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LOCAL MANIFESTATIONS OF CELLULAR IMMUNITY IN BREAST CARCINOMA IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

311.02 – PATHOLOGICAL ANATOMY

Summary of PhD Thesis in Medical Sciences

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The thesis was elaborated within the Department of histology, cytology and embryology and the Morphology Laboratory of the PI State University of Medicine and Pharmacy „Nicolae Testemitanu", Doctoral School in Medical Sciences

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CONTENT

THE RESEARCH CONCEPTUAL FRAMEWORK

Actuality and importance of the researched problems. Relevance and importance of the problem under discussion. Oncological and endocrine diseases represent a challenge for contemporary medicine, reflecting a worrying dynamic regarding their prevalence and incidence, which are continuously increasing. In oncology, breast cancer stands out as the leading neoplasia detected in women, with a global incidence of 11.4%, almost similar to the Republic of Moldova, where it reaches a rate of 11.9% [1]. These statistics not only highlight the magnitude of the problem but also emphasize the acute need to develop effective prevention and treatment strategies [2].

The tumor microenvironment represents a crucial element in the pathogenesis of breast cancer and is characterized by the defective functioning of various cell types, including macrophages, fibroblasts, endothelial cells, and leukocytes [3]. This complex interaction between tumor cells and their microenvironment contributes to the progression and metastasis of the neoplasia. Currently considered an essential factor in carcinogenesis, including breast carcinogenesis, the interrelations in question present complex and multifactorial characteristics, which are influenced by the genetic and phenotypic context of the tumor. According to Weber *et al.* (2006), the tumor stroma not only manifests a distinct genetic signature depending on the carcinoma's phenotype but also plays an active role in promoting neoplastic transformations [4]. The presence of a significant number of lymphocytes in the tumor, particularly T lymphocytes with cytotoxic function, $CD8^+$, is often interpreted as an indicator of an active immune response directed against the neoplasm. However, the specificity and effectiveness of this immune response vary depending on the biological characteristics of the tumor and the composition of the tumor immune infiltrate. In this context, the immune cells involved in the antitumor response also play an essential role in the initiation and perpetuation of chronic inflammation, a process recognized as a central mechanism in the pathogenesis of type 2 diabetes mellitus (T2DM).

Innate immunity represents the body's first line of defense, having a rapid and non-specific character, and involves various cell populations, such as macrophages, neutrophils, dendritic cells, and other cells of the immune system, which recognize pathogens and respond through mechanisms of phagocytosis and the secretion of inflammatory mediators [5].

Diabetes mellitus represents a major public health problem globally, affecting approximately 10% of the population and showing a constant upward trend, especially among the elderly population [6]. In a geriatric context, it presents distinct clinical-biological features, being predominantly represented by T2DM, characterized by a progressive evolution and an increased risk of complications, especially in the absence of adequate therapeutic management [7].

The purpose consisted in studying invasive ductal breast carcinoma associated with T2DM in order to evaluate the tumor's molecular profile and the local cellular immune status**.**

The objectives:

1. Determination and comparative analysis of the morphological changes of the mammary gland in postmenopausal period and of invasive breast carcinoma of NST type, in the context of T2DM;
2. Comparative evaluation of the local cellular immune status, through the quantification of leukocyte subpopulations $CD3^+$, $CD4^+$, $CD8^+$, $CD45^+$, $CD56^+$ and $CD68^+$ macrophages, in non-neoplastic mammary parenchyma with age-related involution changes, and in breast carcinoma, in patients with and without type 2 diabetes mellitus;
3. Analysis of the local angiogenic potential, by determining the expression of the $CD34^+$ marker in invasive breast carcinoma of NST type, depending on the presence of type 2 diabetes mellitus;
4. Characterization of the molecular phenotype of the postmenopausal mammary gland and of invasive breast carcinoma of NST type, by evaluating the expression of ER, PR, HER2, and Ki67 markers, in patients with and without type 2 diabetes mellitus;

5. Establishing the statistical relationships between the local cellular immune status, angiogenesis, and the tumor molecular phenotype, in order to highlight the influence of type 2 diabetes mellitus on the tumor microenvironment.

Scientific research methodology. The biological specimens and clinical data of the patients were selected from the archive of medical records and histopathological blocks of the PI Oncology Institute of the Republic of Moldova, Pathology Service. Tissue fragments were fixed in 10% buffered formalin (pH 7.2–7.4), then processed by dehydration, clearing, and embedding in paraffin, obtaining 3–5 μm sections for HE staining and immunohistochemistry (IHC). The tumor profile was evaluated histopathologically on HE, and the local molecular/ cellular immune profile was established using antibodies for ER, PR, HER2, Ki67, CD3, CD4, CD8, CD34, CD45, CD56 and CD68, with deparaffinization/ antigen retrieval in PT Link (Target Retrieval Solution, 60 min). Data were entered into MS Excel 2017 and analyzed in SPSS (mean, SD, median, IQR, range; Spearman/ Pearson correlations; Mann–Whitney U and t-Student tests), with a significance threshold of $p \leq 0.05$. The research was conducted at the Department of histology, cytology, and embryology, the Laboratory of Morphology of the State University of Medicine and Pharmacy „Nicolae Testemitanu", Republic of Moldova. The study protocol was approved by the Ethics Committee of the State University of Medicine and Pharmacy „Nicolae Testemitanu" (decision no. 7/ 70/ 12.11.2021).

The scientific novelty and originality of the obtained results. For the first time, a complex, comparative study of the local cellular immune status in breast carcinoma in diabetic and non-diabetic patients was performed. The approach allows a deeper understanding of the metabolic impact of diabetes on the tumor microenvironment.

The scientific and applied problem solved. The research elucidated the changes in local cellular immunity in breast carcinoma and the influence of type 2 diabetes mellitus on the tumor microenvironment. By quantifying CD3, CD4, CD8, CD34, CD45, CD56, and CD68 in the intra- and peritumoral stroma, it was confirmed that T2DM significantly modulates the local immune status.

The applied value of the work. The results support the optimization of the clinical management of patients with breast carcinoma, especially in the presence of T2DM, and provide premises for personalized therapeutic strategies. The data show that metabolic disturbances in T2DM can alter local cellular immunity in the tumor microenvironment, justifying the adaptation of treatment and monitoring protocols, with an impact on prognosis and quality of life.

Main scientific results submitted for defense:

1. T2DM constitutes an active factor of remodeling the tumor microenvironment in breast carcinoma, significantly influencing the local cellular immune status and the dynamics of the tumor process;
2. The association of T2DM with breast carcinoma, from a histopathological point of view, correlates with larger tumor sizes, highlighting at the same time a close relationship between histological grade and mitotic activity;
3. T2DM causes significant changes in the T-lymphocyte infiltrate in breast carcinoma, characterized by a tendency to reduce the density of intratumoral $CD8^+$ cytotoxic T lymphocytes, especially in high-grade tumors (G3);
4. Metabolic disturbances in T2DM favor the infiltration of $CD68^+$ macrophages in the tumor microenvironment, both at the intratumoral and peritumoral levels;
5. In the context of T2DM, a dysfunction of $CD34^+$ progenitor endothelial cells is highlighted, with the alteration of tumor angiogenesis;
6. From a molecular perspective, although T2DM does not change the distribution of the molecular subtypes of breast carcinoma, it influences the relationships between hormonal and proliferation markers;
7. T2DM is associated with an increased infiltration of $CD56^+$ cells at the peritumoral level, their density correlating negatively with mitotic activity and HER2 expression and positively with intratumoral vascularization (CD34it).

