MYELOLYMPHOID CELLULAR INFILTRATION - A MODULATOR FACTOR OF TUMORAL INVASIVE PHENOTYPE IN MAMMARY CANCER

SUMMARY

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IASI
2014
Generalities

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Motivation of the doctoral research

Mammary cancer develops in the presence of continued interaction between tumor cells and their contiguous microenvironment. The role of the immune system is not fully explained. There are several researches attempting to evaluate the role of cells of the innate immune system and of the adaptive compartment. While their role in infections and allergic reactions is well documented, for the tumor development many, yet incompletely exploited observations suggest that the immune system might play a dual role, both stimulating and inhibiting the tumor process. The final pro- or anti-tumor result depends on a wide range of factors, belonging both to the tumor and the host.

The practical finding of mammary cancer patients within the same prognostic group according to the classic prognostic factors (number of ganglia with tumor invasions, tumor grade, tumor histology, tumor markers) and following the same treatment but evolving differently suggests that there are other parameters influencing the outcome. Such parameters could have prognostic value, and the tumor microenvironment might be one of them.

The action of tumor cells stimulates the effector differentiation of immune system cells into cellular versions with different actions. For instance, resident macrophages are stimulated to differentiate into M1 or M2 macrophages; neutrophils can differentiate into N1 or N2 neutrophils with different or opposite behaviors towards the tumor. The adaptive immune system also includes elements with diverging actions in the relation between the tumor and its microenvironment. CD8+ cytotoxic T lymphocytes have mostly an anti-tumor role, while CD4+ regulatory T lymphocytes have a typical pro-tumor one. The mechanisms that tilt the pro- and anti-tumor balance and the conditions that precisely specify these differentiation subtypes within the tumor tissue or the lymphoid tissue supporting it are also incompletely understood.

Mammary cancer includes tumors with different histologies, aggressiveness, treatment responses and evolutions. Tumor development is a heterogeneous process, which impedes the evaluation of the relation between the tumor and its microenvironment. To understand this relation, one needs to define histoarchitecture grades within the lesion, allowing the quantification of immune system cells versus the tumor in its various areas: invasive front, peritumoral, intratumoral, and normal surrounding areas.

The evaluation of the tumor microenvironment in the present information age requires the development of computer-aided methods to
generate objective and reproducible results, eliminating from the analysis the subjective human factors (inter- and intra-observer).

Within this context, the evaluation of the immune system cells versus the tumor can result in a better understanding of the pathogenic mechanism and in the identification of prognostic and prediction factors that model the therapy approach in a more efficient way.

Goals of the doctoral research and work plan

- **Goals of the doctoral research**

  This doctoral research aimed at the characterization and quantitative evaluation of myelolymphoid infiltration in mammary cancer and at assessing its influence on tumor development, as a possible prognostic factor, in correlation with the classic prognostic factors (number of ganglia with tumor invasions, tumor grade, tumor histology, tumor markers), the disease-free interval and the general survival.

  As regards the quantitative analysis of myelolymphoid infiltration, the research aimed at the development and optimization of the use of a semi-automatic quantitative analysis technique to generate objective and reproducible data, reducing the subjective human factors. The optimization of the technique concerns two stages: the optimization of the image acquisition parameters and the optimization of the cell delimitation parameters, which subsequently produce automatic statistic data. Another goal was to identify and monitor error factors with a potential impact on the results of quantitative analysis.

- **Work plan**

  The work plan included:

  - The admission of cases based on the selection criteria for patients included in the study.
  - Microtome sectioning surgical exeresis pieces included in paraffin.
  - Staining the sections with hematoxylin and eosin and evaluating the grade of myelolymphoid infiltration.
  - Immune marking of the sections with specific markers for the myelolymphoid infiltration cells. Four immunohistochemical markers were used, representative for the marking of macrophages (CD68), of common T lymphocytes (CD3), of cytotoxic T lymphocytes (CD8), of B lymphocytes (CD20), of T helper cells (CD4), of memory lymphocytes (CD45RO), and of stem cells (CD44).
Immune marked sections were then observed under a microscope, and their images were acquired and fed into a semiautomatic quantitative analysis software.

