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PhD THESIS

Summary

MOLECULAR MARKERS WITH PREDICTIVE ROLE ON RECURRENCES AND METASTASES OF BREAST CANCER

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INTRODUCTION

This doctoral thesis is part of the interest in breast cancer research of the research group of the Histology Discipline from Timișoara and continues some of the ideas that were born over time in this group. Also, through the obtained results, we hope that the work opens new research perspectives for the Ph.D. students who follow in the coming years.

The *motivation for choosing the theme*. Breast cancer is currently the most common neoplasia of the female sex, associated with increased morbidity and mortality in all countries that report this malignant condition on the basis of a national cancer registry. A large number of cases led to the initiation of numerous clinical, imaging, pathological and molecular studies that attempted to gain an in-depth knowledge of the natural evolution of this condition. In the last decades, remarkable progress has been made in the field of early diagnosis through clinical and imaging screening actions, and new therapeutic methods have been introduced. Despite these efforts, a proportional reduction in morbidity and mortality has not been found, most likely due to the heterogeneity of breast cancer. Pathologists who perform the routine diagnosis of breast cancer are used to the heterogeneity of this neoplasia and know that no two breast tumors are identical. So, every tumor has a specific molecular fingerprint, which requires an individualized treatment.

Conventional pathological nor molecular classification provides prognostic information on the possible development of local recurrences and on the prediction of lymph node metastases. This is most likely due to molecular testing of only the primary tumor in most cases. The working hypothesis from which we start in the present research refers precisely to this aspect, namely: the molecular profile of the primary tumor is not necessarily identical to that of lymph node corresponding metastases and distant metastases. This aspect could have major implications on the adjuvant therapeutic strategy, both conventional or/and based on biological agents, like monoclonal antibodies.

The *importance and actuality of the topic*. The natural evolution of breast cancer is complex, and almost every molecular type has been shown to preferentially metastasize. Of utmost importance are the lymph nodal metastases whose presence and molecular profile are decisive for the therapeutic strategy. Although all the publications on this subject point to the importance of lymphatic metastasis, until now the way in which the first tumor cells enter the lymphatic vessels is not known. As can be seen from the above data, there are still many discussions, controversies, and uncertainties regarding the molecular profile and its defined role in the treatment of patients. In addition, although grading systems, gene analysis, and molecular profiling exist, we still lack predictive elements for the existence of metastases by examining the primary tumor. Considering the consensus on the molecular classification from Saint Gallen 2016, and the importance of the subject, in the present work we aimed to investigate an extensive panel of molecular markers to assess whether or not they have a prognostic impact on metastasis.

In the present work, we applied morphological, histochemical, and immunohistochemical methods to all the cases included in the study. We consider the number of cases included in the present study to be representative, ensuring the reproducibility of the results. The recommended methods for molecular diagnosis recommended by the WHO were selected, but also some markers that are currently of uncertain value. All procedures were performed in an automated system that ensured consistency in terms of incubation with the primary antibodies and had the same

incubation time for the visualization agent. These aspects were imported especially for the scoring of the results, with direct implications in scoring interpretation and analysis. For the scoring processes, the methods verified by the international collectives of experts were chosen, such as the interpretations for hormone receptors and HER2. These methods were applied retrospectively to the case series included in this thesis, but can be applied in current use for current pathological and molecular diagnoses, to cases selected in a prospective manner. The mark of originality in the material and methods chapter stands out in the chapter on the tumor microenvironment, where data from the literature is poor or missing so we consider that some methods to be evaluated from this chapter have a mark of originality.

New perspectives and directions developed through this study. The results obtained in the present research reveal a series of perspectives on the application of morphological, histochemical, and immunohistochemical methods in the refinement of histopathological diagnosis, assessment of prognosis, and the effectiveness of therapy. Through the results obtained especially in the application of the molecular classification and the comparison with the immunohistochemical profile of lymph node metastases, one can hope for the refinement and specialization of the therapeutic strategy. Through the results obtained regarding the expression of androgen hormones and mammaglobin A, we believe that new therapeutic possibilities have been opened for hormone-resistant cases to conventional therapy. An additional mention for the elements of the tumor stroma, known as the tumor microenvironment, which on the one hand introduces new prognostic elements, and on the other hand, identifies a number of new therapeutic targets, such as mast cells.

Perhaps the realization of this study would not have been possible without the help of colleagues from the hospitals of Timișoara, Arad, and Cluj, who participated with numerous cases. Thanks to the leadership of the Victor Babeș University of Medicine and Pharmacy in Timișoara for the framework created to carry out this work and in particular, the discipline of Histology, where the immunohistochemical preparations were made. Thanks to the colleagues at the Teodor Andrei Hospital in Lugoj, who indirectly left their mark on this research. We hope that through our results we will bring additional data to the characterization of breast cancer, proposing some elements with a predictive role for the propagation and development of lymph node metastases.

I. GENERAL PART

I.1. BRIEF OF EPIDEMIOLOGY

Cancer incidence and specific mortality are increasing rapidly worldwide. The main reasons for this dramatic change in the pathology of the breast mainly reflect two aspects: the significant increase in the world's population and the increase in average life expectancy, to which are added qualitative-quantitative changes in risk factors consequent to socio-economic development [1]. Clear data on breast cancer incidence and mortality have been published for many regions of the globe, but these aspects are unclear or even confusing for countries that do not even have a national cancer registry. Breast cancer is the most frequently diagnosed neoplasia and the first cause of death in women worldwide.

I.2. THE DIAGNOSTIC AND PROGNOSTIC ROLE OF CONVENTIONAL HISTO-PATHOLOGICAL CLASSIFICATION

Breast carcinoma is the most common malignant tumor of the female sex and the main cause of death from cancer, annually registering over 1,000,000 new cases in the world. Over 100,000 new cases and over 30,000 specific deaths are registered annually in the USA alone. The incidence of breast carcinoma is 91.4%ooo in North America and Europe, intermediate in southern Europe and South America, and low in most African and Asian countries. The diagnosis of a large number of cases in the early stages of evolution is due to the widespread introduction of mammography. Surprisingly, early detection did not significantly change the death rate, which did not change significantly between 1970 and 1990.

The histopathological diagnosis of breast tumor specimens taken by sectorial excision, radical, associated or not with lymph node dissection, as well as by biopsy puncture in some cases, is essential for establishing the diagnosis of malignancy. No oncological treatment is applied in the absence of histopathological confirmation of malignancy. The histopathological diagnosis of the primary tumor and the identification of especially lymph nodal metastases dominated the surgical therapeutic strategy, radiotherapy and chemotherapy, already standardized for this neoplastic condition.

I.3. NATURAL EVOLUTION OF BREAST CANCER

The progression of breast carcinomas is achieved through direct invasion, via lymphatic and blood. In some cases, metastases are already present at the time of diagnosis, and others become clinically manifest months, years, or even decades after the initial therapy.