Implementation of scientific results: The obtained results were implemented in the scientific activity of the Morphology Laboratory of the State University of Medicine and Pharmacy „Nicolae Testemițanu", as well as in the practical activity of the Pathological Anatomy Department of the PI Oncology Institute.

Approval of the results: The obtained results and the basic ideas of the thesis were presented and discussed at the following scientific meetings: National Scientific Conference with International Participation "Cells and tissues transplantation. Actualities and perspectives. The 3rd edition", dedicated to the 80th anniversary of the founding of the State University of Medicine and Pharmacy "Nicolae Testemianu" (Chișinău, 2025); National Scientific-Practical Conference with international participation "History, current affairs and perspectives of the pathological anatomy service in the Republic of Moldova" (Chișinău, 2025); National Congress of Oncology, VIth edition (Chișinău, 2025); Anniversary Congress "80 years of innovation in health and medical education" (Chișinău, 2025); 2nd International Cappadocia Scientific Research Congress (Nevşehir, Turkey, 2022); 2nd International Siirt Conference on Scientific Researches (Siirt, Turkey, 2022); ISPEC 13th International Conference on Engineering & Natural Sciences (Burdur, Turkey, 2022); 4th International Congress of Multidisciplinary Studies in Medical Sciences (Antalya, Turkey, 2022); 3rd International Conference on Medical & Health Sciences (Bingöl, Turkey, 2021); Conference "Integration through research and innovation" (Chișinău, 2021); II International Halich Congress on Multidisciplinary Scientific Research (Istanbul, Turkey, 2021); International Harran Health Sciences Congress-III (Şanlıurfa, Turkey, 2021). The results of this study were discussed and approved at the meeting of the Department of histology, cytology and embryology of SUMPh "Nicolae Testemițanu" (minutes No. 8, of 12.02.2025) and of the Profile Scientific Seminar 311. ANATOMY AND MORPHOLOGY; 351. INTERDISCIPLINARY MEDICINE (minutes no. 01.04.2026).

Publications on the thesis topic. On the topic of the thesis, 40 scientific papers were published, out of which 3 articles in international journals, 4 in national journals, and 33 abstracts in the proceedings of national and international scientific conferences

1. BREAST CARCINOMA ASSOCIATED WITH DIABETES MELLITUS – PATHOLOGIES WITH COMPLEX EVOLUTION AND INCOMPLETELY ELUCIDATED BIOLOGICAL INTERACTIONS

Breast cancer constitutes the uncontrolled proliferation of cells with primary origin in the mammary gland. According to GLOBOCAN 2018 estimates, the incidence is approximately 10%, both globally and in the Republic of Moldova [1]. Among metabolic comorbidities, diabetes mellitus represents a frequently encountered condition in women newly diagnosed with breast cancer [7,8].

1.1. Cellular immunity in the breast carcinoma stroma

The immune system plays a fundamental role in maintaining tissue homeostasis, and its dysfunctions contribute significantly to the initiation and progression of neoplastic processes [9,10].

Tumor-infiltrating lymphocytes (TILs) are the most frequently evaluated intra- and peritumorally in breast cancer, but data regarding their density are uneven and sometimes contradictory due to molecular subtypes and quantification methods. According to Hendry *et al.* (2017), increased TILs are reported more frequently in triple-negative and HER2-positive tumors [11].

Therefore, the evaluation of the stromal lymphocytic infiltrate in breast cancer is performed based on specific criteria, among which the following can be mentioned: the evaluation of TILs must be performed at 20–40x magnifications [12], on high-quality FFPE sections, with an optimal thickness of 4–5 μm , including all mononuclear cells (lymphocytes and plasma cells) [13]. For accuracy, it is recommended to use IHC markers and report the type of infiltrate (minimal/ partial/ subtotal /total), relevant to the degree of tumor involvement [12].

1.2. The molecular profile of breast carcinoma

The estrogen receptor mediates the biological effects of estrogens on cells. Cavener *et al.* (2010) mentioned that signaling through ER receptors influences lipid metabolism and insulin sensitivity,

crucial processes in diabetes mellitus pathology [14] Thus, ER expression in breast pathology becomes an important factor influencing both tumor biology and treatment response [15]. The HER2 marker, also known as ErbB2, represents a transmembrane receptor [16]. Under physiological conditions, HER2 shows low expression in several organs, including breast glandular tissue [17]. In approximately 20% of breast carcinoma cases, HER2 shows amplification, a phenomenon associated with increased tumor aggressiveness [18].

2. MATERIALS AND RESEARCH METHODS

2.1. General research methodology

General research methodology to achieve the purpose and objectives, a methodological plan was developed that included: argumentation of the problem; development and approval of the study protocol; selection and collection of study material; performance of histological and immunohistochemical procedures; collection, analysis, and synthesis of the obtained results; elaboration of recommendations and their application in practice; formulation of conclusions. The work was carried out within the Department of histology, cytology and embryology and the Morphology Laboratory of the State University of Medicine and Pharmacy „Nicolae Testemițanu", Chisinau, Republic of Moldova. The study was approved by the Research Ethics Committee of the State University of Medicine and Pharmacy „Nicolae Testemitanu" (decision no. 7/ 12.11.2021)

2.2. Study Material

The research object served the mammary gland specimens taken postoperatively after total mastectomy within the PI Oncology Institute of the RM during the years 2018-2020. The collected material, invasive ductal breast carcinoma of NST type, from patients who were not subjected to preoperative treatments, was divided into 2 groups: group I included tumors collected from 43 patients with breast cancer without T2DM; group II – 29 breast tumors from patients with T2DM. Mammary gland samples (bilateral), collected at the Center of Forensic Medicine of the RM (2021-2022), from 30 accidentally deceased women, served as a control, the material being collected in the first 24 hours after death.

The cases were selected based on the following inclusion and exclusion criteria:

A. Inclusion criteria:

- confirmed diagnosis of breast cancer (NST ductal type cancer);
- with or without confirmed diagnosis of T2DM, depending on the group;
- age ≥50 years;

B. Exclusion criteria:

- patients diagnosed with type 1 diabetes mellitus;
- autoimmune diseases;
- age under 50 years.

The serum glucose level was evaluated fasting, at admission in venous blood, by colorimetric method (Selectra Pro XL biochemical analyzer, NL) in all patients with tumors.

2.3. Histological and immunohistochemical methods

All sections were initially stained by the classical method. The detailed histopathological characteristic was carried out according to the degree of differentiation (WHO, 5th edition) [19]. The global tumor grade was calculated by summing the scores for each criterion (total 3–9), being classified as follows: grade I (3–5 points), grade II (6–7 points) and grade III (8–9 points). The cases were evaluated independently by two pathologists, and representative sections were selected for immunohistochemical analysis.