The immune marked sections were analyzed under the authority of an anatomopathologist, who helped evaluating the presence and layout of myelolymphoid infiltration, the quality of the immune marking, and identifying the interest areas for analysis.

Defining the selection criteria for interest areas.

Validation of the parameters of the image acquisition technique.

Feeding the acquired images of immune marked paraffin sections into the quantitative analysis software.

Validating the computer-aided quantitative analysis parameters, optimizing the technique, and proper analysis automatically generating statistical data for the relevance analysis.

Feeding obtained data into the database.

Statistical analysis of data.

Interpretation of statistical data and comparing them with the existing publications.

Materials and methods

➢ Research material

The research included 60 mammary cancer patients registered to the Clinic and/or the Oncology Ambulatory Care Unit of the St. Spiridon Emergency Clinical Hospital of Iasi County between 2001 and 2010. The patients were informed on the goals and proceedings of the researched and signed informed consent forms. The study was approved by the Bioethics Committee of the Gr. T. Popa University of Medicine and Pharmacy of Iasi.

➢ Methods used

For the histopathological analysis, the diagnostic blocks included in paraffin were sectioned, stained with hematoxylin and eosin, and then processed using immunohistochemistry techniques. Specifically marked sections were analyzed using a computer-aided quantitative analysis method, which required optimizing the technique.

- Immunohistochemistry (IHC)

Immunohistochemistry is a histological technique for the identification of cell or tissue components using antigen-antibody interaction. Epitopes were visualized by adding a chromogenic substrate (generally
diaminobenzidine - DAB). The set of monoclonal antibodies used in this research is detailed in Table I.

**Table I** Primary antibodies used in the research.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody type</th>
<th>Dilution</th>
<th>Marking type</th>
<th>Primary marked cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>Mouse monoclonal, DAKO Clone KP1, Code-No. IS609</td>
<td>1:1</td>
<td>Cytoplasmic, granular, diffuse</td>
<td>Macrophages, monocytes</td>
</tr>
<tr>
<td>CD3</td>
<td>Mouse monoclonal, DAKO Clone F7.2.38, Code-No. M 7254</td>
<td>1:100</td>
<td>Membrane</td>
<td>Common T lymphocytes</td>
</tr>
<tr>
<td>CD8</td>
<td>Mouse monoclonal, DAKO clone C8/144B, Code-No. M7103</td>
<td>1:100</td>
<td>Membrane</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CD20</td>
<td>Mouse monoclonal, DAKO Clone L26, Code M0755</td>
<td>1:400</td>
<td>Membrane</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>CD4</td>
<td>Mouse monoclonal, DAKO Clone 4B12, Code M7310</td>
<td>1:80</td>
<td>Membrane</td>
<td>T helper cells</td>
</tr>
<tr>
<td>CD45RO</td>
<td>Mouse monoclonal, DAKO Clone UCHL1, Code-No. M 0742</td>
<td>1:100</td>
<td>Membrane</td>
<td>Memory lymphocytes</td>
</tr>
<tr>
<td>CD44</td>
<td>Mouse monoclonal, DAKO Clone DF1485, Code-No. M 7082</td>
<td>1:50</td>
<td>Membrane</td>
<td>Stem cells</td>
</tr>
</tbody>
</table>

- **Computer-aided quantitative analysis technique**

  The principle of the method is the quantitative processing of images (of microscope slides of immunohistochemically marked tissue sections) by automatic separation of colors and of their intensities (using a software), allowing tracing the cell components, under the control of specific image parameter values. The technique further allows automatically quantifying the cell components and automatically performing a statistical analysis using the obtained data.
The HistoFAXS system is a combination of Zeiss hardware modules with two software modules:

1. **HistoFAXS** - the image acquisition module (Fig.17),
2. **HistoQuest** - the immunohistochemical staining analysis and data management module.

![Fig.17](image)

**Fig.17** On the right is a preview of the two areas of interest; the one in a red frame is acquired (using a 20x lens) and viewed on the left side of the image. A field of view (fov) of the acquired region is seen as a detail (at the center of the image).

