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The clinical immunology of the integument

ABSTRACT OF DOCTORAL THESIS

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I. THE CURRENT STATE OF KNOWLEDGE

Chapter 1: Principles of skin immunology

The skin is the largest organ of the human body and represents the most important physical, chemical, and immunological barrier that separates the human body from the environment. Both innate and acquired immunity are activated in the epidermis, dermis, and hypodermis [1-3].

Innate skin immunity is made up of cellular factors (effector cells) and soluble factors - molecules involved in cellular signaling cascades.

Soluble factors of innate skin immunity are represented by PRRs (*pattern recognition receptors*), the complement system, inflammasomes, antimicrobial peptides, and cytokines. PRRs represent an important family of receptors, located on both immune and non-immune cells, with a role in recognizing PAMPs (pathogen-associated molecular patterns: viruses, bacteria, parasites) [4, 5]. At the skin level, the complement system is activated *via* the classical, lectin, or alternative pathway. All three complement pathways lead to the formation of the

membrane attack complex that causes cell death by lysis; in addition, some components of the complement system (C3a, C5a) induce leukocyte recruitment, and C3b induces opsonization and phagocytosis of immune complexes [6]. Inflammasomes are large proteins that assemble in immediate response to PAMPs and DAMPs (danger-associated molecular patterns) and cause caspase-1 activation, IL-18 and IL-1-β synthesis, and pyroptosis (cell death). Cryopyrin is the NLRP-3-associated inflammasome, an intensively studied protein responsible for cryopyrin-associated periodic syndromes responsive to innovative anti-IL-1-β therapy [7, 8]. Antimicrobial peptides (alarmins) represent a family of peptides, most with a cationic structure and an important antibacterial role. They are responsible for the activation of a prompt anti-infectious immunological response and the initiation of skin epithelization mechanisms [9, 10]. Cytokines are proteins with low molecular mass, synthesized mainly by macrophages and T cells (TC), but also by other cell populations, such as keratinocytes, melanocytes, and dendritic cells [11].

The cells involved in innate skin immunity are macrophages, neutrophils, eosinophils, basophils, and mast cells, as well as a separate population of lymphocytes, the NK cells [4].

Antigen-presenting cells (APCs) are either skin residents or infiltrate the skin from the systemic circulation. They are distributed in the epidermis (Langerhans cells), dermis (monocytes, macrophages, and dendritic cells), and hypodermis (monocytes, macrophages, TC) [12]. APCs migrated to regional lymph nodes activate naïve TCs or cause the recirculation of phenotypically and functionally different memory TCs. TC will preferentially accumulate at the site of signaling by APC, but also in the perilesional indwelling skin, in lower titers [13-15]. If within the epidermis, predominate CD8+ TC populations (cytotoxic TC) with anti-infective and anti-tumor potential mediated by MHC I, at the dermal level mainly CD4+ TC are encountered. Cutaneous APCs present CD4+ T-lymphocyte antigen, and these migrate to lymphatic follicles draining regional lymphatics, where they activate B-lymphocyte populations. The interaction between CD4+ TC and LB is made *via* CD40 [16-18].

Immunological escape mechanisms in sexually transmitted infections (STIs) are complex, and innovative therapies in syphilis (linezolid, salvicin), gonorrhea (ertapenem, mupirocin, zoliflodacin, MBX-4132), chlamydia (corallopyronin A, 2-pyridonamine) and herpes simplex (nanocarriers for acyclovir, peptides isolated from Micromonospora species) represent valuable alternatives [19].

Tumor immunogenicity is an essential element in the progression of basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma and consists of a constant interaction between the local antitumor response and tumor cells. The three stages of this process are the elimination phase, the equilibrium phase, and the tumor escape phase. [20, 21]. In SCC, UVB-associated inflammation is characterized by hyperproliferation of keratinocytes, angiogenesis, and cutaneous myeloid infiltration, with the release of pro-inflammatory molecules (especially TNF-α). These initial mechanisms are followed by a massive infiltrate of macrophages, which paradoxically synthesize VEGF (vascular endothelial growth factor) and matrix metalloproteinases. They stimulate tumor vasculature and degrade peritumoral tissue, increasing tumor invasive potential [22-24].

Chapter 2: Topical immunomodulatory therapies

In the last decades, the immunology of the skin has been intensively studied, and its humoral and cellular factors have become targets of immunological therapies. The ability to administer these therapies topically limits possible systemic side effects.

Imiquimod, originally known as the molecule S-26308 or R-837 has a strong immunomodulatory, antiviral, and antitumor role, by binding to the TLR-7 receptor on the surface of immune cells (NK cells, B cells, macrophages, dendritic cells), causing immunogenic cell death, *via* reactive oxygen species. In addition, it induces the production of IL-6, IL-12, and TNF-α, with the activation of the acquired immune response. It is approved for superficial BCC, precancerous lesions such as actinic keratosis, and *condyloma acuminata* [25-27].

5-Fluorouracil (5FU) is a fluorinated derivative of uracil, used in anti-neoplastic therapy for over 50 years. It acts as an antimetabolite by inhibiting thymidine synthetase, *via* its intracellular metabolites. Topical administration in both 0.5% and 5% concentrations is associated with very low systemic absorption [28, 29]. Although 5FU is not a first-line immunomodulatory topical agent, it stimulates TC proliferation and induces local production of type I interferon (IFN-α, IFN-β) [30].

Cryosurgery uses liquid nitrogen to induce temperatures below 0^{o}C and thus generate tissue injury [31-33]. The physical mechanisms of action of cryosurgery are complemented by

immunological ones, which are extremely complex and induce an immunological response, both local and systemic, at the level of the lymph nodes that drain the respective territory [34].