In accordance with the number of markers used in the study, 11 silanized slides were prepared for each case, the sections being made from the same block as for HE. All deparaffinization, retrieval, and visualization procedures were performed automatically, using Autostainer Link 48 (Dako Colorado, USA).

2.4 Quantification methods

The evaluation of receptor expression was performed by a semi-automated technique described by Suciu *et al.* (2014) [20], using Axio Imager A2 (Carl Zeiss, Germany) and Olympus BX53 (Japan) microscopes, by calculating the total score (TS) as the sum of the proportion score and the intensity score. Cases with TS 0–2 were considered negative, and those with TS 3–8 – positive (Figure 1).

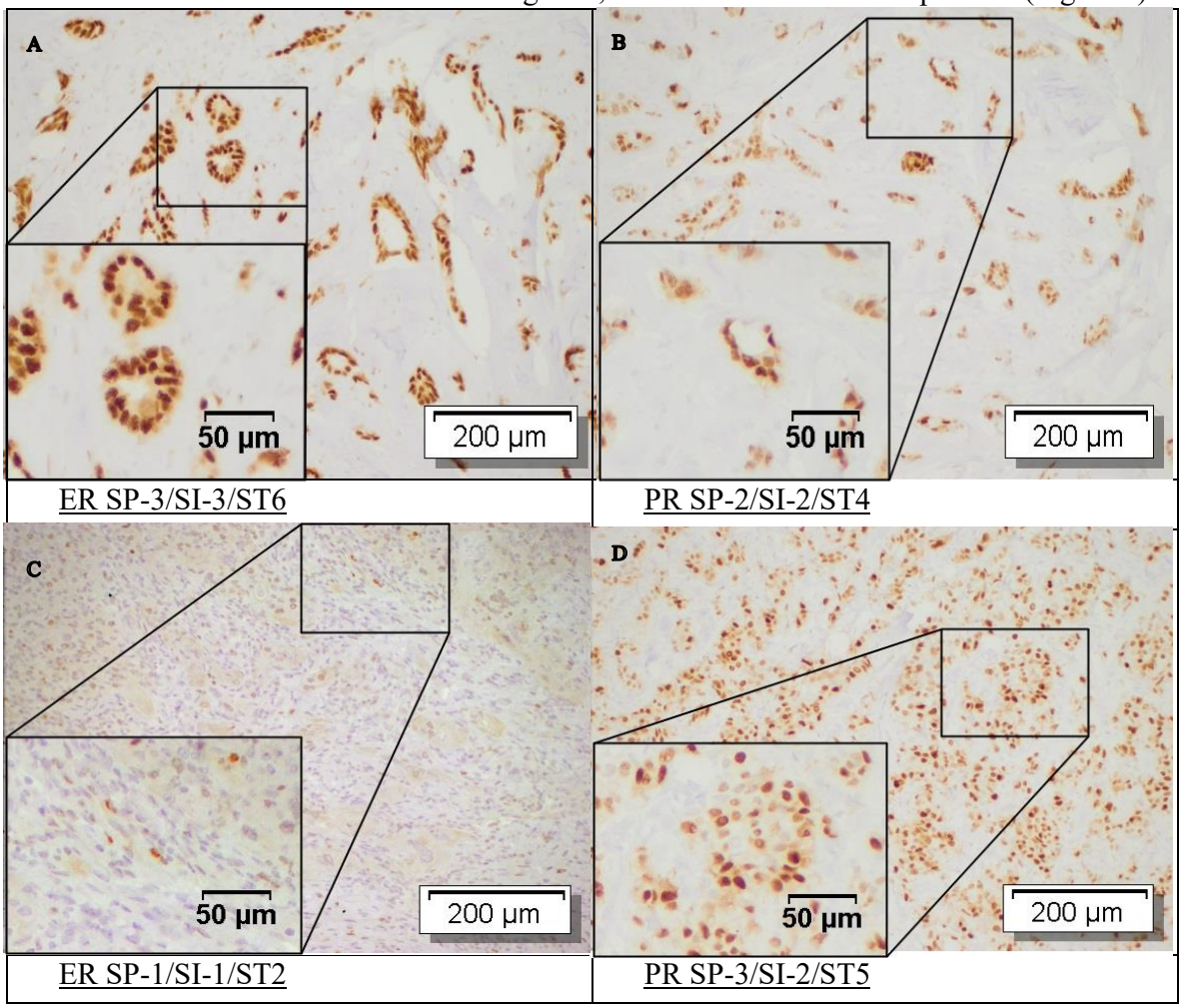

Figure 1. NST type breast carcinomas. Tumor cells immunostained for ER and PR.

HER2 overexpression was evaluated according to ASCO 2018 criteria: 2+ and 3+ tumors were considered HER2 positive. In all HER2 positive cases, dual-color chromogenic *in situ* hybridization techniques were used, run on the BenchMark ULTRA platform. During evaluation of CD3, CD4, CD8, CD34, CD45, CD56, CD68, Ki67 expression, were initially identified the regions of interest, intra- and peritumoral (CDit/ CDpt). The results were presented as the percentage average of 10 fields / 1000 cells. The threshold for Ki67 was ≥14. Subsequently, the cases were divided into groups according to diagnosis, the presence/ absence of T2DM, and molecular subtype (table 1).

2.5. Statistical analysis

The study included 72 patients with breast cancer: 43 BC cases without T2DM and 29 cases were associated with T2DM. The control group included 30 accidentally deceased women, from whom mammary gland samples were collected. The research was retrospective, morphological, based on histological and immunohistochemical evaluation. Hematoxylin-eosin staining and a panel of 11 IHC markers were used: ER, PR, HER2, Ki67, CD3, CD4, CD8, CD34, CD45, CD56, and CD68. The macro-microscopic description was performed according to WHO recommendations (2022), and quantification/ scoring according to Allred *et al.* (1998) and Suciu *et al.* (2014) [20–22]. Depending on the expression of ER, PR, HER2, and Ki67, the tumors were grouped into 5 molecular subtypes based on WHO recommendations.

The data were systematized in MS Access 2016, and the statistical analysis was performed in SPSS v23.0: mean, standard deviation, median, interquartile range, confidence intervals, Spearman/ Pearson correlations, comparisons between groups by Mann–Whitney U and Student's t-tests.