Local invasion may be seen in the breast parenchyma, nipple, skin, pectoral fascia, or other structures of the chest wall. Invasion of the mammary stroma can occur by direct extension, through the intra-mammary lymphatic vessels, and possibly through the tissue spaces known as pseudoangiomatous stromal hyperplasia. The degree of local invasion is generally higher in invasive lobular carcinoma and its variants, probably due to the absence of E-cadherin from the tumor cells. The frequency of microscopic invasion outside the mammary gland was studied on local excision specimens, with safety margins of 2 cm. Among the carcinomas with a diameter of less than 1 cm, 11% presented residual invasive carcinoma and 22% presented residual carcinoma in situ. The importance of microscopic evaluation of local invasion is currently greater, as the diversity of conservative surgical procedures has significantly increased [12, 13].

I.4. MOLECULAR CLASSIFICATION: CHARACTERIZATION AND IMPACT ON THERAPY

Despite the remarkable advances in the field of oncology, both in diagnosis and therapy, breast cancer continues to represent the most common neoplasia in the female, burdened with a high mortality rate. In 2006 alone, over 200,000 new cases and over 40,000 specific deaths were reported in the United States [18].

A significant improvement has been achieved in the last three decades by the evaluation of markers useful in pharmacodiagnosis (hormone receptors, proliferation marker Ki67, and epidermal growth factor receptor, HER2). It has been observed that breast cancer patients can be stratified based on the expression of these markers as

demonstrated by gene analysis and/or immunohistochemistry. Sufficient evidence has been accumulated to demonstrate the direct impact of hormone receptors and HER2 on therapy, with no statistically significant correlation with conventional types of breast carcinoma. These differences are most likely generated by the fact that molecularly distinct proliferative lesions have been grouped into clinical types based on common morphological criteria. In an attempt to resolve this problem, breast malignancies were classified based on gene expression profile and subsequently, immunohistochemical expression of cytokeratin 5 and 8/18, hormone receptors, HER2, and EGFR. This classification, which recognizes luminal, basal-like, HER-2, and unclassifiable types, had an immediate impact on therapeutic strategy, observing that the characterized molecular types respond differently to preoperative chemotherapy and postoperative adjuvant therapy.

I.5. ELEMENTS WITH A PREDICTIVE ROLE FOR LYMPH NODE METASTASES

The progression of breast carcinomas is achieved through direct invasion, via lymphatic and blood vessels spread. In some cases, metastases are already present at the time of diagnosis, and others become clinically manifest months, years, or even decades after the initial therapy.

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Local recurrences after mastectomy appear as superficial nodules in or near the scar or as subcutaneous parasternal nodules. Their malignant nature should always be documented by biopsy, as such nodules may be the result of a foreign body granuloma or other infectious process. Although patients with local recurrence have a higher risk for distant metastases, this event is partially independent and occurs at different times of the disease's evolution. Local recurrences after mastectomy often develop in the same breast area, which has led some authors to recommend en bloc excision of the tumor mass along with the associated ductal system [52].

THE ORIGINAL PART

II.1. MOTIVATION AND GOALS

The scientific objectives that are proposed to be solved in the framework of the research. In the present research, we proposed to solve several objectives, which we consider to be of diagnostic and prognostic importance for the histopathological outcome of breast cancer. These objectives are the following:

- Morphological evaluation and appreciation of the degree of differentiation in all cases included in the present study. Cases with frequently observed histopathological forms and representing the absolute majority in all histopathology laboratories were selected for the study. For the accuracy of the reporting, cases with particular forms, uncertain lesions or with an atypical particular profile were excluded.
- Investigation of the immunohistochemical profile of the cases, according to the recommendations of the St Gallen Conferences, which includes, in addition to conventional markers (ER, PR, HER2, Ki67) and additional markers (EGFR, p53, Bcl-2, cytokeratin 5), which allow refining the classification in five major molecular types of carcinoma. Relatedly, the utility of still uncertain markers for classification, such as E-cadherin, has been tested.
- Comparison of the molecular profile of the primary tumor with the profile of the corresponding lymph node metastases. We believe that from a practical point of view, this part of the thesis has the greatest value through the impact on the therapeutic strategy.
  In most cases, the immunohistochemical evaluation takes into account the receptors for the hormones estrogen and progesterone. Only rarely is the expression of receptors for androgen hormones investigated, most likely because they have no immediate effect on hormone therapy. On the other hand, androgen receptors may represent an attractive therapeutic target in cases resistant to anti-estrogen receptor therapy. For this reason, one of the aims of the study was the immunohistochemical evaluation of androgen hormone receptors and their relationship with histopathological form and prognosis.
- In the same context, we proposed to evaluate the expression of Mammaglobin A, which was shown to have great specificity for normal and pathological breast tissue. Although known for several decades, mammaglobin A has been investigated only from a diagnostic point of view and not much from the perspective of its predictive character in the evolution of neoplasia. For this reason, one of the objectives of the work was the detailed immunohistochemical analysis of the expression of this marker in the cases included in the study.
- For long periods of time, tumor cells represented the main and even the only target of breast oncology research and therapeutic strategy. Practically, even today, the entire therapeutic strategy is directed at tumor cells (as a recent exception we mention anti-angiogenic therapy). For these reasons, in the last part of the present research we focused our observations on the tumor stroma, and in particular on some components that seem to play an important role in the progression of neoplasia and even in the metastasis process. From these points of view, our observations focused particularly on CD34-positive fibroblasts, macrophages, mast cells, and elastic fibers.

II.2. CASE SELECTION

In the present study, 156 patients in whom breast cancer was diagnosed histopathologically were included. The criteria for inclusion in the study were the following: detailed knowledge of staging, knowledge of tumor classification according to the TNM system, and the existence of primary tumor specimens and corresponding axillary lymph nodes. The 156 cases with corresponding paraffin blocks were retrospectively selected from the archive of Timișoara County Hospital (32 cases), Arad County Hospital (50 cases), Chisinau Oncological Institute, Republic of Moldova (46

cases) and Cluj Napoca Oncological Institute (28 cases). All specimens were re-stained in the automated standardized system, both for the primary tumor and for axillary lymph nodes with or without metastases. The patients were aged between 34 and 82 years. The characteristics of the cases included in the study are presented in Table 10. The cases diagnosed with invasive breast carcinoma of ductal NOS or lobular type, particular forms (mucinous, medullary, papillary) with or without local recurrence, with and without axillary lymph nodes metastases were selected.

II.3. PRIMARY PROCESSING OF SPECIMENS AND USUAL STAINS

The primary processing of the biological material was identical for all specimens. The sampled tissues were fixed for 24-48 hours in 10% buffered formalin, Ph 7.2-7.4. The material was embedded in Paraplast High Melt (Leica Biosystems). In order to avoid possible divergences related to material processing, the primary tumor and lymph node metastasis were included in the same block. Serial sections (made with the Shandon microtome, HM355S Automatic Microtome, Thermo Scientific, USA) with a thickness of 3-5 micrometers were displayed on silanized slides (S3003, Dako, Denmark). All sections were initially stained by the classical method, with hematoxylin-eosin using Harris hematoxylin (HHS32, SigmaAldrich) and eosin CS701 (Dako, Denmark). In the group with breast pathologies, the type and histological grade of the carcinoma, and the presence of metastases in the ipsilateral axillary lymph nodes were determined.