II. PERSONAL CONTRIBUTIONS

Chapter 3: Hypothesis and general objectives

In the Romanian academic medical environment, as part of my professional training, I met numerous patients for whom the diagnosis of basal cell carcinoma (BCC) was confirmed dermatoscopically and histopathologically. Basal cell carcinoma represented the most frequent neoplasia that we encountered in clinical practice, for which we supported in the research group of the Dermatology I Clinic, Colentina Clinical Hospital, the hypothesis of association with immunodepression and the role of a clinical marker of immunodepression.

In addition, there is a global tendency to avoid surgical excision, and this was often reflected in the demands of patients diagnosed with BCC on our ward, who requested alternative methods of treatment. So, starting from these needs from our current practice, within the research group, a combined, conservative treatment was developed, which combines ablation by physical methods, with cryosurgery and the application of immunomodulatory and chemotherapeutic topicals. This original method of treatment is based on the discovery of some molecules that can stimulate the skin's innate immunity.

Research Study 1 hypothesized the role of BCC as a clinical marker for immunodepression, while the second study looked at the effectiveness of a conservative ablative immuno-cryosurgery treatment (CAICT) in BCC. The primary objective of the first, original research study was the assessment of immunosuppressive comorbidities in patients diagnosed with BCC, in correlation with demographic and histopathological characteristics of BCC. The secondary objectives were: a) the evaluation of the distribution by age, sex, and environment of origin of the patients, the identification of the distribution of BCC locations considering the particularities of the skin phototype of our country, as well as b) the identification of the macroscopic and microscopic characteristics of BCC for the studied population, representative for the Southern region of Romania.

Research Study 2 primary objective was to test an effective CAICT for BCC, with therapeutic results comparable or superior to surgical excision, devoid of the potential side effects associated with surgery and adapted to patients with multiple comorbidities (which often contraindicate surgery), the elderly or those reluctant to classical surgical excision. The secondary objectives of this study were to evaluate the cosmetic results after CAICT and compare CAICT with classic surgical excision from the perspective of therapeutic response, recurrences, local and systemic adverse events (AE), and cosmetic results (RC).

Chapter 4: The general research methodology

Study 1 was retrospective, non-interventional, and was performed on 275 patients between the ages of 31 and 92 diagnosed with BCC, admitted to the Dermatology Clinic I, the Colentina Clinical Hospital, and a private academic clinic, between October 1, 2019, and October 1, 2023. Digital medical records were analyzed for patients with a histopathologically confirmed diagnosis of BCC and included demographic variables, skin phototype, detailed patient medical history, and microscopic and macroscopic features of BCC. Subjects were divided into immunosuppressed and non-immunosuppressed patients according to associated comorbidities. Subjects were divided into immunosuppressed (IP) and non-immunosuppressed (NIP) patients according to associated comorbidities. The following comorbidities were considered causes of immunosuppression: primary immunosuppression, diabetes mellitus, chronic kidney disease, organ transplantation, immunosuppressive medication, history of solid or hematogenous cancer 5 years before BCC diagnosis, chronic infections, NYHA IV heart failure, asplenia. All patients over 18 years of age with a histopathologically confirmed diagnosis of basal cell carcinoma were included in this research study. Patients under 18 years of age, those with Gorlin syndrome, *xeroderma pigmentosum*, and patients with incomplete data in the clinical examination record were not included in the study. This retrospective study was approved by the Colentina Clinical Hospital.

Study 2 was prospective, clinical, comparative, interventional, and bicentric, by creating a group of 179 patients diagnosed with BCC within the framework of Dermatology I Department Colentina Clinical Hospital and a private academic clinic; 129 patients opted for CAICT and 50 patients required radical surgery. In establishing the diagnosis of BCC, the local

clinical examination, the dermatoscopic examination, and the histopathological examination of certainty were taken into account. Before treatment, standardized BCC dermoscopy criteria were applied. Patients over 18 years of age with clinical, dermatoscopic, and histopathological diagnoses of BCC were included in this research study. Patients with: age under 18 years, Gorlin syndrome, recurrent BCC, ulcerated BCC, history of severe immunosuppression in the last 2 years, pregnancy (pregnant patients), inability to understand/ follow treatment indications/ give informed consent were not included in the study. The demographic characteristics of the patients, the anatomo-clinical characteristics of the BCC, as well as the adverse events (AE), and the cosmetic results of the treatments, were evaluated.

CAICT consisted of a protocol that included ablative CO_2 laser, cryosurgery, 5FU, and topical imiquimod. The novelty of this protocol lies in the combination of therapeutic options, otherwise mono-therapeutic, in a conservative approach in several stages. CAICT was started with full CO_2 laser ablative therapy and laser hemostasis under topical anesthesia with lidocaine 2.5% and prilocaine 2.5%. One to three laser passes were used with power adjusted to 5–7 W/cm^2 (beam diameter 1 mm), with a clinical safety margin of 5 mm around the suspicious area. The angiogenic component guided the laser depth and indicated tumor removal. If laser was unavailable, electroablation was used instead. Laser ablation was followed by an occlusive chemowrap dressing with 5FU for 24 hours. Chemowrap consists of applying a 5% 5FU cream under a self-adhering compression bandage. After removal of the 5FU chemowrap, an antibiotic powder containing zinc bacitracin 250 IU and neomycin sulfate 5000 IU was applied for 5 days by the patients, twice a day. After a 7-day break, patients presented for focused cryosurgery. A liquid nitrogen cryosurgery gun was used, with a slot of 1 mm diameter, at -196°C, with a freezing duration of 10 seconds, 2 cm from the surface of the lesion. Immediately after cryosurgery, a chemowrap with 5% 5FU was performed for 24 hours. Eight occlusive applications of 5% imiquimod cream were then made, one application every two days. Patients were instructed to apply imiquimod cream at night, followed by an occlusive dressing, and to remove the imiquimod cream in the morning by washing thoroughly with soap and water. Imiquimod applications were followed by a four-week healing phase. A second cryosurgery session was subsequently performed, immediately followed by a chemowrap with 5% 5FU for 24 hours. After another four-week healing phase, clinical and digital dermoscopic evaluation

was performed and remission was assessed. Tumor remission after CAICT was defined by the absence of dermoscopic criteria suggestive of residual tumor on digital dermoscopy.