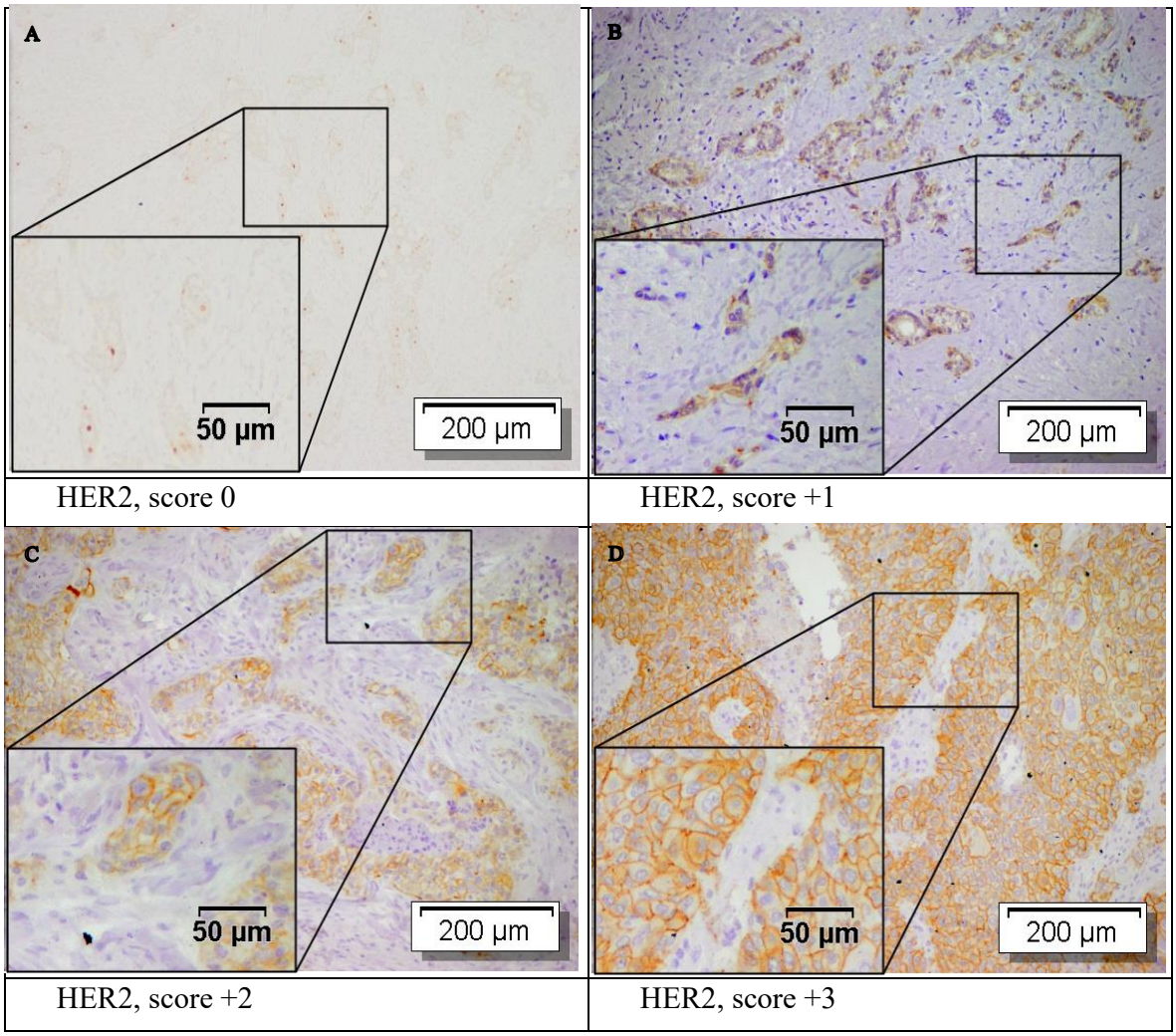

Figure 2. Invasive ductal breast carcinoma of NST type. Note: tumor cells immunostained for HER2 (scores 0 -+3). IHC staining, counterstained with Lille's hematoxylin (20x).

Table 1. Distribution of collected specimens based on morphological and immunohistochemical diagnosis

Molecular subtype	BC (43 cases)			BC+ T2DM (29 cases)			Total (72 cases)
	G1	G2	G3	G1	G2	G3	
Luminal A	2 (4.7%)	2 (4.7%)		1 (3.4%)	2 (6.9%)		7 (9.7%)
Luminal B, HER2-		14 (32.5%)	4 (9.3%)		12 (41.3%)	2 (6.9%)	32 (44.4%)
Luminal B, HER2+	1 (2.3%)	3 (7.0%)	5 (11.6%)		5 (17.2%)		14 (19.4%)
HER2+		3 (7.0%)	3 (7.0%)			3 (10.3%)	9 (12.5%)
Triple-negative		2 (4.7%)	4 (9.3%)		1 (3.4%)	3 (10.3%)	10 (13.9%)
Total	**3 (7%)**	**24 (55.8%)**	**16 (37.2%)**	**1 (3.4%)**	**20 (68.9%)**	**8 (27.6%)**	**72 (100%)**

Note: G1-G3 – tumor differentiation grade; BC – breast cancer; T2DM – type 2 diabetes mellitus

3. MORPHOLOGICAL CHARACTERISTICS OF THE MAMMARY GLANDS IN NORMAL AND PATHOLOGICAL CONDITIONS

The morphological analysis of the mammary glands represents a fundamental element in understanding the mechanisms of mammary carcinogenesis

3.1. Morphology of the mammary gland in normal conditions

In the collected samples, changes such as periductal fibrosis, glandular involution, increased proportion of stromal connective tissue, reduction in the amount of adipose tissue, vascular sclerosis, calcium deposits, and discrete lymphocytic infiltrates were highlighted. Periductal fibrosis was identified in 15 cases (50%) of all analyzed samples, and the involution of glandular components in 11 cases (36.7%). Sclerosis of the stromal connective tissue was present in 14 cases (46.7%), the decrease in adipose tissue in 11 cases (36.7%), and vascular sclerosis in 13 cases (43.3%). The thickening of vascular walls and/or the reduction of the vascular lumen diameter, associated with calcium deposits, were found in 11 cases (36.7%). Lymphocytic infiltrate was present in 15 cases (50%) among the examined cases.

3.2. The structure of breast carcinoma in the absence of carbohydrate metabolism disorders

The study included patients with NST breast carcinoma, with an average age of 63.7±6.9 years. Peripheral glycemia constituted 5.04±1.10 mmol/L (Me 4.7 mmol/L), and the macroscopic tumor size varied between 0.70–5.00 cm, with an average of 2.10±0.80 cm (Me 2.0 cm). When stratified by age, the tumor sizes were: 51–60 years: 1.98±0.2 cm (Me 2.0; 15 cases/34.9%); 61–70 years: 2.21±0.3 cm (Me 2.1; 23 cases/53.5%); 71–80 years: 2.0±0.3 cm (Me 2.0; 5 cases/11.6%). Although a tendency towards larger tumors is observed in the 61–70 years group, the differences were not statistically significant: 51–60 *vs* 61–70 (p=0.792), 61–70 *vs* 71–80 (p=0.888), 51–60 *vs* 71–80 (p=0.951).

3.3. Morphology of breast carcinoma associated with diabetes mellitus

Subsequently, the morphological characteristics of breast carcinoma associated with diabetes mellitus were analyzed. The average age of the patients was 64.5±7.9 years, and the average glycemia was 9.9±3.1 mmol/L (Me 9.0 mmol/L). The tumor sizes varied between 1.0–12.0 cm, with an average of 2.9±2.2 cm (Me 2.2 cm); by age groups: 51–60 years (10 cases; 34.5%) – 2.67±1.3 cm, 61–70 years (15 cases; 51.7%) – 2.92±1.5 cm, 71–80 years (4 cases; 13.8%) – 3.1±1.4 cm. Although descriptively larger tumors are observed in the 71–80 years group, the association between age and tumor size was very low and not significant (r=-0.04; p=0.84). According to the pT category, pT1 was identified in 13 cases (44.8%), pT2 in 12 cases (41.4%), pT3 in 3 cases (10.3%), and pT4 in 1 case (3.4%), indicating the predominance of early/intermediate stages (pT1–pT2).