II.4. EVALUATION OF THE DEGREE OF DIFFERENTIATION, G AND THE NOTTINGHAM PROGNOSTIC SCORE

We performed the histopathological grading using the Scarff-Bloom-Richardson system, recommended by WHO (2003), which includes (Ellis et al, 2003) [64]: A. The formation of tubular structures (as an expression of glandular differentiation): 1. in the majority of the tumor (>75%) - 1 point; 2. in 10-75% surface – 2 points; 3. less than 10% or absent – 3 points. B. Nuclear pleomorphism: 1. small nuclei, with minimal variations in shape and size – 1 point; 2. nuclei with moderate variation in size and shape – 2 points; 3. nuclei with marked variation in size and shape – 3 points. C. Mitotic figures (at Plan Fluor 40x/0.75 WD-0.44 objective) - average calculated at 10 fields): 1. up to 5 mitoses per field – 1 point, 2. 6-10 mitoses – 2 points, 3. more than 11 mitoses – 3 points

Grading of carcinoma (sum of A+B+C): G1 (highly differentiated tumor) – 3-5; G2 (tumor with an average degree of differentiation) – 6-7; G3 (poorly differentiated tumor) – 8-9.

II.5. IMMUNOHISTOCHEMICAL METHODS

All dewaxing, unmasking, and visualization procedures were performed automatically, using the Leica Bond-Max apparatus (Leica Microsystems GmbH, Wetzlar, Germany), automated staining: dewaxing in 2 baths of Bond Dewax Solution (code AR9222) of 5 minutes each, followed by 3 baths in 100%, 90% and 70% alcohol for 2 minutes each, rehydration for 5 minutes in distilled water. To block endogenous peroxidase activity, sections were treated for 5 min with Dako REAL™ Peroxidase-Blocking Solution (S2023, Dako). Nuclei were counterstained with Mayer's hematoxylin, modified after Lille (HMM500, ScyTek Laboratories, Inc.). Subsequently,

the histological pieces were dehydrated manually in increasing solutions of ethyl alcohol (70-100%), clarified with xylene, and mounted. To mount the parts, we used the Leica CV Mount solution (Leica Biosystems, code 14046430011).

II.6. METHODS OF INTERPRETATION AND QUANTIFICATION

The quantification of cells labeled with ER, PR, AR, and Ki67 was performed based on the semi-automatic method proposed by Suciu et al (2014) [66]. This method consists in quantifying at least 10 fields at x40 objective with the highest number of labeled tumor cells (or at least 1000 tumor cells in total), completed by evaluating the mean in percentage. To facilitate the numerical evaluation, selected tumor fields were captured as JPEG, and inverted with the program NIS-elements D2.30 (Nikon Instruments Europe BV). Quantification was performed on a Nikon Eclipse 80i microscope with a Nikon DS-Fi1 video camera (Nikon Instruments Europe BV). Overall images were obtained with the AxioZo-om.V16 stereomicroscope, PlanNeoFluor Z1x/0.25 objective, with AxioCam MRc video camera (Carl Zeiss, Germany).

Antibody expression scoring. Numerical evaluation of hormonal markers for ER, PR, and AR was completed by calculating the Allred score [67], which included combining the percentage of immunolabeled cells with the intensity of nuclear staining. The Allred score is a semiquantitative method of assessing hormone receptor expression and has been shown to correlate with prognosis and response to hormone therapy [68, 69].

II.7. MICROSCOPIC IMAGE ACQUISITION AND ANALYSIS

The examination was carried out with the help of the Nikon Eclipse600 and 80i optical microscopes, the images being captured with the help of the Coolpix950 digital camera. Microscopic image analysis was performed with the LuciaNet program (Nikon), on acquired JPEG images, at x400 calibration.

II.8. STATISTICAL ANALYSIS

Statistical tests were performed in SPSS 22.0 software for Windows 8 (SPSS Inc, Chicago, IL). A p-value <0.05 was considered significant. The final reporting was carried out in descriptive terms for all cases and statistics for the subgroups defined above. Continuous variables were presented as mean, and categorical variables were presented numerically and as percentages.

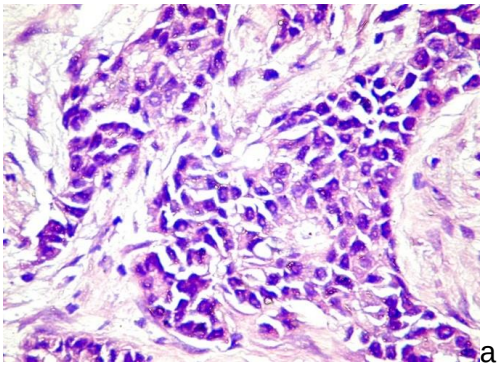
III. RESULTS

III.1. REPORTING OF HISTOPATHOLOGICAL RESULTS AND GRADE OF DIFFERENTIATION

For the histopathological diagnosis and determination of the degree of differentiation, we examined the stained sections with the routine hematoxylin-eosin method, performed according to the protocol specified above. As can be seen from Table 1, most of the cases in this study were invasive ductal carcinoma, characterized by major architectural and cellular changes. The lobulation and terminal lobular units of the normal mammary tissue were no longer identified, being replaced by an irregular proliferation, which in some cases still outlines the formation of glands, but in the majority, the cells proliferate in the form of cords, trabecula or islands of different sizes (fig. 1a). In most cases, structural differences between luminal and basal cells were no

longer identified, with the exception of ductal carcinoma in situ lesions. Tumor cells presented very varied aspects, with sometimes severe non-clear changes, which are reflected in the assessment of the degree of differentiation (fig. 1b). The tumor stroma was quantitatively variable, some cases showing reduced cellular-fibrillar amounts, arranged among the islands of tumor cells (fig.1c), and others showed important amounts of stroma consisting mainly of fibrillar elements (fig.1d). Although our observations are based on the general criteria of histopathological diagnosis in breast cancer, the morphological and architectural heterogeneity, not only of the disposition of tumor cells but also of the tumor microenvironment, attracted our attention. The histopathological form of carcinoma did not correlate statistically significantly with any of the clinical-pathological prognostic factors, including the prediction of lymph node metastases (p=0.24).