For the surgical subgroup, an elliptical excision was performed with a standard scalpel, with a minimum safety margin of 5 mm under local anesthesia with 1% lidocaine and/or sedation. Subcutaneous and superficial sutures were performed. Patients were reevaluated at 10–14 days (depending on the location of the BCC) for suture removal and wound verification.

All patients in the study were informed in detail about the procedures and the study, about the right to data confidentiality, how the data are stored and used for research purposes, and signed an informed consent after fully understanding all the details, risks, and possible side effects. This research study did not present any potential psychological, physical, or legal risk to participating patients and was developed in accordance with the principles, norms, and ethical values of the Helsinki Declaration of Human Rights. The current research study was approved by the Scientific Research Ethics Committee of the "Carol Davila" University of Medicine and Pharmacy.

Chapter 5: Study 1: Basal cell carcinoma - a clinical indicator of immunodepression

In this study, we included 133 men and 142 women, representing 48.36% and 51.64% of the patients, respectively. The patients included in the study presented an average age of 65.2 years, with a minimum age of 31 years and a maximum age of 92 years.

Most patients (194) presented secondary diagnoses (comorbidities), representing 66.44% of all patients ($p<0.001$, 1 sample prop test). Among them, immunosuppressed patients (IP) represented 81.44% ($p<0.001$, 1 sample prop test). The distribution of comorbidities among the 158 IPs reveals an uneven distribution, and diabetes was the most prevalent ($p<0.001$, Chi-Square-Goodness-Fit). The second most common comorbidity was a history of cancer (≈30%), followed by patients with a history of antirheumatic immunosuppressants (≈10%). Patients with chronic kidney disease (CKD) and those with chronic infections showed approximately equal prevalences (≈8–9%). Regarding the history of neoplasia, breast cancer was the most prevalent (2.27%). Patients undergoing immunosuppressive therapies most frequently presented arthritis

(62.5%) and systemic lupus erythematosus (37.5%), and those with chronic infections presented viral hepatitis (62.5%) and pulmonary tuberculosis (37.5%). None of the patients had HIV infection, asplenia, NYHA IV heart failure, or had undergone organ transplantation.

We performed a comparative analysis of the average age, age groups, distribution by sex, skin phototype, and environment of origin for IP and NIP. IPs showed a mean age approximately 5 years older than NIPs ($p=0.0003$, Mann Whitney T). The comparative age group distribution of IP and NIP shows the predominance of the middle age group (55-74 years) in both subgroups, with a higher weight in IP ($p<0.0001$, Chi-squared T) and population under 54 years at NIP, compared to IP ($p<0.0001$, Fisher T). There is no statistically significant difference between the distribution of skin phototype II ($p=0.6303$, Fisher T) and type III ($p=0.6885$, Chi-squared T) or between urban areas of origin ($p=0.852$, Chi-squared T), respectively rural ($p=0.8097$, Fisher T) between IP and NIP. The distribution of comorbidities according to gender does not reveal statistically significant differences between IP and NIP ($p=0.6802$, Chi-squared T). Among the IPs, a predominance of women was noted among those with a history of neoplasia and under treatment with immunosuppressants and a predominance of men for the rest of the comorbidities, statistically significant ($p<0.001$, Fisher T). Diabetes mellitus was the most common comorbidity, followed by history of malignancy, in both women and men. Men with BCC had more chronic infections and none were on immunosuppressant treatment.

Among the 275 patients diagnosed with BCC included in this study, 262 patients presented with one carcinoma, 11 patients presented with 2 carcinomas, and 2 patients presented with 4 carcinomas, totaling 292 carcinomas included in the study. The average BCC size in IPs was 1.44 cm, and in NIPs was 1.47cm. However, this difference was not statistically significant ($p=0.2577$, Mann-Whitney Test). Furthermore, the distribution of BCC sizes by size group did not differ between IP and NIP ($p=0.0806$, Chi-Square Test). The distribution of BCC sizes by group among the various comorbidities in IPs revealed a high share of patients with diabetes with BCC below 10mm and between 10-30mm and a high share of those with a history of cancer at sizes greater than 30mm. However, the differences were not statistically significant ($p=0.09581$, Fisher Exact Test). Also, no statistically significant difference was revealed between BCC sizes in patients with a history of cancer, compared to other IPs ($p=0.1724$, Fisher

Test). Analysis of the distribution of BCC localizations in IPs and NIPs shows increased weights of BCC in photo-exposed areas for both analysis groups. The proportions of BCC localizations on the trunk and nasal pyramid were similar between IPs and NIPs. Higher percentages of BCC were noted at the level of the cheeks, respectively at the level of the scalp in IPs. However, there is no statistically significant difference between the locations of carcinomas both in IPs and NIPs ($p= 0.3328$, Fisher Exact Test with simulated p-value).

However, particularities with high statistical value were highlighted in the comparative analysis of BCC locations at IPs. Thus, among patients with a history of cancer in the last 5 years, the most frequent localization was on the nasal pyramid (approximately 30%), followed by BCC of the trunk and cheeks. No patient with a neoplastic history had BCC on the scalp or neck. Patients under immunosuppressants showed BCC of the trunk (about half) and cheeks. Patients with diabetes showed a predilection of BCC on the trunk (more than 39%), followed by the nasal pyramid and cheeks. The trunk, followed by the cheeks, represented the most frequent locations in patients with chronic kidney disease; however, no CKD patient presented neck, ears, or scalp BCC. Patients with chronic infections most frequently presented carcinomas on the trunk (about half) and on the nasal pyramid. The localization of BCC in patients with a neoplastic history presents particularities compared to the other IPs, with statistically significantly higher frequencies on the nasal pyramid and cheeks and lower on the trunk ($p=0.01047$, Monte Carlo Simulation Test).