4. THE INFLUENCE OF T2DM ON THE TUMOR MICROENVIRONMENT

4.1. Identification of CD3, CD4, CD8, CD34, CD45, CD56, CD68 expression in the intact mammary gland

The evaluation of $CD3^+$ T lymphocytes highlighted a mixed population of the compartments, without significant quantitative differences between the ductal epithelium and the periductal stroma (intraepithelial: 21.7±18.5; Me=16; 0–55 *vs* periductal: 22.2±15.7; Me=16.5; 14–54). T helper lymphocytes ($CD4^+$) significantly predominated in the periductal stroma (20.2±8.1; Me=21) compared to the ductal epithelium (8.5±6; Me=8) (p=0.011), confirming $CD4^+$ as the majority population of the periductal infiltrate, while the distribution of suppressor T lymphocytes (CD8+) was similar in both areas (10.8 *vs* 10.2; p=0.25). The CD34 marker showed a strict topography: absent vascularization in the ductal epithelium, but present and variable periductally (24.8±11.2; 16–45; p=0.01). $CD45^+$ cells had a relatively uniform distribution (periductal: 36.2±13.1 *vs* intraepithelial: 30.3±14.2; p=0.48); NK cells ($CD56^+$) showed a tendency of intraepithelial accumulation (15.3±11.7 *vs* 5.5±3.7; p=0.14), and macrophages ($CD68^+$) were numerically denser periductally (29.5±10.3 *vs* 11.8±8.1), without statistical significance (p=0.33).

4.2. Evaluation of local cellular immunity and angiogenic potential in the stroma of NST-type tumors

The comparative analysis highlighted a peritumoral predominance of the immune infiltrate and vascular density, with distinct clinicopathological correlations between compartments. The $CD3^+$ infiltrate was higher peritumorally (33.6±16.3) than intratumorally (25.1±15.6), at the limit of significance (p=0.05); intratumorally, $CD3^+$ correlated inversely with Ki67 (r_s=−0.37; p=0.02), and peritumorally it correlated positively with Ki67 (r_s=0.35; p=0.03) and negatively with age (r_s=−0.45; p=0.01). $CD4^+$ had a significantly higher density peritumorally (29.4±15.2) compared to intratumorally (17.5±13.4) (p=0.0005), and the intratumoral component correlated positively with age (r_s=0.36; p=0.03). Intratumoral $CD8^+$ correlated positively with glycemia (r_s=0.37; p=0.02), and peritumorally it showed positive correlations with ER and HER2 (r_s=0.33). Microvascular density ($CD34^+$) was higher peritumorally (14±8.5) *vs* intratumorally (10.5±8.3) (p=0.003), and intratumoral vascularization correlated with Ki67 (r_s=0.49), pT (r_s=0.54) and lymphovascular invasion (r_s=0.34; p=0.03). $CD45^+$ was denser peritumorally (58.7±29.6) than intratumorally (40.5±29) (p=0.01), and the intratumoral level correlated with pT and proliferative activity. $CD56^+$ was reduced intratumorally (2.5±1.7) compared to peritumorally (5.0±4.8) (p=0.013), correlating positively with HER2 status (r_s=0.36) and inversely with tumor size (r_s=−0.31; p=0.05). $CD68^+$ was higher peritumorally (12.4±12.2) *vs* intratumorally (9.5±7.5) (p=0.02) and correlated with mitotic activity (r_s=0.44), pT (r_s=0.51) and HER2 (r_s=0.34), suggesting the association of macrophages with a more aggressive tumor phenotype.

4.3. Quantitative characteristics of targeted markers in breast carcinoma associated with T2DM

The evaluation of intratumoral (it) and peritumoral (pt) compartments highlighted the following: CD3 – the average density was 17.9±15.9 (it) and 35.7±18.9 (pt) (p=0.98); CD3it correlated positively with age (r_s=0.47; p=0.01), and CD3pt correlated with tubule formation (r_s=0.48), the Nottingham score (r_s=0.44) and HER2 expression (rs=0.33). CD4 – the average density was 7.8±5.5 (it) and 14.8±14.3 (pt) (p=0.09); CD4it correlated negatively with ER (r_s=−0.32; p=0.04), and CD4pt had an association at the limit of significance with perineural invasion (r_s=0.27; p=0.08). CD8 – average values were 15.2±11.3 (it) and 28.0±18.3 (pt) (p=0.02); CD8it correlated positively with perineural invasion (r_s=0.42), and mitotic activity showed a tendency of inverse correlation with intratumoral cytotoxic infiltration (r_s=−0.29). CD34 (microvascular density) – 12.8±12.7 (it) and 16.0±7.2 (pt) (p=0.19); CD34it correlated negatively with tumor grade (r_s=−0.33), Nottingham score (r_s=−0.34) and Ki67 (r_s=−0.37; p=0.02). CD45 – the average density was 43.1±35.1 (it) and 50.1±34.6 (pt); CD45pt correlated positively with age (r_s=0.37) and degree of differentiation (r_s=0.48), and negatively with ER/PR (r_s=−0.41; p=0.01). CD56 – the density was significantly higher peritumorally (12.92±7.62) compared to intratumorally (0.58±0.42) (p=0.03); CD56it correlated negatively with lymphovascular invasion (r_s=−0.35), and CD56pt correlated negatively with mitotic activity (r_s=−0.37) and HER2 (r_s=−0.38). CD68 – the average density was 10.61±9.85 (it) and 16.3±16.1 (pt) (p=0.05); CD68it correlated positively with age (r_s=0.40) and pN nodal extension (r_s=0.42; p=0.01), suggesting a more intense macrophage response in advanced tumors.

Statistical correlations between analyzed immunohistochemical markers in breast cancer associated with diabetes mellitus. The analysis of the interdependence of biological markers in breast carcinoma associated with diabetes mellitus highlighted suggestive relationships for tumor progression. A strong co-expression between ER and PR was confirmed (r_s=0.78; p=0.001), suggesting the maintenance of the hormonal axis in this context, with potential response to endocrine therapy. Ki67 correlated inversely with PR (r_s=−0.49; p=0.007), indicating the association of PR loss with increased proliferation and a more aggressive phenotype. In addition, a positive correlation was highlighted between HER2 and Ki67 (r_s=0.52; p=0.004), suggesting the potentiation of proliferation in a diabetic metabolic context; no significant correlations were identified between HER2 and ER/PR (p=0.80/ p=0.13) nor between Ki67 and ER (p=0.12).