Cell contours are traced automatically on the color overlay image (Fig.21). The color overlay image is the area of interest for the quantitative analysis with the cell contours overlaid automatically, based on the software’s interpretation guided by a set of parameters modulated by the analyst. The processing of this type of image is achieved by changing the specific parameters for each marker (nuclei and positive cells for the marker used). The analyst must know the way of changing the software-defined default parameters to obtain correct cell delimitation. When the correct cell delimitation is obtained, the software automatically generates statistical data displayed as histograms and scatter plots.
Fig. 21 Tracing the contours of cell components on the color overlay image in a tissue section marked for estrogen receptors, acquired using a 20X lens.

A cut off must be placed, separating the populations of positive and negative cells. The cut off can be positioned manually or automatically. This research demonstrated the advantage of using automatic positioning, but with a comparative qualitative analysis of the studied image vs. the software-generated results, as for less than 10% positive cells, modified results are generated. Based on the positioning of the cut off, the software generates tables mentioning the absolute number and the percentage of positive and negative cells, and the total number of cells in the analyzed area.

**Results of the analysis of the myelolymphoid infiltration in mammary cancer**

To analyze the myelolymphoid infiltration, serial sections were made (12 slides with 2-3 sections per slide) from each block of each patient included in the doctoral research; hematoxylin staining was subsequently used for the overall evaluation of the slide. Then the immunohistochemistry technique was applied, using the 7 markers: D68, CD3, CD8, CD20, CD4, CD45RO and CD44.

Slides with immunohistochemically marked section were initially acquired in full as an overall image using a 2.5X lens. Subsequently, each section on the blade was acquired using a 20X lens.
Section images were imported in the HistoQuest analysis software. An anatomopathologist assisted the analysis of the whole surface of the section, and the choice of 12 areas of interest for each section: 3 invasive front areas, 3 peritumoral areas, 3 intratumoral areas, and 3 normal tissue areas, defined as follows:

**Invasive front areas (FI)** are on the tumor’s perimeter. Half of the selected area belongs to the tumor, and the other half to the peritumoral tissue, where groups of tumor cells are identified, proving the tumor invasion.

**Intratumoral areas (IT)** are chosen in the middle of the tumor, where tumor cells are well represented, showing specific modifications for the tumor process: large nuclei with visible nucleoli, scarce cytoplasm.

**Peritumoral areas (PT)** are chosen at the periphery of the tumor. Half of the selected area belongs to the tumor, and the other half to the peritumoral tissue. Peritumoral tissue must be free of tumor invasion elements (tumor cells); it must show a clear separation between the two compartments, to make the difference with the invasive front.

**Normal areas (N)** are chosen at some distance from the tumor periphery; they contain normal components of mammal tissue, without visible effects of the tumor invasion.

Statistical analysis on the 4 areas showed that the value of the average percentage, for the CD68+ phenotype and for the CD8+ cytotoxic T lymphocytes, decreasing from the invasion front towards the peritumoral and intratumoral areas, with a slightly higher average in the normal area. Unlike the CD68+ and CD8+ profile, for the other two markers, the CD3 and CD20, the decrease of the percentage is from the invasive front towards the peritumoral and intratumoral areas, with the smallest percentage in the normal tissue (Fig. 69). In the three tumor compartments (invasive front, peritumoral, intratumoral), for all the markers, the highest values were in the invasive front, and decreased in the peritumoral area, with the lowest value in the intratumoral area.

The frequency of both CD8+ T lymphocytes and CD3+ T lymphocytes showed a downward gradient from the invasion front to the peritumoral and tumor areas. The maximum difference between the magnitude of the total CD3+ T population and the CD8+ T subset follows this gradient, and is visible in the invasive front (25% and 15% respectively).

Looking at the differences between the values of the CD3 percentage (all T lymphocytes) and CD8 percentage (cytotoxic T lymphocytes),
Fig. 69 Variation of the average percentages for markers CD68, CD8, CD3, and CD20 for all the areas of interest: invasive front (FI), peritumoral (PT), intratumoral (IT) and normal area (N).

which are a subpopulation of the CD3+ cells, we see a difference of approximately 2% in the normal compartment, most probably owing to CD4+ T cells. For the same two markers, the highest increase is seen in the invasive front both for CD3+ and CD8+, with a difference of roughly 10% between the two population, probably owing to CD4+ T lymphocytes. The increase of this population of T cells, most probably from 2% in the normal region to 10% in the invasive front demonstrate a chemoattraction to the tumor for this population, which is very likely actively involved in the facilitation of tumor growth.