Therefore, this study concluded that more than 80% of the comorbidities of patients with BCC are immunosuppressive comorbidities. In addition, we showed that among the immunosuppressive comorbidities, diabetes is the most frequent, followed by the history of neoplasms, and the fewest cases identified were those of immunosuppressive treatments, chronic kidney disease, and chronic infections. The fact that skin phototype did not vary between patients and that the anatomical distribution of carcinomas did not vary on photo-exposed or intermittently exposed areas between immunocompetent and immunosuppressed patients eliminates the possibility of study bias, related to solar radiation as a potential risk factor for certain age groups.

I find it remarkable that among the patients with a history of cancer, females dominated and that among them the BCC localization on the nose was the most frequent, unlike the other

immunosuppressed patients. In addition, these patients had more carcinomas on the nose and cheeks and fewer on the trunk. This study did not conclude the existence of more aggressive or larger BCCs in immunocompromised patients. This observation opens the perspective of further research on cellular infiltrates in certain categories of immunosuppressed patients.

It should be noted that the ulcerated form of basal cell carcinoma had a high frequency in patients with a history of chronic kidney disease, which is why I propose to perform a prospective study regarding the histopathological and cytological profile of these patients. In conclusion, in this study, we showed that patients with basal cell carcinoma predominantly have immunosuppressive comorbidities and that, unlike patients with SCC, these patients do not develop more aggressive subtypes and BCC does not appear earlier in their life.

Because BCC differs between patients with secondary immunodeficiencies and immunocompetent patients in terms of sex, histopathological subtype, and localization, and multiple BCCs are more common in immunocompromised patients, I consider BCC a clinical marker of immunosuppression. Understanding the relationships between basal cell carcinoma and visceral/ hematogenous neoplasia can represent the promoter of early cancer screening campaigns for patients diagnosed with basal cell carcinoma in our country and beyond.

Chapter 6: Study 2: Comparative evaluation of a conservative ablative immuno-cryosurgery treatment versus surgical excision in basal cell carcinoma

179 patients with histopathologically confirmed non-ulcerated BCC were included in the study: 129 patients opted for CAICT (72.07%) and 50 patients for radical surgery. Among the patients with CAICT, 61 (47.29%) patients chose this treatment because they presented surgical anxiety (rejection of surgical intervention), 19 (14.73%) patients presented comorbidities (insulin-requiring diabetes mellitus, cardiac arrhythmias, anticoagulation, liver diseases, history of neoplasia, etc.) and considered the risks associated with CAICT to be lower, and 49 (37.98%) patients considered age a contraindication to surgery. In addition, most patients considered CAICT for its potential cosmetic advantages as well.

A total of 113 patients completed the full CAICT regimen, 16 dropped out and 10 were excluded from the final analyses due to loss to follow-up. Therefore, 103 patients in the CAICT subgroup were analyzed. The patients presented the following reasons for abandoning CAICT: prolonged duration of treatment, emigration, accessibility (for those from the territory), respectively the discovery of secondary diagnoses and subsequent loss from records. From the surgical subgroup, 4 patients were lost to follow-up and 46 patients were analyzed. A total of 149 patients completed the follow-up procedures and were included in the final statistical analysis.

Regarding patient age, patients in the CAICT subgroup had a mean age of 67.68 years (CI: 65.56, 70), with a minimum age of 31 years and a maximum age of 91 years. Those in the surgical group had a mean age of 61.64 years (CI: 58.03, 65.35), with a minimum age of 31 years and a maximum of 82 years. Consequently, the age of patients in the conservative group was statistically higher (p-value=0.007, Mann-Whitney U Test).

Regarding associated comorbidities, 40.31% of CAICT patients and 68% of surgery patients had no comorbidities. Therefore, patients in the CAICT subgroup presented statistically significantly more comorbidities (p=0.0001, Chi-Squared Test). The histopathological subtypes (superficial, nodular, others) revealed a predominance of the superficial subtype (S) in the conservative subgroup and a predominance of forms other than nodular and superficial (O) in the surgical subgroup. This difference was statistically significant (p=0.005, Chi-Squared Test). The analysis of BCC sizes according to skin phototype revealed a higher mean BCC size in both the conservative subgroup (p=0.011, Mann-Whitney U Test) and the surgical one (p=0.013, Mann-Whitney U Test), in patients with skin phototype II.

Of the 113 patients who underwent CAICT, 112 showed remission at the end of treatment, thus representing a clearance rate of 99.11%. An 82-year-old patient with a superficial BCC on the posterior thorax did not respond to CAICT and was referred to the radiotherapy service. Among the 112 patients with a favorable clinical response to CAICT, 102 performed the re-evaluations according to the recommendations (91%) and 10 were lost to follow-up (LFU). Only one patient had recurrence at follow-up, hence we calculated a recurrence rate of 0.98% of conservative treatment. The recurrent BCC belonged to a 58-year-old female patient with a pigmented BCC of 50 mm at diagnosis who presented with clinical

and histopathological recurrence 48 months after completion of CAICT. Recurrent BCC was removed by surgical excision. The average duration of follow-up of patients in the conservative subgroup was 21.11 +/- 14.57 months. 8.93% of patients were LFU, 22 (19.64%) were followed for less than 12 months, 40 (35.71%) were followed between 12 and 24 months, and 40 (35.71%) were followed for more than 24 months. Telephone follow-up revealed no additional recurrences.