4.4. Evaluation of correlations between cellular immunity markers and angiogenic potential in breast tumors with and without associated T2DM

Breast carcinoma without diabetes mellitus. The analysis of correlations between cellular immunity markers and angiogenic potential in breast carcinoma without diabetes highlighted coordinated interactions in the tumor microenvironment. The intratumoral immune response was dominated by the T helper component, supported by the correlation between CD3it and CD4it (r=0.48; p=0.01), and the T infiltrate was closely associated with CD8it (r=0.62; p=0.01) and CD68it (r=0.60; p=0.01), suggesting the collaboration of lymphocytes and macrophages in the neoplastic focus. The vascular component correlated positively with immune infiltration: CD34it associated with CD3it (r=0.43; p=0.01) and especially with CD4it (r=0.59; p=0.01), indicating that angiogenesis facilitates intratumoral immune recruitment; additionally, CD34it correlated with CD56pt (r=0.36; p=0.03), suggesting influences on the peritumoral compartment. At the tumor periphery, increased leukocyte recruitment was found, through CD3pt–CD4pt (r=0.36) and CD3pt–CD45pt (r=0.40; p=0.02) associations, as well as communication between compartments: CD4pt correlated with CD8it (r=0.31; p=0.05). In contrast, CD8it was inversely associated with CD34pt (r=−0.34; p=0.03), suggesting an antagonistic relationship between intratumoral cytotoxic activity and peritumoral neoangiogenesis.

Breast carcinoma associated with T2DM. The analysis of correlations in breast carcinoma associated with T2DM indicates a remodeling of immune and vascular interactions under the influence of metabolic disorders. The CD3it infiltrate depends on the CD4it (r=0.46; p=0.01) and CD8it (r=0.48; p=0.01) subpopulations and associates synergistically with macrophages (CD3it–CD68it: r=0.49; p=0.01), suggesting an active role of macrophages in T lymphocyte recruitment. Communication between compartments is supported by the CD3it–CD45pt relationship (r=0.38; p=0.02), and intratumoral neoangiogenesis facilitates immune infiltration (CD34it–CD3it: r=0.38; p=0.02; CD34it–CD8it: r=0.49; p=0.01). Peritumorally, CD3pt remains associated with CD4pt (r=0.32) and CD8pt (r=0.37), and CD8it correlates with both CD68it (r=0.45) and CD56pt (r=0.41; p=0.01), suggesting interactions between cytotoxic lymphocytes and NK cells. A mechanistic core seems to be formed by the CD34it–CD68it axis (r=0.51; p=0.01) and the links to the periphery (CD34it–CD56pt: r=0.44; p=0.01; CD56pt–CD68it: r=0.50; p=0.01), and the CD45pt–CD8it association (r=0.34; p=0.04) reconfirms the importance of the peritumoral leukocyte infiltrate for the level of intratumoral cytotoxic response.

Comparatively, in the normal mammary gland CD3/CD8 predominates stromally, and CD4/CD56 are more evident epithelially; in non-diabetic carcinoma, active CD3/CD8 intratumoral infiltration is noted, with peritumoral predominance of CD4/CD45. In the presence of T2DM, this pattern is disrupted by the redistribution of CD8 and CD68 to the peritumoral stroma, concomitant with the increase in CD34 vascularization and CD56 density at the tumor periphery, suggesting adaptive/compensatory mechanisms in an altered metabolic context.

5. CHARACTERIZATION OF THE EXPRESSION OF HORMONAL MARKERS (ER, PR), HER2 AND Ki67 IN THE INTACT MAMMARY GLAND AND IN BREAST CARCINOMA

In breast cancer, the expressions of markers for ER, PR, HER2, and Ki67 represent key characteristics of the tumor and are the basis of predictive prognosis [8].

5.1. Characteristics of the control cases

In the unaffected mammary gland, ER receptors were predominantly localized in the luminal epithelial cells, similarly PR are localized in the luminal epithelial cells and are essential for proliferation and tissue remodeling processes [23]. During menopause, along with the general reduction of hormonal activity, PR expression also decreases significantly [23].

5.2. Breast tumors without associated T2DM

The analysis of molecular markers (ER, PR, Ki67, HER2) in the tumors of non-diabetic patients highlighted a marked heterogeneity. ER expression was on average 57.2%±41.6% (Me=80%), with

43.3% hormone-independent cases, and 32.6% reached Allred 8; PR had lower values (35.2%±35.0%, Me=30%) and was absent in 37.2% of cases. A strong ER–PR co-expression was confirmed (rs=0.74; p=0.001). Proliferation was high-variable, with Ki67=35.2%±19% (Me=35%, range 10–70%), the majority of cases falling into moderate-high proliferation. HER2 was negative in 81.4% (most frequently score 1+: 46.5%), and 3+ was identified in 18.6%. The ER–HER2 (p=0.31) and ER–Ki67 (p=0.29) relationships were insignificant, and the HER2–Ki67 association was weakly positive, without statistical significance (r_s=0.18; p=0.17).

5.3. The phenotype of NST breast tumors associated with T2DM

Breast tumors were predominantly hormone-dependent, with ER 60.2%±37.8% (Me=80%); Allred scoring highlighted strong expression (Allred 8 in 26.7%, respectively 7 in 33.3%). PR had lower values (39.7%±38.3%, Me=35%), being absent in 36.7%; although ER–PR correlated positively (r_s=0.77; p=0.01), PR was significantly lower than ER (p=0.00084), suggesting that sensitivity to hormone therapy is dominated by ER. Ki67 showed marked variability (30.6%±17.8%, Me=27.5%), and 76% of the tumors had Ki67≥20%. The HER2 status was predominantly low/absent (83.3%, Me=1), while 16.7% had 3+. Molecular relationships showed strong ER–PR correlations (rs=0.78), an insignificant ER–HER2 association, an inverse ER–Ki67 trend (r_s=−0.30; p=0.06), and a significant inverse PR–Ki67 correlation (r_s=−0.49; p=0.001); conversely, HER2–Ki67 was positively correlated (r_s=0.52; p=0.001), indicating that HER2 overexpression in T2DM is associated with increased proliferation.

5.4. Comparative characteristics of breast tumors, with and without T2DM association

The comparative analysis of tumor biomarkers between the non-diabetic and diabetic groups highlighted differences in expression and interdependence with prognostic relevance. For ER, the non-diabetic group had 57.2%±41.6% (Me=80%), with 30.2% ER-negative cases, and the diabetic group 60.2%±37.8%, with a lower proportion of ER-negative cases (23.3%); in both groups, a strong positive ER–PR correlation was maintained (r_s=0.74 *vs* r_s=0.77), suggesting hormone dependence regardless of metabolic status. For PR, the averages were comparable (35.2% *vs* 39.7%, p=0.78), but in the diabetic group, a significant negative PR–Ki67 correlation was highlighted (r_s=−0.49; p=0.001), absent in the non-diabetic group, indicating the association of PR-positivity with lower proliferation in the context of T2DM. Ki67 was slightly lower in the diabetic group (30.6%±17.8%) compared to the non-diabetic group (35.2%±19%), at the limit of significance (p=0.06). A distinct element was the strong positive HER2–Ki67 correlation in the diabetic group (r_s=0.52; p=0.001), absent in the non-diabetic group, suggesting a more proliferative/aggressive phenotype of HER2-positive tumors in the presence of metabolic disorders. HER2 expression did not differ significantly between the groups (p=0.12), and HER2 3+ cases were similar (18.6% *vs* 16.7%). Overall, although NST tumors are predominantly hormone-dependent in both groups, in T2DM a closer association between the HER2 axis and proliferation is highlighted, with a potential negative impact on the prognosis for HER2-positive subtypes.

