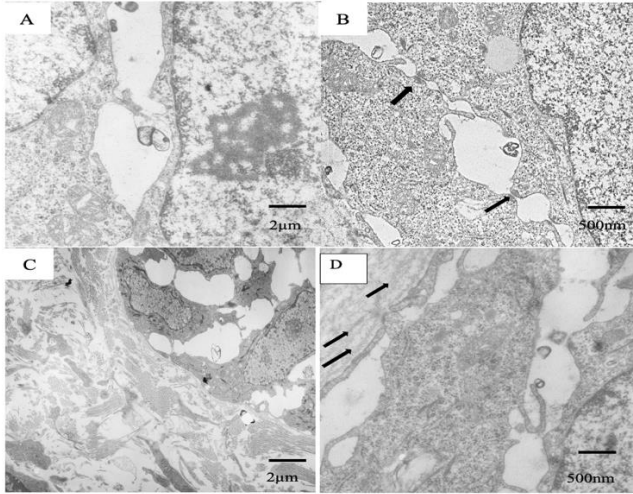
Figure 3.28 **Ultrastructural changes of tumour cells and stroma in mixed basal cell carcinoma**

(A, B) Fragments of two tumour cells, showing dilated intercellular spaces; only primitive cell contacts are preserved (arrows). In the cytoplasm, small mitochondria, free ribosomes, individual tonofilaments, × 15,000. **(C)** Basal part of the tumour surrounded by a stroma containing collagenous fibres, × 4,000. **(D)** The lamina densa of the basement membrane shows a heterogeneous appearance and multilamination (arrows), × 15,000.

4 Discussion

In this study, we have (1) assessed the incidence and prevalence of 79 primary and recurrent BCCs that developed in the head and neck region; (2) performed clinical and dermatoscopic control of patients, analysing the most frequent dermatoscopic signs and localisations depending on the type of BCC; (3) determined the level of vitamin D in the blood serum of all BCC patients included in this study; (4) performed immunohistochemical analysis of major constituents of the BM – collagen type IV and laminin enveloping the tumoural compartment, which enclose the tumour and stop its spread, and (5) analysed the expression of α-SMA, PDPN, VDP and SHh found in a bulk of the tumour and surrounding stroma in different subtypes of BCC, reflecting the complexity of the biology and signalling in this neoplasm.

The results of the given study have to be viewed in light of some limitations. A moderate sample size of the study cohort may be assumed as a potential limitation. The disease relapse was monitored over a 2-year follow-up period only. Simultaneously, the relevance of the results that are obtained and conclusions that are drawn out is enhanced by the prospective nature of the present research. Only a conventional chemiluminescence immunoassay was used to measure the total vitamin D serum level in BCC patients. Finally, the size of BCC tissue obtained by surgical excision in the head and neck region was strongly associated with the treatment necessity, and, therefore, explains some difficulties related to the number of histopathology and immunohistochemistry assays performed.

A high frequency of BCC constituting approximately 80 % of all nonmelanoma skin cancers, and commonly appearing on the head and neck – body areas exposed to the sun has been demonstrated previously (Rubin et al., 2005; Carucci et al., 2008; Fernanda et al., 2017). We found that the gender distribution is similar to the results of Mawardi and co-authors (Mawardi et al.,

2016) and shows a female predominance, while the age at diagnosis was similar when comparing this study with previous ones. When evaluating the skin phototype of the patients included in the particular study, we found that skin phototype II was the most common. It is characteristic of the people living in the Baltic region. At the same time, it is recognized that individuals with skin phototype I–III have a higher risk of developing skin cancer (Wright et al., 2011).

In this study, we have demonstrated that BCC can cause serious damage due to its local recurrence, and the midface is more susceptible to tumour development. Areas of the nose, cheeks, and eyelids that are chronically exposed to sunlight were more frequently affected by both primary and recurrent tumours than other areas of predilection for BCC. According to our results, most of the mixed-type BCCs are localized in the nasal and nasolabial skin area, and most of these tumours are larger in size than tumours of other subtypes. These results are consistent with those demonstrated by Mawardi and co-authors (Mawardi et al., 2016) when local but not distant recurrence and aggressiveness of BCC were investigated.