Of the surgery subgroup, 46 patients were followed up and four were LFU. We encountered no recurrence in the surgical subgroup during follow-up. The average duration of follow-up of surgically treated patients was 18 +/- 9.77 months. 8% of patients were LFU, 6% were followed for less than 12 months, 64% were followed for 12 to 24 months, and 22% were followed for more than 24 months. Telephone follow-up revealed no further recurrences.

Considering that 71.42% of conservatively treated patients and 86% of those who underwent surgery were followed up over 12 months, we appreciate that the follow-up process achieved its goal in this study, especially as the telephone follow-up did not reveal new recurrences. We found no statistical dependencies between clearance rate and treatment ($p=1$, Proportion Test), or between recurrence rate and treatment ($p=1$, Proportion Test). Therefore, we did not find that radical surgical excision is superior to this conservative treatment in terms of clearance or recurrence rates. Furthermore, histopathological subtypes were not correlated with cure or recurrence rates ($p=1$, Proportion Test).

A majority of 70.8% of patients in the CAICT subgroup did not experience any AE during treatment. Only 14.16% had mild and 15% moderate local AE (pain and itching, especially after laser/ cryosurgery and/ or imiquimod). Chemowraps with 5-FU were well tolerated. No severe local or systemic AE were encountered in the CAICT subgroup. Two patients presented with secondary impetiginization that responded promptly to a topical antibiotic powder containing zinc bacitracin 250 IU and neomycin sulfate 5000 IU for 5 days. One patient presented with a blister after cryosurgery that was punctured with a sterile needle. None of these three patients dropped out of CAICT. Within the surgical subgroup, 76% of patients had no AE, 14% had mild local AE, and 10% had moderate local AE (after surgery or suture removal). We did not encounter any wound dehiscence or wound impetigo.

In both subgroups, all reported AE were expected and in a causal relationship to treatment. No severe AE were noted in any of the subgroups. No statistical difference was found between the two subgroups regarding AE.

Within the CAICT subgroup, 89.32% of patients declared a very satisfactory CR, and 10.68% moderately satisfactory. In addition, 50% of patients in the surgical subgroup declared a very satisfactory CR, 34.78% moderate, and 15.22% poorly satisfactory. Therefore, telephone self-assessed CR revealed a higher CR ($p<0.001$) in the conservatively treated subgroup. We can consider that the healing process was superior in the CAICT subgroup because no hypertrophic/ keloid scar was encountered after cryosurgery or ablative laser. Overall, pigmentary changes and visible scarring were significantly more prominent after standard surgery.

Therefore, in this research study, we evaluated an effective immunological and cryosurgery ablative conservative treatment for BCC to obtain therapeutic results comparable to classical surgical excision. We studied a treatment free of the potential AE associated with surgery and adapted it to patients with multiple comorbidities that contraindicate surgery, the elderly, or those reluctant to surgery. We also evaluated the cosmetic results after this treatment and compared conservative treatment with surgical treatment in terms of therapeutic response, recurrences, side effects, and cosmetic results.

This conservative treatment was based on the synergism of potentiation between some treatments approved for BCC in monotherapy but with therapeutic responses inferior to classical surgical excision. The protocol we chose was, therefore, a quasi-experimental one, and patients diagnosed with BCC chose their desired treatment after understanding alternatives and signing the patient's informed consent. Most patients chose conservative treatment due to surgical anxiety or advanced age, and the treatment dropout rate was low. Among the causes of abandonment were not the AE, but some personal reasons, related to accessibility or emigration.

Patients who opted for conservative treatment were approximately 6 years older and had more comorbidities compared to those who chose surgical excision. Conservatively treated patients more frequently presented with superficial BCC, and the locations of choice were the trunk and nose. The nodular form and other forms (mixed, pigmented, infiltrative) were more common in operated patients, mostly on the trunk and cheeks.

Patients operated on for basal cell carcinoma had larger carcinomas (with approximately 5.5 mm). For the conservatively treated subgroup, lesions of intermediate size prevailed, with an average size of approximately 14mm, and for the surgical one, the average size was 16mm. However, the proportion of patients with larger carcinomas was higher in the surgical group.

Patients preferred conservative treatment regardless of the size of the tumor, which can often be a psychological factor for the patient to opt for a classic surgical excision. This difference is most obvious in sizes below 10mm, where over 80% of patients opted for conservative therapy.

We recorded over 99% remission in conservatively treated patients, and all surgical excisions showed free margins of tumor tissue and carcinomas were completely excised. Patient follow-up was an overwhelmingly important element of this study for both subgroups. Among patients treated conservatively and in remission, 9% were lost to records, and 91% presented for reevaluations as indicated. Among those reevaluated, we recorded a recurrence rate of less than 1%, in only one patient.

In addition to the increased therapeutic success rates, the increased duration of follow-up is an important element of this study. We recorded a mean follow-up of 21 months for CAICT patients and 18 months for operated patients. Thus, over 70% of conservatively treated patients and over 84% of operated patients were followed for more than 1 year. Telephone follow-up did not record recurrences for any of the patients in the two subgroups.

We did not highlight dependencies between the clearance rate and the treatment chosen by the patients, or between the recurrence rate and the treatment. Therefore, we did not find that radical surgical excision is superior to this conservative treatment in terms of clearance or recurrence rates.

We have also had excellent results in terms of AE. For none of the subgroups we encountered systemic AE, but only local ones: mild and moderate. More than 70% of patients treated conservatively did not experience any AE. About equal percentages experienced mild and moderate stinging/ pruritus/laser pain/ cryosurgery and/or imiquimod side effects. Chemowraps with 5-FU were well tolerated by patients. 76% of operated patients had no local adverse reaction, 14% had mild and 10% had moderate local AE (after surgery or suture

removal). We did not encounter any wound dehiscence or wound impetigo. We found no statistically significant differences between sexes, size groups, or age groups in the distribution of local AE between subgroups.