However, we identified diffusely arranged lymphocytes in medullary carcinomas. They were characterized by large tumor cells with bulky nuclei and prominent nucleoli, with a major change in the nucleocytoplasmic ratio and quantitatively reduced tumor stroma. In 9 cases, the diagnosis was metaplastic carcinoma (fig. 1e), where the main characteristic observed was the tendency of the tumor cells to become spindle-shaped, as well as the reduced amount of stroma in the tumor area.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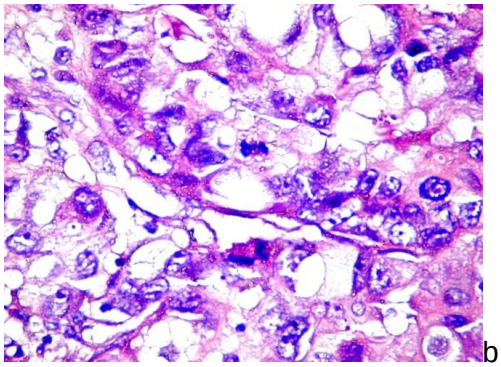
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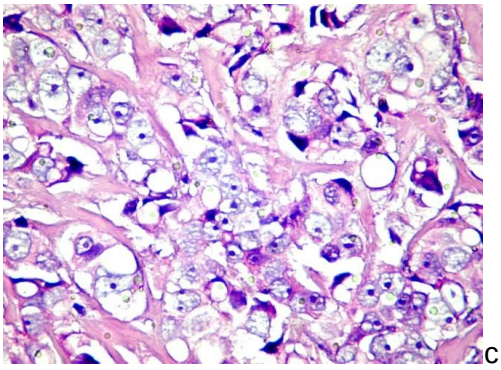
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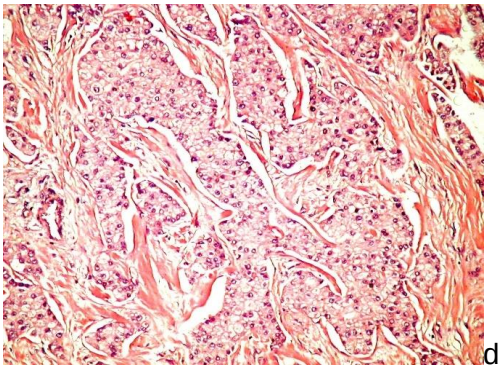
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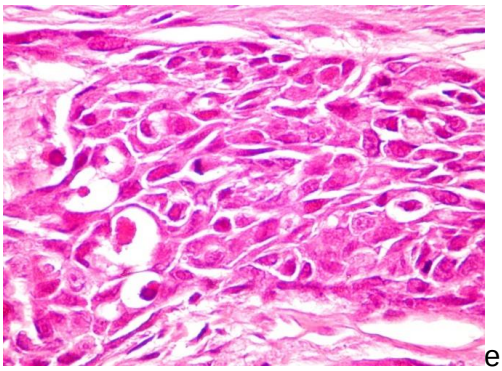

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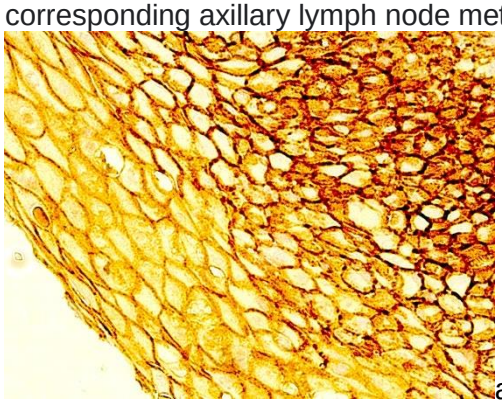
Fig. 1. Disorganized architecture, tumor cells arranged in branched and anastomosed trabeculae. Invasive ductal carcinoma (a, x200). Metaplastic carcinoma (b, x200). Solid invasive ductal carcinoma with cells arranged in small compact clusters and relatively well-represented stroma (c, x200). Invasive ductal carcinoma with rich tumor stroma (d, x100). Metaplastic carcinoma (e, x200). Hematoxylin-eosin staining.

III.2. MOLECULAR CHARACTERIZATION OF PRIMARY TUMORS

The advantages of immunohistochemistry are especially related to the potential application to all patients and the significantly lower cost of the investigation. That is why in the first stage we carried out the qualitative validation of the immuno-histochemical methods selected for molecular classification, and the verification of accuracy by studying the internal and external control sections. To achieve this objective, we tested all the cases included in the present study for the expression of ER, PR, HER2, Ki67, EGFR, cytokeratin 5, p53, and Bcl2. In addition to the markers that have become classic in defining the molecular types of breast cancer, we also added E-cadherin, controversial in value from this point of view.

We studied E-cadherin, which is expressed more intensely the more differentiated the tumor is. The final reaction product is intensely stained, with significant membrane enhancement (fig. 8), with various patterns of distribution of the final reaction product. The positive control was represented by the epidermis, with a typical membrane reaction, more intense in the cells of the deep layers (fig. 8a). The intensity of the reaction was variable in the tumor cells from weak and heterogeneous (fig. 8b), to intense and homogeneous (fig. 8c). We did not observe significant differences in expression between primary tumors and E-cadherin expression in the corresponding axillary lymph node metastases (Fig. 8d).



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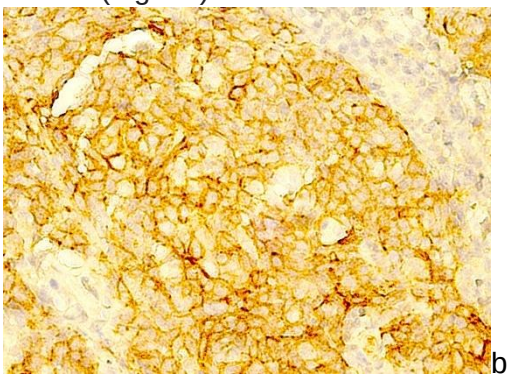

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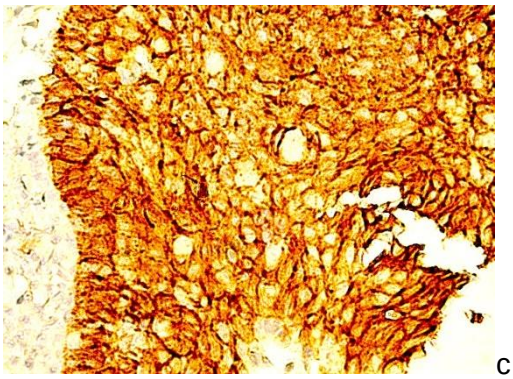
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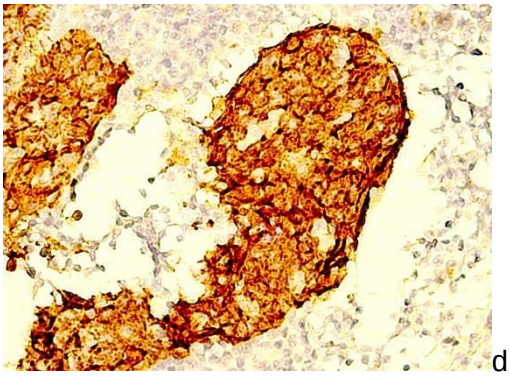
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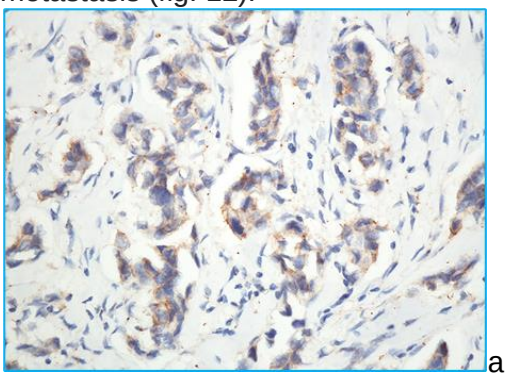
Fig. 8. E-Cadherin expression in breast carcinoma, distribution patterns. External positive control, the epidermis (a). Positive reaction with weak intensity and heterogeneous distribution of the final reaction product (b). Intensely positive immunoreaction with homogeneous distribution (c). Lymph node metastasis with all cells intensely positive, as in the primary tumor in the previous image. Original magnification x400.