Most studies evaluating tumor immunity focussed on CD8+ cytotoxic T cells, with their cytotoxic activity killing the tumor. Recent studies indicate that CD4+ T cells (in their instance of Th1 differentiation), B cells, macrophages and dendritic cells have important contributions to the anti-tumor immune response, by secreting immunostimulator factors or antigen presentation mediated functions. On the other hand, in the lesions, imunosuppressive cell subtypes can be documented, including regulatory T cells, myeloid derived suppressor cells, and M2 macrophages, which play an important role in oncogenesis and in tumor progression.
Fig. 75 Average percentages for the 4 markers (CD20, CD3, CD8, CD68) vs. the Her2neu status.

Regarding the analysis of the infiltration versus the Her 2 status, this research demonstrates that the infiltration is better represented in the Her2+ subplot than in the Her2- one; the difference between the CD3+ and CD8+ populations in the invasive front is 12% for the Her2+ patients, and 5% in the Her2- patient, which is more than twofold, with the difference interpreted as CD4+ T cells population. This finding is also supported by Perez and co-workers, which assert that Treg FOXP3 cells are represented in higher numbers in the Her2+ group than in the Her2- group (Perez et al., 2007). This cell population inhibits the action of CD8+ T lymphocytes, a subset with both cytolytic and cytokine complex roles, and the impact of the interaction between Treg and T cytotoxic can be differentiated between Her2+ and Her2- mammary cancers. Thus, Perez et al. sustain that a higher proportion of CD8+ Y lymphocytes is a positive prognostic factor only in non amplified Her2 cases, and not in amplified Her2 cases. In our lot, we have also observed a heterogeneity in the representation of this cell population vs. the Her2 status (Fig. 75). Increased inflammatory infiltration was associated with RE- and Her2+ (in accordance with Tsanq et al., 2014)
Fig. 76 Representation of the absolute values for the 4 markers in the 4 areas of interest, vs. the status of estrogen receptors.

The analysis of the myelolymphoid infiltration vs the receptor status pointed out that it is more present in RE- than in RE+, which is also confirmed by Calabro and co-workers (Calabro et al., 2009) (Fig. 76). For the subgroup with positive estrogen receptors, the CD20+ B lymphocyte and CD3+ T lymphocyte populations double in the invasive front, peritumoral and intratumoral areas, compared to the normal area. As regards the layout of CD8+ T population in the lesion, we noted that the RE+ sub-lot in tumor areas systematically shows higher values than the normal area; on the contrary, higher values of this population were found throughout the RE- breast mass. Such a contrasting image of RE+ and RE- cases suggests fundamentally different functions of the CD8+ T cells population, probably rather pro-tumor than anti-malignant. A similar image of heterogeneity of the CD8+ T infiltration was also reported in publications of Calabro et al.

Tumor infiltration of axillary lymph nodes is the most important prognostic factor in operable mammary cancer, and it is significantly correlated with both the disease-free survival and the overall survival (Jatoi et al., 1999, Wilking et al., 1992).

For the N0 sub-lot, the accumulation of CD68+ cells in the normal area is accompanied by the decrease of the common T lymphocyte population.
For this sub-lot (N0) the correlations between CD3+, CD8+ and CD20+ (values of cellular density in the tissue) have been strongly significant in the analysis of each subgroup of histological area; the correlations are maintained for N1, with the mention that CD68+ is more strongly correlated with CD20+ in the intratumoral region, B lymphocytes are quantitatively correlated with cytotoxic T lymphocytes, and the bibliography classifies them as beneficial for the N0 group of patients.

![Graphic representation of the variation of absolute value averages for each marker (CD68, CD8, CD3, CD20) vs. N for each of the four identified areas (FI, PT, IT, N).](image)

**Fig. 79** Graphic representation of the variation of absolute value averages for each marker (CD68, CD8, CD3, CD20) vs. N for each of the four identified areas (FI, PT, IT, N).