Analysing the dermatoscopic and histopathological correlations, it becomes clear that the aggressive potential of the tumour is not acquired only by tumour growth over time. As part of the study, very small tumours were diagnosed in terms of size, which were histologically confirmed to be completely aggressive. Our study confirmed, and our data agree with other authors, that the localisation of aggressive tumours should be considered, as they are often located in areas crossed by large arteries (Lammers et al., 2011). In recent years, the diagnosis of skin tumours has been improved using a non-invasive and inexpensive in vivo dermatoscopic examination method. This modern and convenient examination method allows to characterize the characteristics of the tumour instrumentally, thereby contributing to the accuracy of its diagnosis.

The most common dermatoscopic finding of mixed BCC is the presence of arborized blood vessels and short fine telangiectasias. Shiny white areas, white streaks, chrysalides, milky pink to red background featureless areas, ulceration and multiple small erosions were also observed more frequently than other findings. The above-mentioned dermatoscopic criteria, which we found to be appropriate criteria in the study of aggressive BCC, correlate with the results of other authors and correspond to the peritumoural inflammation that is common in aggressive forms of BCC (El-Sayed et al., 2020; Zalaudek et al., 2010).

According to the results of the present research, 94.9 % of the Latvian cohort of patients with primary and recurrent BCC of the head and neck were found to be deficient in vitamin D. In total, 5.1 % of the examined patients had a sufficient level of 30.0–40.0 ng/mL. These data are in agreement with the results of the vitamin D deficiency study conducted in Latvia in 2015 by specialists from the Latvian Osteoporosis and Bone Metabolic Diseases Association, Rīga Stradiņš University, and Riga East Clinical Hospital which is the only study that has been done before in Latvia (Bouillon et al., 2020, Mukane et al., 2015). Similar results come from the neighbouring countries reporting vitamin D deficiency when carrying out a large Swedish cohort study and confirming the average estimated level of vitamin D as 19.9 ng / mL (Samuel et al., 2008), thus being slightly higher than that determined in the Latvian cohort of BCC patients. In the present study conducted using a medium-sized cohort of BCC patients, a hierarchical cluster stratified into three major branches was recognized. The first branch was represented by males with low serum vitamin D levels and large, mostly high risk and recurrent BCC tumours. Therefore, it can be said with certainty that male sex and low blood vitamin D levels are risk factors for the development of aggressive types of BCC. The second branch was represented by both males and females with greatly varying serum vitamin D

levels and small-sized primary, however, high risk BCC tumours. Finally, the third branch was represented by males and females with varying serum vitamin D levels and small-sized primary and less aggressive BCC tumours.

In the studies of other authors, both tumour proliferation markers and those significant for differential diagnosis between BCC and SCC are analysed (Alhumaidi, 2012; Ramezani et al., 2020). In contrast, in our work, the local aggressiveness and invasiveness of BŠK were intensively investigated using specific immunohistochemical markers. Previous studies have demonstrated that the disappearance of type IV collagen in the basement membrane *lamina densa* is significant for tumour cell invasion (Fang et al., 2014), and changes in type IV collagen expression, as well as infiltrative tumour growth, are analysed in aggressive bladder tumours (Miyake et al., 2017). Our evidence regarding the entire basement membrane structure, as indicated by the expression of collagen and laminin, aligns with findings reported in the available literature (Quatresooz et al., 2003; Gozdzialska et al., 2016; Khlebnikova et al., 2020).The level of expression of type IV collagen and laminin varied from low to moderate and high; however, two-thirds of the BCC samples had low expression. The immunohistochemical expression of the investigated BM molecules was either disrupted or absent in BCC regions showing aggressive growth. These results align with findings from other authors (L. Marasà 2008, Peterson et al., 2015).

Apart from the assessment of collagen type IV impairment, the expression of the podoplanin marker was estimated and ultrastructural peculiarities of BCC at the invading cone was analysed. The studies exploring the expression of tumour-associated genes in squamous cell carcinoma have already suggested the role of podoplanin in normal and malignant homeostasis of the epidermis (Acton, 2012; Baars et al., 2015). Previous studies have highlighted the role of podoplanin in the induction of collective and single tumour cell migration (Wicki and Christofori, 2007). In this study, we

demonstrated irregularly shaped invasive tumour cords highly decorated with the anti-podoplanin antibody along the basal aspect and reflecting aggressive neoplastic potential. Additionally, we proved the increase of podoplanin expression demonstrated at the invading cone and paralleled by a decrease of cytoplasmic tonofilaments, simplification of cellular junctions and the discontinuity of the BM assessed ultrastructurally.