The CR was superior in CAICT patients from the point of view of the patient's self-assessment. Almost 90% of patients appreciated a very satisfactory result, and the remaining 10% moderately satisfactory. Among the operated patients, 50% declared themselves very satisfied, 35% moderately and 15% slightly satisfied. Although the skin surgical techniques were extremely laborious, patients rated the post-excisional scar as a disadvantage of surgical excision, although we did not encounter keloid formation. In general, pigmentary changes and visible scarring were significantly more prominent after surgical excision.

This study revealed a conservative treatment for non-ulcerated basal cell carcinoma, effective in both less aggressive and more aggressive forms, with a low drop-out rate and minimal local AE. This treatment is not inferior to surgical excision in terms of recurrence, but it is superior to it in terms of the cosmetic result appreciated by the patient.

Chapter 7: Conclusion and personal contributions

The skin represents the most important physical, chemical, and immunological barrier of the human body and uses mechanisms of innate immunity and acquired immunity for this purpose. In the general part of the doctoral thesis, I revealed the main immunological mechanisms involved in skin immune responses. Cellular factors and soluble factors of innate skin immunity as well as acquired immunity simultaneously contribute to the immunological function of the skin. Skin immunological responses in sexually transmitted infections, in some skin cancers (BCC, SCC, melanoma), or patients with altered immunity (immunosuppressed) are particularly complex and represent the target of topical immuno-modulating therapies or focused cryosurgery. Topical therapies with Toll-like receptor modulators (imiquimod), calcineurin inhibitors, and 5FU have changed the therapeutic approach in many dermatological pathologies. These molecules work both individually and synergistically and their use opens up new treatment perspectives, including in some skin cancers. They are today approved for some

forms of skin cancer, but there is growing interest in other forms as well (off-label), especially in patients with a debilitating condition. I believe that the association between cryosurgery and ablative methods can increase the favorable results of topical immunomodulatory therapies, constituting a complex research direction.

In these research studies, we highlighted the deep immunological function of the skin from two different points of view:

- in the first study we revealed the “immune signal” function in patients with immunodepression;

- in the second study we showed the therapeutic response to an innovative combined immunomodulatory therapy.

Both studies were conducted on patients diagnosed with the most common skin neoplasia (basal cell carcinoma), and the results opened up numerous perspectives for further research.

The first study retrospectively (cross-sectionally) evaluated the comorbidities among 275 patients diagnosed with basal cell carcinoma (292 carcinomas) and established correlations between the demographic characteristics of the patients (age, sex, background), the histopathological characteristics of the carcinomas (size, histopathological subtype, aggressiveness) and patients' history of comorbidities. We have shown that most patients with BCC have immunosuppressive comorbidities and that they are more frequently associated with multiple carcinomas. Therefore, it is my opinion that patients presenting with BCC carcinomatosis should be evaluated with high caution for causes of immunosuppression.

I believe that a prospective study on patients with multiple BCCs would bring more information in this direction. In addition, we showed that the localization of carcinomas on the nasal pyramid was the most frequent in patients with a neoplastic history, in contrast to other immunosuppressed patients, and that the ulcerated form of BCC had a high frequency in patients with a history of chronic kidney disease.

Considering these conclusions, I aim to carry out two prospective studies on groups of patients with a neoplastic history, respectively with a history of chronic kidney disease, in which

to study the anatomo-clinical particularities of BCC. Therefore, this study, although it was not prospective (representing a disadvantage), may constitute the premises for the implementation of screening programs in Romania for patients diagnosed with basal cell carcinoma. Given these observations, I consider this study to have achieved its primary and secondary objectives.

In the second research study, we included patients diagnosed with non-ulcered basal cell carcinoma who chose between classical surgical excision and a conservative ablative immunological and cryosurgery treatment, which was based on the potentiation synergism between some standardized treatments for BCC in monotherapy, but with inferior therapeutic results. For this conservative treatment, we recorded a remission rate of over 99% and a recurrence rate of under 1%, as well as cosmetic results superior to classical excision. The objectives of this study were also achieved from the perspective of minimal adverse events experienced by patients, comparable to those of operated patients. The disadvantages of this method consisted of repeated doctor visits, but no patient abandoned the treatment for this reason. The financial considerations associated with this conservative therapy (especially the costs of imiquimod therapy) may represent a challenge for Romanian patients diagnosed with non-ulcerated basal cell carcinoma. I believe that national public health services can support these methods in the future, given the many associated advantages.

The general part of this Ph.D. thesis constituted an exhaustive review of the literature, within which I included, as elements of originality:

a. a review article on soluble factors involved in skin innate immunity;

b. a review article, referring to the immunological escape mechanisms in sexually transmitted infections;

c. particularities of a skin disease with complement deficiency, in a case report article;

d. Figures 1.1-1.8, 2.2, and 2.5 of this Thesis represent own, original creations, created in Bio Render software.

Personal contributions of the Ph.D. Thesis consist of two original studies:

1. The first study characterized the immunosuppression status in patients with histopathologically confirmed BCC. In this study, we collected all patient data, respecting the

inclusion and exclusion criteria, created the database, performed the statistical analysis, figures, tables, and graphs, and demonstrated that:

a. Men with basal cell carcinoma have a higher average age ($p=0.0432$, Unpaired T-test);

b. 81.44% of the comorbidities of patients with basal cell carcinoma represent causes of immunodepression ($p<0.001$, 1 sample prop test);

c. The distribution of comorbidities among immunosuppressed patients is uneven, and diabetes mellitus was the most prevalent, followed by the history of neoplasms, treatment with antirheumatic immunosuppressants, chronic kidney disease, and chronic infections ($p<0.001$, Chi-Square-Goodness-Fit) ;

d. Immunosuppressed patients had an average age of approximately 5 years older ($p=0.0003$, Mann Whitney T);

e. Women with BCC had a more frequent history of neoplasia and immunosuppressive treatments, and men most frequently presented a history of CKD, diabetes, and chronic infections ($p<0.001$, Fisher T);

f. immunosuppressed patients do not show more aggressive BCC ($p=0.3589$, Chi-Squared Test) or larger sizes ($p=0.2577$, Mann Whitney Test);

g. patients with a history of cancer present more frequently BCC on their nose and cheeks ($p=0.01047$, Monte Carlo Simulation Test);

h. patients with a history of chronic kidney disease presented more frequently the ulcerated subtype of BCC ($p=0.0064$, Fisher Exact Test with simulated p-value).