5.5. The structure and molecular phenotype of breast carcinoma

The structuring of molecular subtypes was performed by integrating ER/PR expression, HER2 status, and Ki67 index. In the non-diabetic group, the distribution was heterogeneous, with the predominance of the Luminal B/HER2-negative subtype (48.8%; 21 cases), followed by Triple-negative (20.9%); the Luminal B/HER2$^+$ and Luminal A subtypes constituted 11.6% and 9.3%, respectively, and pure HER2-positive was the rarest (7%). In the group with T2DM, the predominance of Luminal B/HER2-negative was maintained (58.6%; 17 cases), followed by Triple-negative (17.2%), and Luminal A and Luminal B/HER2$^+$ each had 10.3%; the pure HER2-positive subtype was rare (3.5%; 1 case). The statistical comparison of the groups did not reveal significant differences in the distribution of subtypes (p=0.48), suggesting that T2DM can influence the microenvironment and aggressiveness, but does not decisively change the molecular affiliation defined by standard IHC markers

6. THE INFLUENCE OF METABOLIC DISORDERS FROM T2DM ON THE LOCAL CELLULAR IMMUNE STATUS IN BREAST CARCINOMA

This chapter integrates and discusses the results obtained regarding the influence of type 2 diabetes mellitus (T2DM) on the morphological, immunological, angiogenic, and molecular characteristics of invasive breast carcinoma of the NST (*No Special Type*) type. The association of T2DM did not correlate strongly with classic tumor stage parameters, but it was accompanied by an accentuation of lymphovascular and perineural invasion, suggesting that metabolic disturbances particularly influence tumor dissemination capacity and the remodeling of the local microenvironment.

From a molecular perspective, the T2DM group maintained a predominance of hormone-dependent phenotypes, with no statistically significant differences in the global distribution of molecular subtypes compared to the non-diabetic group. The biological importance of diabetes does not reside as much in changing the affiliation to a certain molecular subtype, but rather in shifting the relationships between tumor markers. Thus, a strong ER–PR co-expression was maintained, an inverse association between PR and Ki67 was highlighted, and the positive relationship between HER2 and Ki67 was more accentuated in the context of T2DM, suggesting that tumor proliferation becomes more closely linked to the HER2 axis in the presence of metabolic disorders.

The analysis of histological grade highlighted a smaller proportion of G3 tumors in the diabetic group, but this observation does not exclude more aggressive biological behavior. The results indicate that tumor aggressiveness, in the context of T2DM, must be assessed not only through histological grade but by integrating proliferative markers, the molecular profile, and the specific characteristics of the tumor microenvironment. In this regard, T2DM appears to primarily influence the network of interactions between biological markers, rather than exclusively the isolated quantitative expression of a single marker. Data regarding local cellular immunity support the existence of a compromised anti-tumor response in the presence of type 2 diabetes mellitus. In patients with T2DM, a tendency towards a reduction in the density of intratumoral $CD8^+$ cytotoxic T lymphocytes was observed, along with the redistribution of the immune infiltrate toward the peritumoral compartment. This reorganization of the infiltrate suggests a decrease in the efficiency of the cytotoxic immune response directly against tumor cells. Furthermore, the inverse relationship between intratumoral CD4 and ER expression suggests additional interdependencies between local immune status and the hormonal profile of the tumor. Regarding $CD45^+$ and $CD56^+$ cells, the results show that T2DM influences not only their density but especially their distribution within the tumor microenvironment. The peritumoral accumulation of $CD56^+$ cells, associated with reduced intratumoral density, supports the hypothesis of insufficient penetration of the effector component into the tumor focus. Consequently, in a diabetic context, the presence of these cells at the tumor periphery may reflect an incompletely efficient immune response rather than adequate anti-tumor immune control.

Tumor angiogenesis presented features suggestive of dysfunctional vasculature. In the T2DM group, intratumoral $CD34^+$ microvascular density was inversely associated with tumor grade, Nottingham score, and the Ki67 index, which supports the existence of a disorganized vascular architecture and inefficient tissue perfusion. In this context, type 2 diabetes mellitus may favor the installation of a hypoxic tumor microenvironment, capable of supporting neoplastic progression and further altering the local immune response.

An important result of the study is the role of $CD68^+$ macrophages in the context of T2DM. The intratumoral component of macrophages was associated with nodal extension, while the peritumoral component was associated with mitotic activity and tumor size, supporting their involvement in local and regional disease progression. These observations confirm that, under metabolic disturbance conditions, the macrophage infiltrate acquires a pro-tumor biological significance, contributing to the perpetuation of chronic inflammation and the remodeling of the breast carcinoma microenvironment.

Overall, the results demonstrate that type 2 diabetes mellitus does not act merely as a metabolic comorbidity, but as an active factor in remodeling the breast carcinoma microenvironment. Its influence is exerted by altering the balance between local cellular immunity, angiogenesis, and the relationships between hormonal and proliferative markers. This interpretation supports the need for an integrated histopathological and immunohistochemical evaluation in patients with T2DM-associated breast carcinoma, with potential prognostic and therapeutic impact.

GENERAL CONCLUSIONS

1. The postmenopausal mammary gland exhibits involutive changes associated with physiological processes of senescence and tissue remodeling. Invasive breast carcinoma of the NST type, in the context of type 2 diabetes mellitus, presents more aggressive morphological and proliferative characteristics, expressed by increased tumor sizes, a more advanced histological score, and accentuated proliferative activity compared to cases without metabolic comorbidity.

2. The postmenopausal mammary parenchyma is characterized by a particular status of immune control, evidenced by the predominant distribution of $CD3^+$ and $CD8^+$ lymphocytes at the level of the glandular stroma, concomitantly with the preferential localization of $CD4^+$ and $CD56^+$ leukocytes in the epithelial compartment. In contrast, breast carcinoma associated with type 2 diabetes mellitus presents a profoundly altered local cellular immune status, characterized by a significant increase in the population of intratumoral $CD68^+$ macrophages and by immune correlations suggestive of the development of an immunosuppressive tumor microenvironment, favorable to neoplastic progression.

3. In breast carcinoma associated with type 2 diabetes mellitus, tumor angiogenesis is characterized by a disorganized microvascular architecture and deficient vascular functionality, aspects highlighted by significant statistical associations between $CD34^+$ microvascular density and markers of tumor aggressiveness and proliferation. These correlations are suggestive of compromised tissue perfusion and the installation of a hypoxic tumor microenvironment, favorable to neoplastic progression.

4. The postmenopausal mammary gland presents a reduced expression of steroid receptors (ER, PR), as well as HER2 and Ki67 markers. The differences between breast carcinoma, with and without associated diabetes mellitus, are not reflected in the quantitative levels of ER, PR, or HER2 expression, but in the statistical association patterns of these markers with proliferative activity (Ki67), which, overall, indicate a more aggressive tumor profile.