Our study based on careful statistical assessments of the markers studied provided the meaningful evidence – the appearance of strong stromal α-SMA expression levels demonstrated in the recurrent BCC should be interpreted with caution considering the relapsing nature of the tumour. The development of α-SMA-rich phenotype cells in the stromal component of aggressive BCC variants – micronodular and morphoea type – was previously demonstrated (Mercut et al., 2014), and our immunohistochemical findings support this evidence.

Activation of SHh signalling in the TME and its association with tumour growth and metastatic activity has been shown in studies examining the morphogen expression in neoplasms (Sahin et al., 2015; Song et al., 2018). Previous studies have suggested enhancement of resting fibroblasts stimulation and conversion into myofibroblasts by SHh, leading to the accumulation of collagen and dermal thickening in mice (Horn et al., 2012). In this study, we have assessed the levels of SHh in primary and recurrent, and in five different subtypes of BCC by use of immunohistochemistry and statistics. We proved the increase of strong levels of SHh expression in both – tumoural and stromal compartments. Furthermore, when specifying the subtypes of BCC analysed, we found the increase of strong levels of SHh expression in aggressive variants – infiltrative, the mixed, and micronodular. These results are in agreement with the data demonstrated by Casas and colleagues (Casas et al., 2017).

By contrast, among the other subtypes of BCC, previously considered as aggressive variants, we found upregulation of SHh paralleled by downregulation of α-SMA expression in the superficial subtype of the tumour assumed to be nonaggressive. This leads us to propose that SHh participates in normal and affected epidermal homeostasis, but the molecular pathway of the signalling is not completely understood. Finally, we may suggest that the assessment of morphological and immunohistochemical characteristics of primary and recurring forms of skin cancer and possible changes in the properties of a tumour is important for determining prognostic factors and choosing an adequate method for treating a disease.

In the present study, the serum levels of vitamin D were explored in the Latvian cohort of patients who presented with different primary and recurrent BCC of the head and neck; cutaneous DBP and SHh indices found in these subjects were investigated, and established correlations were analysed. Simultaneously, a correlation between the tumour size and serum vitamin D levels was established, and this observation is in agreement with data from other studies (Kim et al., 2019). Furthermore, the association between high DBP and low SHh tissue expression found in smaller tumours with a favourable course, such as superficial and nodular BCC, was demonstrated. Importantly, low expression of DBP and high expression of SHh were observed in mixed and infiltrative BCCs which may correlate with the assumption of a deficiency in the protective effect of vitamin D in patients with high-risk tumours and a tendency to relapse after treatment. In turn, the tissue expression of DBP is likely to be associated with favourable prognostic characteristics, such as small tumour size, and low invasiveness. This is consistent with the evidence that DBP-positive tumours are associated with a reduced risk of metastasis and mortality from various cancers. Recent studies that are in line with the results of the present research demonstrate a significantly low to no DBP immunohistochemical

staining in advanced tumours (Tang et al., 2012). The use of hierarchical clustering analysis has shown that susceptible male individuals with low blood vitamin D levels are at risk of developing aggressive and recurrent BCC.

The heterogeneity of BCC histopathology has been demonstrated by other authors and us (Bartoš et al., 2016). In addition, a high proportion of mixed BCC, often with aggressive growth and requiring surgical excision with controlled margins, was demonstrated, and analysed both by other authors and in this study (Wu et al., 2014). Despite the high incidence of BCC, there are very few studies describing the characteristics of different tumour subtypes and possible differences between them. There are various treatment methods for BCC, ranging from minimally invasive methods to surgical therapy, in which tumour histopathological findings are one of the determining factors in choosing an appropriate treatment method (Drucker et al., 2018). Specialists involved in the diagnosis and treatment of BCC need to be aware of the successful and safe treatment of patients due to frequent recurrences even in the case of complete primary surgical tumour removal (Paoli et al., 2019).

Conclusions

1. In a cohort of seventy-nine BCC patients on the head and neck (46 women, 33 men; mean ages 70 and 64 years), predominant skin phototypes were Fitzpatrick scale II and III, unlike Europe, where phototypes I and II are common. Sun-exposed areas like the nose and cheeks were primary sites for both primary and recurrent BCC, aligning with global trends. Particularly concerning is the nasal region's BCC recurrence within two years, underscoring the need for precise surgical removal and histopathological assessment, especially for mixed morphological types. BCC commonly presented as a slow-growing skin-colored nodule or erythematous spot with erosion or ulceration.