III.3. COMPARISON BETWEEN THE MOLECULAR PROFILE OF THE PRIMARY TUMOR AND LYMPH NODE METASTASES

Among the 156 cases included in the present study, 80 presented lymph-node metastases (51.28%). Metastases were present in the majority of cases in the subcapsular space and rarely occupied most of the lymph node parenchyma.

To compare the molecular profile of the primary tumor and lymph node metastases, we applied the same methods to both specimens, respectively: ER, PR, HER2, Ki67, cytokeratin 5, p53, EGFR, E-cadherin and Bcl-2. We applied the same evaluation and scoring criteria as routinely used for primary tumors. Ki67 was assessed by the semi-automated method [66] to avoid over-interpretation in the case of lymph node metastases.

HER2 was positive in 31 of the cases. It was the most stable form on our material, with only four cases being discordant (3 from 4 where minor discordances). One of them was negative in the primary tumor and positive with +3 in the lymph node metastasis (fig. 12).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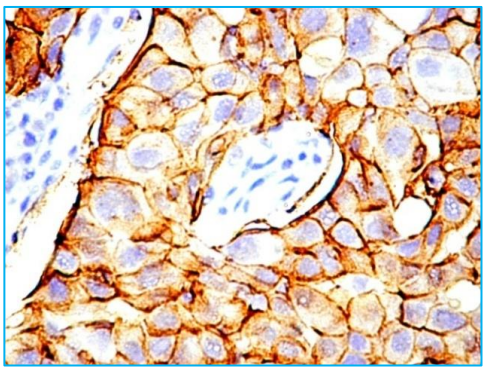
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Fig. 12. HER2 expression negative (+1) in the primary tumor (a) and positive in the lymph node (b).

Our results signal major molecular profile discrepancies between the primary tumor and the corresponding lymph nodr metastasis. In our case series, there were no significant changes in the cases initially diagnosed as HER2 and unclassifiable. After

examining the immunohistochemical expression of the mentioned markers, the number of luminal A carcinomas decreased by 3% and the basal-like ones by 2%, at the expense of the increase in Luminal B type cases.

III.4. IMMUNOHISTOCHEMICAL EXPRESSION OF ANDROGEN HORMONE RECEPTORS IN MOLECULAR TYPES OF BREAST CARCINOMA

Normal breast tissue adjacent to the tumor was available in 111 of the 124 cases. The final reaction product for AR was strictly confined to the nuclei of glandular luminal epithelial cells.

In ductal carcinoma in situ with luminal profile (ER/PR positive) we found intense reaction for AR in all tumor cells and the intensity of the reaction was closer to normal tissue than to the aspects observed in invasive carcinoma. In the invasive carcinoma the intensity of the immunoreaction was usually weaker than in the normal breast tissue and the distribution of the final product of reaction had an increased degree of heterogeneity. In invasive ductal carcinoma, the positive cases relatively consistently present more than 50% of the tumor cells stained at the nuclear level with moderate and strong intensity (fig. 19a) and in most cases marked with +2 and +3 according to the Allred scoring system. Invasive lobular carcinoma was significantly more heterogeneous, some cases being marked with +1 (fig. 19b) and in most cases with +2 (fig. 19c).

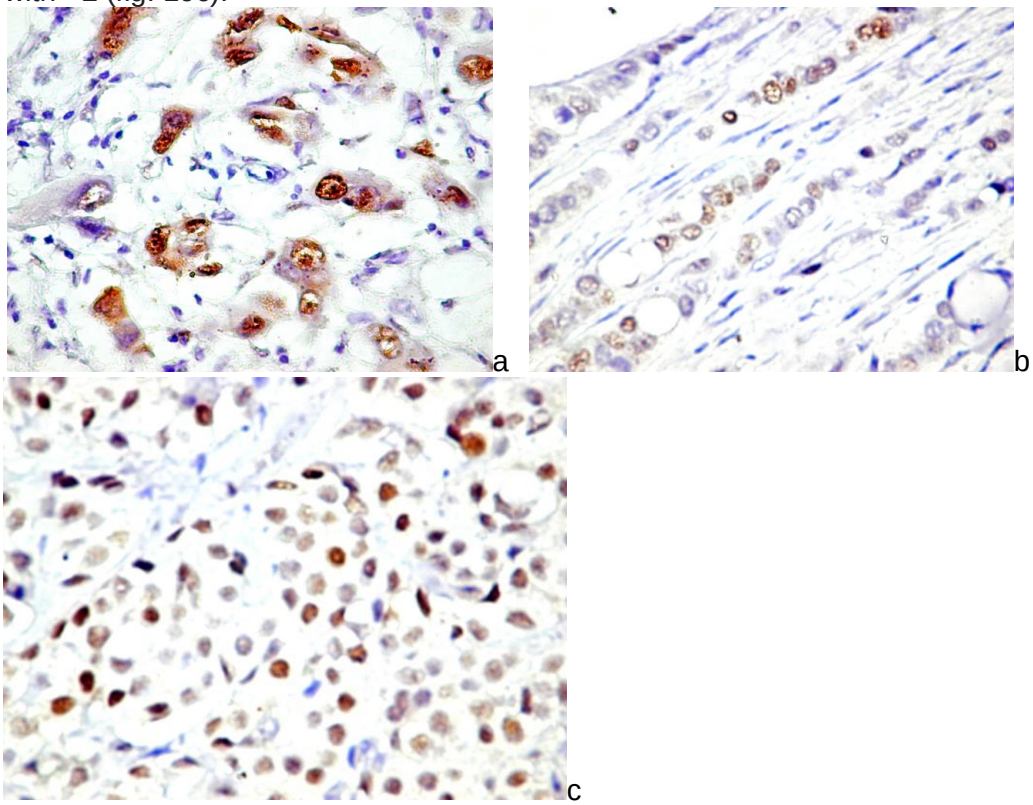


Fig. 19. Intense expression for AR in high-grade invasive ductal carcinoma (a). Invasive lobular carcinoma with poor (b) and moderate (c) reaction. Magnification a-c, x400.

Overall, we identified immunohistochemical expression for AR in 92 (74.19%) of the 124 breast cancer cases included in this part of the study. We found significant differences in positive reaction between pathologic types of breast carcinoma with the

highest values for invasive ductal and medullary carcinoma as seen in Table 28. The incidence of positive reaction decreases with increasing grade of differentiation. We found AR-positive tumors in 21 (91.30%) of 23 G1 cases, 65 (80.24%) of 81 G2 specimens, and 6 (30%) of 20 G3 cases. We note the marked decrease in AR expression in undifferentiated carcinomas.