2. The second study evaluated a conservative ablative immuno-cryosurgery treatment for non-ulcerated BCC compared with standard surgical excision. I actively participated in the procedures (both conservative and surgical excisions), I participated in the monitoring of patients, adverse events, and the healing process, in the process of *follow-up*, we created a database, further processing (statistical analysis, tables, graphs, figures) and demonstrated that:

a. most patients (72%) prefer a conservative therapy;

b. the age of patients in the conservative subgroup was significantly higher (p-value=0.007, Mann-Whitney U Test);

c. patients in the conservatively treated subgroup presented statistically significantly more comorbidities (p=0.0001, Chi-Squared Test);

d. patients in the surgical group presented carcinomas approximately 5.5 mm larger (p=0.0001, Mann-Whitney U Test);

e. the success rate of conservative treatment was 99.11%, and that of surgery was 100%;

f. 91% of conservatively treated patients showed up for follow-up, the average duration of follow-up was 21 months and the recurrence rate was 0.98%;

g. 92% of surgically treated patients showed up for follow-up, the average duration of follow-up was 18 months and they did not show any recurrence;

h. We found no statistical dependencies between clearance rate and treatment (p=1.2, sample Test for equality of proportions with continuity correction), between recurrence rate and treatment (p=1.2, sample Test for equality of proportions with continuity correction), or between local adverse events and treatment (p=0.676, Pearson's Chi-squared test);

i. the cosmetic results were superior in conservatively treated patients (p<0.001, Fisher's Exact Test).

References

1. Bonefeld, CM; Geisler, C. The role of innate lymphoid cells in healthy and inflamed skin. Immunol. Lett. 2016, 179, 25–28. https://doi.org/10.1016/j.imlet.2016.01.005.
2. Matsui, T. & Amagai, M. Dissecting the formation, structure and barrier function of the stratum corneum. Int. Immunol. 27, 269–280 (2015).
3. Rajsheker, S. et al. Crosstalk between perivascular adipose tissue and blood vessels. Curr. Opin. Pharmacol. 10, 191–196 (2010).
4. Meyer-Hoffert, U. Innate Immunity in the Skin. J. Innate Immun. 2012, 4, 223–224.https://doi.org/10.1159/000336867.

5. Ermertcan, A.; Ozturk, F.; Gunduz, K. Toll-like receptors and skin. J. Eur. Acad. Dermatol. Venereol. 2011, 25, 997–1006.https://doi.org/10.1111/j.1468-3083.2011.04049.x.
6. Palianus, J.; Meri, S. Complement System in Dermatological Diseases—Fire Under the Skin. Front. Med. 2015, 2, 3. https://doi.org/10.3389/fmed.2015.00003.
7. Latz, E.; Xiao, TS; Stutz, A. Activation and regulation of the inflammasomes. Nat. rev. Immunol. 2013, 13, 397–411. https://doi.org/10.1038/nri3452.
8. He, Y.; Zeng, MY; Yang, D.; Motro, B.; Núñez, G. NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. Nature 2016, 530, 354–357.https://doi.org/10.1038/nature16959.
9. Yamasaki, K.; Gallo, RL; Kenshi, Y.; Richard, LG Antimicrobial peptides in human skin disease. Eur. J. Dermatol. EJD 2007, 18, 11–21.https://doi.org/10.1684/ejd.2008.0304.
10. Herman, A.; Herman, AP Antimicrobial peptides activity in the skin. Skiing. Res. Technol. 2019, 25, 111–117.https://doi.org/10.1111/srt.12626.
11. Scurtu LG, Simionescu O. Soluble Factors and Receptors Involved in Skin Innate Immunity-What Do We Know So Far? Biomedicines. 2021 Nov 29;9(12):1795. doi: 10.3390/biomedicines9121795.
12. Kashem SW, Haniffa M, Kaplan DH. Antigen-Presenting Cells in the Skin. Annu Rev Immunol. 2017 Apr 26;35:469-499. doi: 10.1146/annurev-immunol-051116-052215. Epub 2017 Feb 6.
13. Kashem, SW, Haniffa, M. & Kaplan, DH Antigen-presenting cells in the skin. Ann. rev. Immunol. 35, 469–499 (2017).
14. Ho AW, Kupper TS. T cells and the skin: from protective immunity to inflammatory skin disorders. Nat Rev Immunol. 2019 Aug;19(8):490-502. two: 10.1038/s41577-019-0162-3.
15. Jiang, X. et al. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. Nature 483, 227–231 (2012).
16. Sabat R, Wolk K, Loyal L, Döcke WD, Ghoreschi K. T cell pathology in skin inflammation. Semin Immunopathol. 2019 May;41(3):359-377. two: 10.1007/s00281-019-00742-7.
17. Halle S, Halle O, Forster R. Mechanisms and dynamics of T cell-mediated cytotoxicity in vivo. Trends Immunol. 2017;38:432–443.

18. Becattini S, Latorre D, Mele F, Foglierini M, De Gregorio C, Cassotta A, Fernandez B, Kelderman S, Schumacher TN, Corti D, Lanzavecchia A, Sallusto F. T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. Science. 2015;347:400–406.