2. Based on the results of dermatoscopic examination, the development of high-risk BCC can be predicted. The presence of arborizing vessels, short fine telangiectasias, shiny white areas, ulceration, and white streaks or strands extending beyond the clinical borders of the lesion on dermatoscopy indicates an aggressive course of BCC. Moreover, this signifies the necessity of dermatoscopic evaluation of excision margins during the preoperative period.

3. Morphological tumour diversity was determined in the study patient cohort; high-risk BCC are tumours of infiltrative, micronodular, and mixed type, while nodular and superficial BCC are tumours of low risk of recurrence. The coexistence of two or more different histopathological types of BCC in one anatomical area is very rare. The loss of the basement membrane integrity, which was confirmed by performing immunohistochemical reactions with anti-laminin and type IV collagen antibodies and studying the features of tumour development ultrastructurally, was more often observed in infiltrative and micronodular BCC; they are also characterized by increased peritumoural and stromal

Results of the clinical data of patients with BCC *a*-SMA expression. The increase in podoplanin expression in the basal layer of the tumour suggests the invasiveness of neoplastic cells. SHh is an essential factor that regulates tumour cell behaviour and stromal transformation, possibly through both autocrine and paracrine pathways. High-risk BCC is characterized by increased SHh expression and tumour invasion into the stroma when targeted therapy should be considered.

4. A vitamin D deficiency was determined in the Latvian cohort of BCC patients. When studying the level of vitamin D in the blood serum, no statistically reliable differences were found between genders, primary and recurrent BCC, as well as between tumour morphological types. A higher level of vitamin D in the blood serum correlates with the appearance of smaller neoplasms and a more favourable prognosis of the disease. In smaller and less aggressive tumours like superficial and nodular BCC, a connection between high-VDP and low-SHh tissue expression supports the link between vitamin D, its metabolic proteins (like VDP), and the SHh signalling pathway.

5. Three clusters were identified in the analysis of the Latvian cohort of BCC patients: 1) primarily males with low vitamin D levels, exhibiting large, high-risk recurrent BCC tumours; 2) both genders with varying vitamin D levels, demonstrating small-sized primary BCC tumours, yet with high risk; and 3) both genders with diverse vitamin D levels, presenting small-sized primary BCC tumours that are less aggressive. These findings underscore the heterogeneity and complexity of these tumours themselves and their correlations with vitamin D.

Practical recommendations

1. For patients with cutaneous BCC, it can be recommended to perform a dermatoscopic examination for the purpose of diagnosis of the formation.

2. For patients with a history of cutaneous BCC, it is recommended to perform dermatoscopy in the postoperative period 1×6 years to detect tumour recurrence in time, especially if histologically there was a suitable BCC tumour with a tendency to aggressive growth.

3. For patients with cutaneous BCC, the factors of disease recurrence, the obtained clinical, dermatoscopy and immunohistochemistry results are recommended.

4. The complex of morphological structure and biological properties of the skin BCC, as a treatment method can be used for treatment. Minimally invasive treatment (imiquimod, cryotherapy, etc.) can be applied to histologically confirmed superficial BCC without signs of infiltrative growth.

5. Histopathology departments should perform serial specimen cutting, this approach was the accuracy of diagnosis, especially in case of mixed and aggressive type of BCC.

List of publications

Publications:

1. Moisejenko-Golubovica, J., Volkov, O., Ivanova, A., Groma, V. 2020. Analysis of the occurrence and distribution of primary and recurrent basal cell carcinoma of head and neck coupled to the assessment of tumour microenvironment and Sonic hedgehog signaling. Romanian journal of morphology and embryology, 61(3), 821–831.

2. Moisejenko-Golubovica, J., Volkovs, O., Ivanova, A., Petrošina, E., Groma, V. 2021. What We Need to Learn When Exploring the Mixed Basal Cell Carcinoma of Head and Neck. Proceedings of the Latvian Academy of Sciences. Section B, Natural Sciences, 75, 75–85.

3. Moisejenko-Goluboviča, J., Groma, V., Svirskis, Š., Ivanova, A. 2022. Serum Vitamin D Levels Explored in the Latvian Cohort of Patients with Basal Cell Carcinoma Linked to the Sonic Hedgehog and Vitamin D Binding Protein Cutaneous Tissue Indices. Nutrients, 14(16), 3359.