III.5. DIFFERENTIAL EXPRESSION OF MAMAGLOBIN A IN THE PRIMARY TUMOR AND LYMPHONICAL METASTASES

Histopathological reporting. In 41 cases we diagnosed invasive ductal carcinoma, lobular carcinoma in 3 cases, papillary carcinoma in 2 cases and mucinous carcinoma in one case. Four cases were well differentiated, G1, 27 moderately differentiated (G2) and 16 were poorly differentiated (G3). Ductal carcinoma in situ was identified in association with invasive ductal carcinoma in 25 cases, atypical ductal hyperplasia in 4 cases and apocrine metaplasia in two cases. Normal breast tissue adjacent to the malignant tumor was present in 34 of our specimens.

Mammaglobin expression. The final reaction product for mammaglobin A was intensely stained, with a cytoplasmic granular distribution pattern and restricted to epithelial cells. Normal breast tissue adjacent to the tumor was positive for mammaglobin in all 34 cases. But on average less than 50% of the epithelial cells were stained (fig. 21a). Occasionally, some terminal ductal-lobular units showed positive homogeneous reaction. With the exception of breast tissue, only skin sweat glands showed positive reaction. In both cases, the apocrine metaplasia presented all cells intensely colored (fig. 21b). Three of the four atypical ductal hyperplasia lesions were also positive.

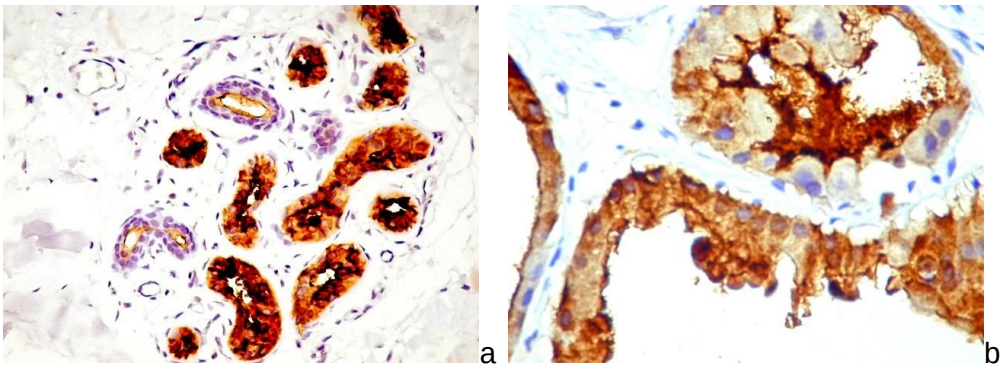

a b

Fig. 21. Normal breast tissue. Ductal-lobular terminal unit with heterogeneous positive reaction (a, x100). Apocrine metaplasia with uniformly stained epithelial cells (b, x400). Immunoreaction for mammaglobin A.

Ductal carcinoma in situ (DCIS) was identified in association with invasive ductal carcinoma in 25 cases, and expressed mammaglobin in 22 cases (88%). Usually, the number of positive cells in DCIS was higher than in the adjacent invasive carcinoma. We identified two patterns of distribution of the final reaction product: diffuse, with all positive tumor cells (fig. 22a), and heterogeneous with positive and negative areas in the same gland (fig. 22b).

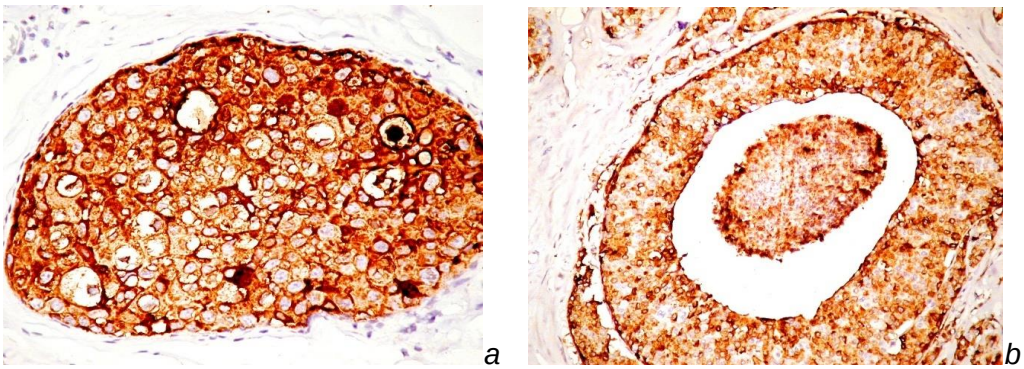
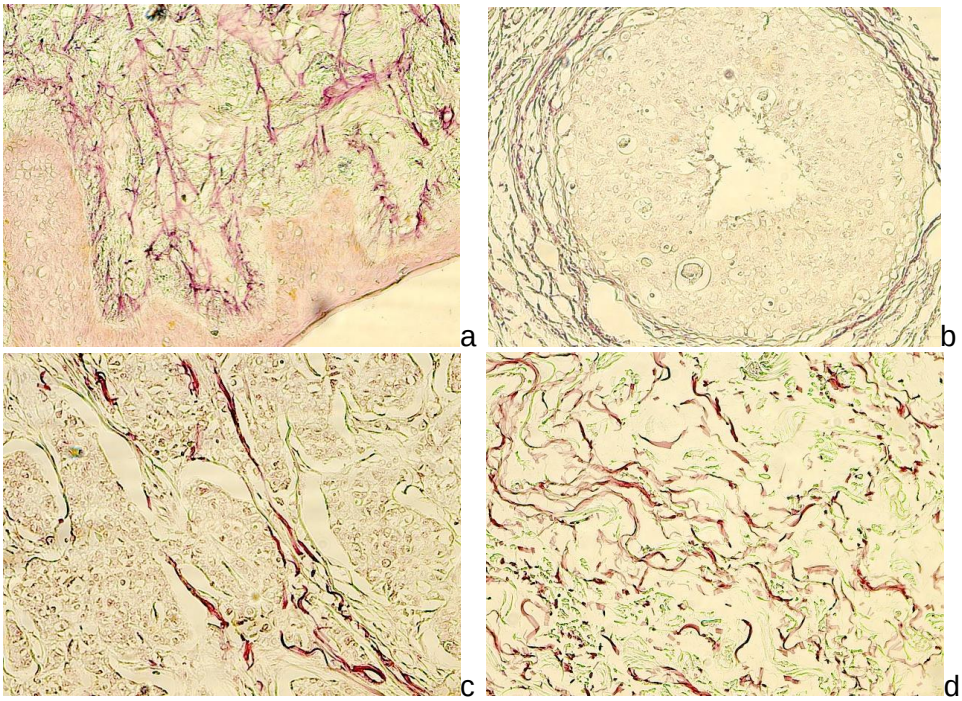


Fig. 22. DCIS, diffuse (a), and heterogeneous (b) distribution pattern. Anti-mammaglobin, x400.