19. Scurtu LG, Jinga V, Simionescu O. Fascinating Molecular and Immune Escape Mechanisms in the Treatment of STIs (Syphilis, Gonorrhea, Chlamydia, and Herpes Simplex). Int J Mol Sci. 2022 Mar 24;23(7):3550. doi: 10.3390/ijms23073550.
20. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331(6024):1565–70.
21. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006;6(10):715–27.
22. Pettersen JS, Fuentes-Duculan J, Suarez-Farinas M, Pierson KC, Pitts-Kiefer A, Fan L, Belkin DA, Wang CQ, Bhuvanendran S, Johnson-Huang LM, et al. Tumor-associated macrophages in the cutaneous SCC microenvironment are heterogeneously activated. J. Investig. Dermatol. 2011;131:1322–1330. two: 10.1038/jid.2011.9.
23. Moore RJ, Owens DM, Stamp G., Arnott C., Burke F., East N., Holdsworth H., Turner L., Rollins B., Pasparakis M., et al. Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. Nat. Med. 1999;5:828–831. two: 10.1038/10552.
24. Bald T., Quast T., Landsberg J., Rogava M., Glodde N., Lopez-Ramos D., Kohlmeyer J., Riesenberg S., van den Boorn-Konijnenberg D., Homig-Holzel C., et al . Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in melanoma. Nature. 2014;507:109–113. doi: 10.1038/nature13111.
25. Papakostas, D.; Stockfleth, E. Topical treatment of basal cell carcinoma with the immune response modifier imiquimod. Future Oncol. 2015, 11, 2985–2990.
26. Wagstaff AJ, Perry CM. Topical imiquimod: a review of its use in the management of anogenital warts, actinic keratoses, basal cell carcinoma and other skin lesions. Drugs. 2007;67(15):2187-210. two: 10.2165/00003495-200767150-00006.
27. Garcia-Mouronte E, Berna-Rico E, de Nicolas-Ruanes B, Azcarraga-Llobet C, Alonso-Martinez de Salinas L, Bea-Ardebol S. Imiquimod as Local Immunotherapy in the Management of Premalignant Cutaneous Conditions and Skin Cancer. Int J Mol Sci. 2023 Jun 29;24(13):10835. doi: 10.3390/ijms241310835.

28. Wigmore PM, Mustafa S, El-Beltagy M, Lyons L, Umka J, Bennett G. Effects of 5-FU. Adv Exp Med Biol. 2010;678:157-164. doi:10.1007/978-1-4419-6306-2_20.
29. Levy S, Furst K, Chern W. A novel 0.5% fluorouracil cream is minimally absorbed into the systemic circulation yet is as effective as 5% fluorouracil cream. Knife. 2002;70(2 Suppl):14-21.
30. Tian J, Zhang D, Kurbatov V, et al. 5-Fluorouracil efficacy requires anti-tumor immunity triggered by cancer-cell-intrinsic STING. EMBO J. 2021;40(7):e106065. doi:10.15252/embj.2020106065.
31. Hoffmann NE, Bischof JC. The cryobiology of cryosurgical injury. Urology. 2002 Aug;60(2 Suppl 1):40-9.
32. Bischof JC. Quantitative measurement and prediction of biophysical response during freezing in tissues. Annu Rev Biomed Eng. 2000;2:257-88.
33. Hoffmann NE, Bischof JC. Cryosurgery of normal and tumor tissue in the dorsal skin flap chamber: Part I--thermal response. J Biomech Eng. 2001 Aug;123(4):301-9.
34. S. Gazzaniga, A. Bravo, SR Goldszmid, et al., Inflammatory changes after cryosurgery-induced necrosis in human melanoma xenografted in nude mice, Journal of Investigative Dermatology. 116 (5) (2001) 664–671.

Published scientific papers

1. **Lucian G. Scurtu**, Olga Simionescu. Soluble Factors and Receptors Involved in Skin Innate Immunity-What Do We Know So Far? Biomedicines. 2021, 29;9(12): 1795. ISI (Web of Science) and PubMed indexing. IF=4.757. https://pubmed.ncbi.nlm.nih.gov/34944611/

2. Lucian G. Scurtu, Viorel Jinga, Olga Simionescu. Fascinating Molecular and Immune Escape Mechanisms in the Treatment of STIs (Syphilis, Gonorrhea, Chlamydia, and Herpes Simplex). International Journal of Molecular Sciences. 2022, 23(7): 3550. ISI (Web of Science) and PubMed indexing. IF=5.6. https://pubmed.ncbi.nlm.nih.gov/35408911/

3. **Lucian G. Scurtu**, Marian Petrica, Francesca Scurtu, Anca Angela Simionescu, Marco I. Popescu and Olga Simionescu. Basal cell carcinoma—a clinical indicator of immunosuppression. Frontiers in Medicine. 2024, 11: 1381492.ISI (Web of Science) and PubMed indexing. IF=3.9. https://doi.org/10.3389/fmed.2024.1381492

4. **Lucian G. Scurtu**, Marian Petrica, Mariana Grigore, Alina Avram, Ion Popescu, Olga Simionescu. A Conservative Combined Laser Cryoimmunotherapy Treatment vs. Surgical Excision for Basal Cell Carcinoma. Journal of Clinical Medicine. 2022, 11(12): 3439. ISI (Web of Science) and PubMed indexing. IF=3.9. https://pubmed.ncbi.nlm.nih.gov/35743507

5. **Lucian G. Scurtu**, Mariana Costache, Daniela Opriș-Belinski, Olga Simionescu. Hypocomplementemic Urticarial Vasculitis Associated With Hashimoto's Thyroiditis and Hepatitis B Virus Infection: A Case Report. Cureus. 2022, 14(9):e29643. ISI (Web of Science) and PubMed indexing. IF=1.2. https://pubmed.ncbi.nlm.nih.gov/36320953/