Reports and theses at international congresses and conferences:

1. Moisejenko -Golubovica, J., Ivanova, A., Groma, V. 2016. Features of the pathogenesis and development of locoregional recurrence of basal cell carcinoma on the neck and face area. Russian Congress of Molecular oncology. – Vol. 4, No 1, Suppl., p.65. – 2nd Russian Congress of Molecular oncology: Programme and abstract book.

2. Moisejenko-Golubovica, J., Groma, V., Ivanova. 2017. Kolagēna un laminīna ekspresijas atšķirības bazālajā membrānā bazālo šūnu karcinomu gadījumā galvas un kakla rajonā. III Pēterburgas starptautiskais onkoloģijas forums "Baltās naktis", lpp. 214, nr. 1728.

3. Moisejenko-Golubovica, J., Groma, V., Ivanova. 2017. Surgical multistage treatment for aggressive and neglected basal cell carcinoma. 9th Baltic Congress of Maxillofacial and Plastic Surgeons, p. 47: Programme and abstract book.

4. Moisejenko-Golubovica, J., Groma, V., Ivanova, A., Karl, R. 2017. An immunohistochemical study of laminin and collagen IV in basal cell carcinoma. 13th Congress of the EADO, JEADV, 31 (Suppl.3), 3–100, p. 87 (P124).

5. Moisejenko-Golubovica, J., Ivanova, A., Zarins, J., Groma, V. 2017. Case report: coexistence of solid and adenoid basal cell carcinoma at the same anatomical site. 13th Congress of the EADO, JEADV, 31 (Suppl.3), 3–100, p. 87 (P125).

6. Moisejenko-Golubovica, J., Volkov, O., Ivanova, A., Groma, V. 2018. Results of immunohistochemical analysis of Sonic Hedgehog, a key factor in the pathogenesis of basal cell carcinoma. Russian Congress of Molecular oncology. – Vol. 5, No 4, Suppl.1, p. 50 – 4th Russian Congress of Molecular oncology: Programme and abstract book.

7. Moisejenko-Golubovica, J., Groma, V., Ivanova, A., Zabludovska, K. 2017. Peculiarities of the pathogenesis and locoregional recurrence in case of head and neck basalioma. RSU Scientific Conference, lpp.133: programme and abstract.

8. Moisejenko-Golubovica, J., Groma, V., Ivanova, A., Volkovs, O., Skuja, S. 2017. Kolagēna un laminīna ekspresijas atšķirības bazālajā membrānā mutes dobuma plakanšūnu vēža un bazaliomas gadījumā. RSU zinātniskā konference, programma un tēžu grāmata, lpp.196.

9. Moisejenko-Golubovica, J., Ivanova, A., Muceniece, J. 2019. Basal cell nevus syndrome – diagnosis and treatment. RSU Scientific Conference, programme and abstract.

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Acknowledgements

Annex

Decision of RSU Ethics Committee

RSU ĒTIKAS KOMITEJAS LĒMUMS NR. 11 / 08.09.2016.

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Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1. Profesors Olafs Brūvers	Dr.theo.	teologs
2. Profesore Vija Sīle	Dr.phil.	filozofs
3. Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
4. Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5. Profesore Regīna Kleina	Dr.med.	patalogs
6. Profesors Guntars Pupelis	Dr.med.	ķirurgs
7. Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs
8. Docente Iveta Jankovska	Dr.med.	
9. Docents Kristaps Circenis	Dr.med.	

Pieteikuma iesniedzējs: **Dr. Jeļena Moisejenko-Goluboviča (Latvijas universitāte)** Medicīnas fakultāte

Pētījuma nosaukums: „ Bazālo šūnu karcinomas patoģenēzes un lokoreģionālo recidīvu attīstības īpatnības galvas un kakla rajonā"

Iesniegšanas datums: 08.09.2016.

Pētījuma protokols: Izskatot iesniegtos pētījuma dokumentus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot, bez kāda apdraudējuma veselībai un dzīvībai, pacientu objektīvā stāvokļa izvērtējumu, ievācot pacientu anamnēzes datus un analizējot iegūtos (izoperētos) BŠK audu materiālus, veicot pacientu medicīniskās dokumentācijas (medicīnas vēstures) izpēti, visu iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst medicīnas pētījuma ētikas prasībām.

Izskaidrošanas formulārs: ir

Piekrišana piedalīties pētījumā: ir

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers   Tituls: Dr. miss., prof.

Paraksts

Ētikas komitejas sēdes datums: 08.09.2016.

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