III.6. THE PECULIARITIES OF THE MICROENVIRONMENT IN PRIMARY TUMORS OF THE BREAST

Elastic fibers were identified in normal breast tissue adjacent to the tumor in all cases. They were arranged isolated or in small groups, without forming thick bundles.

In the case of malignant tumors, the reactions for elastic fibers showed a great heterogeneity of distribution (fig. 27a). Thus, in some cases of carcinoma in situ, the elastic fibers formed a fine continuous layer around the malignant lesion (fig. 27b). In many cases, we identified elastic fibers in the vicinity of the proliferation and invasion front (fig. 27c). Depending on the disposition and density of the elastic fibers, we scored the elastosis associated with the malign tissue from 0 to +3, according to the definitions above (fig. 27 d, e, f).



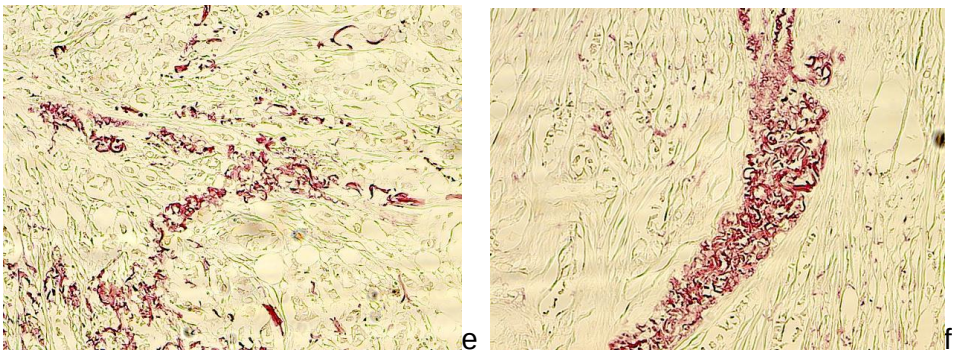
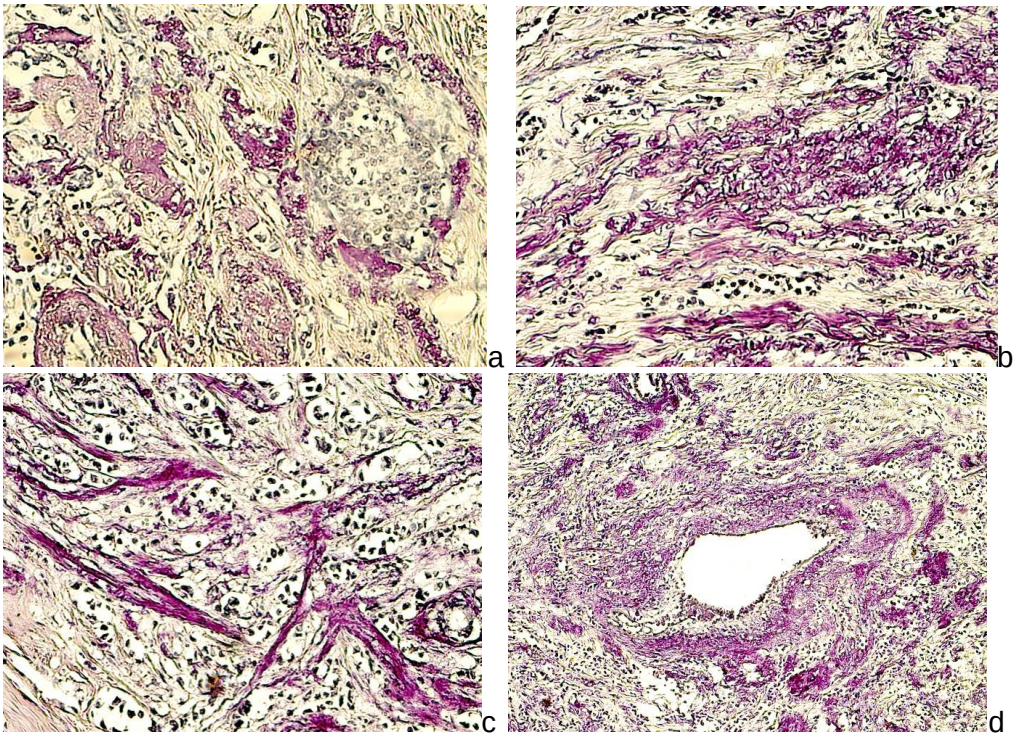


Fig. 27. Fine network of elastic fibers in the papillary dermis bordering the breast tumor (a, x200). Ductal carcinoma in situ, showing fine elastic fibers on the outline (b, x200). Elastosis marked +1 (c, x200). Elastosis marked +2 (d and e, x200). Elastosis marked +3 (f, x200). Orcein staining, Unna-Taenzer variant.

A particular aspect, in addition to the disposition of the elastic fibers, was the presence of an amorphous material, located only in the immediate vicinity of the malignant cells and which we did not observe in the adjacent normal breast tissue (fig. 28a). This material is in direct continuity with the elastic fibers arranged in the form of thick bundles (fig. 28b). We did not observe this aspect in the cases marked with +2 (fig. 28c), but it is relatively frequently observed around malignant ducts and glands (fig. 28 d, e, f). We have not found the presence of this material identified in both staining for elastic fibers reported in the literature, nor do we know its significance. We can only assume that these are fibrillar precursors possibly secreted by the tumor cells, but which do not aggregate as fibers, possibly through a defect in elastin synthesis.



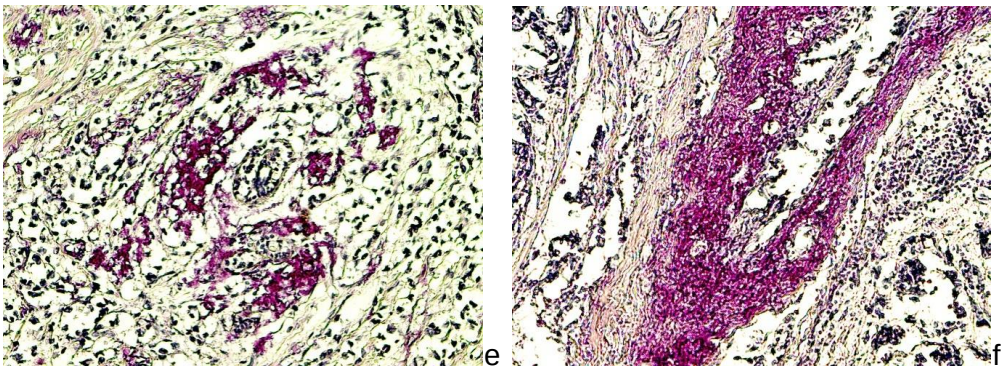


Fig. 28. Amorphous material in the immediate vicinity of tumor cells (a). Continuity with thick bundles of elastic fibers (b). Elastosis marked +2 (c). Amorphous material around a duct (d). Detail of amorphous material around a malignant gland (e). Elastosis grade +3 (f). Weigert elastica staining. X200.