5. The tumor microenvironment in invasive breast carcinoma of the NST type associated with type 2 diabetes mellitus is characterized by a complex remodeling, resulting from the dynamic interaction between immune, vascular, and hormonal components. This remodeling occurs in the absence of changes in the distribution of molecular subtypes, yet it exerts a significant impact on tumor proliferative behavior.

RECOMMENDATIONS

1. It is recommended to include the evaluation of immunological markers (CD3, CD4, CD8, CD68) in the histopathological analysis of breast carcinoma, particularly in patients with type 2 diabetes, to characterize the tumor immune status.

2. The concurrent assessment of angiogenic potential (CD34) and cellular immune infiltrate is recommended for a comprehensive evaluation of the tumor microenvironment.

3. The integration of immunological and molecular parameters (ER, PR, HER2, Ki67) into the prognostic stratification of patients with T2DM-associated breast carcinoma is proposed.

4. Monitoring tumor immunological particularities in patients with T2DM is recommended to individualize therapeutic management.

5. It is essential to consider the impact of metabolic disturbances on the tumor microenvironment when developing personalized therapeutic strategies.

6. The development of integrated histopathological and immunohistochemical evaluation protocols, including markers for cellular immunity and angiogenesis in T2DM-associated breast carcinoma, is recommended.

7. The use of immunological markers as potential indicators of tumor progression and treatment response in patients with metabolic comorbidity is proposed.

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ADNOTARE

Brînza Dumitru

„Manifestările locale ale imunității celulare în carcinomul mamar la pacienții cu diabet zaharat de tip 2”

Teză de doctor în științe medicale, Chișinău, 2026

Structura tezei: introducere, 6 capitole, concluzii generale, recomandări practice, bibliografie cu 330 titluri, 16 anexe, 110 pagini de text de bază. Rezultatele au fost publicate în 40 lucrări științifice.

Cuvinte cheie: cancer mamar, profil hormonal, HER2, Ki67, diabet zaharat tip 2 (DZT2), imunitate locală, T limfocite, macrofage, angiogeneză.

Scopul lucrării: Evaluarea complexă a carcinomului mamar invaziv de tip ductal (NST) asociat cu DZT2, prin prisma profilului molecular al tumorii și a statusului imun celular local, comparativ cu pacienții non-diabetici.

Metodologia cercetării: Studiu retrospectiv, comparativ, pe un lot de 102 specimene: 72 paciente cu cancer mamar (43 fără diabet și 29 cu DZT2) și un lot de control de 30 cazuri (țesut mamar neafectat). S-au utilizat metode histologice clasice (HE) și imunohistochimice (panel de 11 anticopi: ER, PR, HER2, Ki67, CD3, CD4, CD8, CD34, CD45, CD56, CD68).

Rezultatele obținute: Analiza morfologică a demonstrat că tumorile asociate cu DZT2 prezintă caracteristici mai agresive, având dimensiuni mai mari și stadii patologice mai avansate comparativ cu lotul non-diabetic. S-a constatat că diabetul zaharat nu este doar o comorbiditate, ci un factor activ care remodelează microambianța tumorală.

Evaluarea statusului imun a relevat modificări semnificative induse de DZT2. În tumorile pacientelor diabetice, s-a observat o diminuare a densității limfocitelor T citotoxice ($CD8^+$) și a macrofagelor ($CD68^+$) în zona intratumorală, comparativ cu stroma peritumorală. În schimb, stroma peritumorală la diabetici este caracterizată printr-o infiltrare mai intensă cu celule NK ($CD56^+$) și o densitate vasculară ($CD34^+$) crescută, sugerând o adaptare compensatorie. Spre deosebire de pacientele non-diabetice, unde vascularizația intratumorală corelează pozitiv cu agresivitatea, la diabetici aceasta corelează negativ cu gradul histologic și Ki67, indicând o angiogeneză paradoxală sau ineficientă.

Din punct de vedere molecular, tumorile asociate cu DZT2 sunt preponderent hormon-dependente (ER/ PR pozitive), iar subtipul Luminal B/HER2-negativ este cel mai frecvent. Deși diabetul nu alterează distribuția globală a subtipurilor moleculare, acesta modulează relațiile dintre markeri: s-a evidențiat o corelație pozitivă puternică între HER2 și Ki67 la diabetici, sugerând un comportament mai agresiv al tumorilor $HER2^+$ în context metabolic alterat.

Concluzii: Dereglările metabolice din DZT2 influențează profund interacțiunea dintre tumoră și gazdă, favorizând un profil imun local modificat ce pot contribui la progresia neoplazică și necesită o abordare terapeutică personalizată.

ANNOTATION

Brinza Dumitru

„Local manifestations of cellular immunity in breast carcinoma in patients with type 2 diabetes mellitus”

PhD, Thesis in Medical Sciences, Chisinau, 2026

Thesis structure: Introduction, 6 chapters, general conclusions, practical recommendations, bibliography with 330 titles, 16 annexes, 110 pages of core text. The results were published in 40 scientific papers.

Keywords: breast cancer, hormonal profile, HER2, Ki67, type 2 diabetes mellitus (T2DM), local immunity, T lymphocytes, macrophages, angiogenesis.

The purpose of the study: A complex evaluation of invasive ductal breast carcinoma (NST) associated with T2DM, through the lens of the tumor's molecular profile and local cellular immune status, compared to non-diabetic patients.

Research methodology: A retrospective, comparative study on a batch of 102 specimens: 72 patients with breast cancer (43 without diabetes and 29 with T2DM) and a control group of 30 cases (unaffected breast tissue). Classical histological (HE) and immunohistochemical methods were used (a panel of 11 antibodies: ER, PR, HER2, Ki67, CD3, CD4, CD8, CD34, CD45, CD56, CD68).

Results obtained: Morphological analysis demonstrated that T2DM-associated tumors exhibit more aggressive characteristics, showing larger sizes and more advanced pathological stages compared to the non-diabetic group. It was established that diabetes mellitus is not merely a comorbidity but an active factor that remodels the tumor microenvironment. The evaluation of immune status revealed significant changes induced by T2DM. In the tumors of diabetic patients, a decrease in the density of cytotoxic T lymphocytes ($CD8^+$) and macrophages ($CD68^+$) was observed in the intratumoral area compared to the peritumoral stroma. Conversely, the peritumoral stroma in diabetics is characterized by a more intense infiltration of NK cells ($CD56^+$) and increased vascular density ($CD34^+$), suggesting a compensatory adaptation. Unlike non-diabetic patients, where intratumoral vascularization correlates positively with aggressiveness, in diabetics, it correlates negatively with histological grade and Ki67, indicating paradoxical or inefficient angiogenesis. From a molecular perspective, T2DM-associated tumors are predominantly hormone-dependent (ER/PR positive), and the Luminal B/HER2- subtype is the most frequent. Although diabetes does not alter the global distribution of molecular subtypes, it modulates the relationships between markers: a strong positive correlation was highlighted between HER2 and Ki67 in diabetics, suggesting more aggressive behavior of HER2-positive tumors in a context of altered metabolism.

Conclusions: Metabolic disturbances in T2DM profoundly influence the interaction between tumor and host, favoring a modified local immune profile that may contribute to neoplastic progression and necessitates a personalized therapeutic approach.