According to the score defined above, of the 156 cases included in the study, 39 (25%) presented elastosis. Of these, 11 cases were scored with +1, 14 cases with +2 and 14 cases with +3. The data regarding elastosis did not correlate either with the age of the patients or with their menopausal status. We did not observe a statistically significant correlation with the histopathological form, but elastosis correlates with a reduced degree of differentiation ($p<0.0024$). Although a significant number of cases with elastosis were not associated with lymph node metastases, we obtained no significant correlation, and apparently, elastosis is not a useful predictor of lymph node metastases.

FINAL CONCLUSIONS AND ORIGINAL CONTRIBUTIONS

Breast cancer is the most common neoplasia of the female sex as we have shown in the general part. The rapid transition to the molecular stage of the diagnosis of neoplasia had a major impact not only on the prognosis but also on the therapeutic strategy. Breast neoplasia enjoys great interest from this point of view and is the first human tumor to be classified on a molecular basis, with practical applicability.

In the present work, we investigated 156 cases of breast cancer selected according to the criteria given in the material and methods. The main requirement for inclusion in the study was the correctness of establishing the tumor stage, based on the elements included in the TNM system. Although the specimens came from several different centers, morphologically and molecularly we did not find significant differences between the four series, so we considered the series of patients as homogeneous. The majority of cases of breast carcinoma were of the invasive ductal type, without other specifications. Most tumors were detected in locally advanced stages of clinical evolution, which once again draws attention to the need for early detection through screening and self-examination actions. From our data, histopathological form is not a useful predictor of lymph node metastases. Most cases were moderately differentiated, G2. We obtained a statistically significant positive correlation only for G3, which appears to be a useful predictive factor for lymph node metastases.

Lymph node metastases were identified in 80 of the 156 cases (51.27%), being characterized by tumor cells present in the subcapsular sinus or partially or completely replacing the lymphoid tissue. We point out the possibility of metastases in atrophic lymph nodes, an aspect not mentioned in the specialized literature. It is an original observation, and on this subject, no article has been published to date. We consider

the examination of atrophic lymph nodes mandatory, under the same conditions, even if the altered nodal microenvironment does not yet allow the explanation of this process. This observation raises new issues in the genesis of malignant adenopathy, most likely involving the reticular cells of the stroma, deeply neglected from this point of view until now. Except for the diagnosis of the primary tumor and lymph node metastases, the conventional histopathological examination brings little useful information for the prognosis and the establishment of the therapeutic strategy in breast cancer.

Based on the results obtained, we consider extensive molecular classification mandatory, based on a spectrum of useful antibodies that includes ER, PR, HER2, Ki67, CK5, EGFR, and p53. Other cytokeratins, E-cadherin, and Bcl-2 do not add value to molecular diagnosis classification. Ki67 performance is mandatory for differentiation of luminal types and possibly monitoring of postoperative chemotherapy. Our data support the use of the expanded antibody panel for defining the molecular type of breast cancer. Luminal A type cases represented 46.79%, Luminal B 17.94%, HER2 19.87%, basal-like 10.25%, and unclassifiable 5.12%. The molecular type of breast carcinoma does not correlate statistically significantly with the prediction of lymph node metastases, and the basal-like type showed lymph node metastases as well as the other types. E- and P-cadherins are differentially expressed in molecular forms of breast cancer and our results do not support the introduction of these markers into the current panel of molecular diagnosis and classification. On the other hand, E and P-cadherins are useful to demonstrate the epithelial-mesenchymal transition. Currently, the molecular classification does not replace but complements the conventional morphological one.

Our results indicate major molecular profile discrepancies between the primary tumor and the corresponding lymph node metastasis, which account for nearly 20% of cases. In our case series, no significant interconversion changes occurred in cases initially diagnosed as HER2 and unclassifiable. After examining the immunohistochemical expression of the mentioned markers, the number of luminal A carcinomas decreased by 3% and the basal-like ones by 2%, at the expense of the increase in Luminal B type cases. The increase in the metastatic capacity of tumor cells induces major changes in the molecular profile. Our data support synchronous molecular examination of the primary tumor and lymph nodes or distant metastases, with the outcome having a major impact on therapeutic strategy. Based on the data from the literature, we consider this study as original, being the first in the country presented on this subject. As a priority, it is the first study to investigate this aspect based on the expanded panel used for molecular classification.

In this study, we demonstrated that androgen receptors are expressed in 74.19% of all breast cancer cases. We found a statistically significant correlation between AR expression, tumor histopathological type, degree of differentiation, and lymph nodes status. Among AR-positive tumors, 52.17% of cases co-express ER and 59.78% co-express PR, but without significant correlation. We identified no significant correlation between AR expression, distant metastases, and HER2 overexpression, but only an inverse correlation with triple-negative breast cancer molecular profile. However, our results argue for the evaluation of an experimental model of anti-androgenic medication in cases resistant to conventional hormone therapy.

Elastosis associated with breast cancer was observed in 25% of cases, an aspect confirmed by two staining methods. We propose an original elastosis scoring system and describe for the first time an amorphous material with a similar orcein

affinity to elastin, of unknown origin and of uncertain significance. Elastosis correlated with a reduced degree of differentiation, but not with the other clinical-pathological prognostic parameters. Under these conditions, elastosis was not a predictive factor for lymph node metastases.

The density of mast cells in the tumoral and peritumoral areas does not correlate with the molecular profile of the breast carcinomas included in the present study. Conversely, the density of mast cells in the tumoral area, but not peritumoral, correlates statistically significantly with lymph node metastases, independent of the molecular type. Under these conditions, we believe that the density of mast cells in the tumor area is a useful predictor for lymph node metastases. Although the presence of mast cells in the breast tumor microenvironment has been well known for many years, our data highlight for the first time the role of tryptase-positive mast cells in the prediction of lymph node metastases.

In our study, CD68-identified macrophages and tumor-associated CD34-positive fibrocytes have no predictive value for lymph node metastases. We believe that the involvement of these cells in the phenomenon of metastasis has been overestimated in the past.

Mammaglobin A is expressed in 74.19% of primary breast tumors and in 58.06% of corresponding lymph node metastases. Through the reaction with mammaglobin, lymph node micrometastases, lymphovascular and peri-nervous invasion from invasive ductal carcinoma are easily identified. The immunohistochemical expression of mammaglobin A correlates with the degree of differentiation, with lymph node metastases, but not with the other clinical-pathological prognostic parameters. The negative character of the expression is a potential indicator of lymph node metastasis, the intensity of the reaction decreasing proportionally with the increase in the degree of differentiation. Our data support that mammaglobin expression defines a subgroup of patients with a better prognosis and is a useful method for the diagnosis of lymph node metastases.

Summarizing the above data, we report significant molecular profile differences between the primary tumor and lymph node metastases, the association of AR with tumors that predominantly express ER, the negative reaction for mammaglobin, and the high number of mast cells that have predictive value for lymph node metastases. The molecular profile of the primary tumor is not necessarily the same as the profile of lymph node metastases, and we found discordances in almost 20% of the cases. This finding could have major importance in the therapeutic strategy of patients with breast cancer.

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