There are researches that used different approaches to the analysis of the lymphocyte infiltration, namely intratumoral (in contact with tumor cells) and peritumoral (in the stroma not in contact with tumor stroma) by using semi-quantitative scoring. For instance, a 2014 study (Chen Z et al., 2014) reveals a significant independent impact of the parameter representing the proportion of CD8+ T lymphocytes in the infiltration, both on DFS and on the survival, for a large lot of patients in N0. This causality was distinctly created from many other biological factors, and was more spectacular in triple negative cases. We must mention that this research uses its own nomenclature, and what it defines as *intratumoral* is the equivalent of the invasive front and peritumoral area in our research, while *peritumoral* is equivalent to our normal area (Chen Z et al., 2014).
According to modern hypotheses of cancer immunoediting, cancer progression has 3 phases: elimination, equilibrium, and escape. In the initial elimination and equilibrium phases, cancer cells are predominantly attacked by CD8+ T lymphocytes or by other antitumoral factors; on the other hand, in the escape phase the tumor inhibits the CD8+ T lymphocytes either directly (reducing the expression of MHC-I, HLA-G type immunomodulating and other) or through the suppressor action of Treg FOXP3+ lymphocytes, myeloid suppressor cells, neutrophils, M2 macrophages, CD4+ Th2 cells, and cytokines (Chen Z et al., 2014).

This vision includes the possibility of continuous variation of the tumor, both genetic and antigenic, and encompasses positive and negative feedback type effects between the polyclonal tumor and the immune system (*immunosculpting*).

The analysis of myelolymphoid infiltration versus G, the tumor differentiation grade shows a growth for CD8+ cytotoxic T lymphocytes for the G3 sub-lot, in all the three tumor compartments (FI, PT, IT), compared to G1 and G2. For the CD3+ T lymphocyte and the CD20+ B lymphocytes, the
behavior was similar, with a gradual increase from the G1 sub-lot to G2 and G3 Fig. 81). Concordantly, others support the association between the increase of inflammatory cellular infiltration and the increase of the tumor differentiation grade (Mohammed et al., 2012).

The analysis of the myelolymphoid infiltration in mammary cancer was first performed for the entire lot of patients using the CD68 marking - usually assimilated to the revealing of the monocytes-macrophages population, CD3 - marking for common T lymphocytes, CD8 - for cytotoxic T lymphocytes, and CD20 - for B lymphocytes. The overall population of T lymphocytes includes several lineages (such as CD8+ cytotoxic T, CD4+ T helper, T\(\delta\) – typical for negative co-receptors, NKT), with a heterogeneous representation in various tumor lesions. We had expected the proportion of the marked CD8 cells subset systematically follow the CD3 marking. However, we noted a very disperse difference between the CD3 and CD8 marking values (as an independent parameter for viewing the non-CD8+ T compartment) between different tissue compartments. The maximum values of these differences were found in the invasive front area. This difference was interpreted as strongly representative for the proportion of the Th (CD4+) cellular compartment. Markings in CD4 are currently seen as uncomfortable for the paraffin tissue sections, thus this marker was not included in the initial set. Subsequently, to clarify such discrepancies, one of the few available CD4 dedicated staining was introduced. Given the inherent difficulties of the immunohistochemical technique, the CD4 marking being significantly affected by the tissue specimen fixation conditions and by the thermal preparation time, the statistic analysis for this direct marking was carried out on a sub-lot of 10 cases (Fig. 88). The idiotype used for marking does not distinguish between the CD4 expression in T lymphocytes versus other cell lineages, such as macrophages and dendritic cells.

The analysis of the CD4+ marking showed an increased accumulation for the CD4+ cell population in the invasive front, compared to the CD3 marking (varying in the same direction, but at a higher magnitude than the discussed CD3 - CD8 difference); this phenomenon might be explained by the marking of both CD3+ (Th) cells and CD68+ cells (mostly resident macrophages). An additional cell group could be involved in the explanation of the very high proportion of CD4 markings versus others, namely the dendritic cells (Fig. 88).

Note that CD3 in the IF area has a negative correlation (r = -0.825, p = 0.022) with CD4 for the FI, which could be explained that an increased intensity of the CD4 marking in the FI does not involve the CD3+ T cells, but
rather the increase of the number of macrophages and dendritic cells in FI. Another possible explanation is the increase of the number of CD4+ cells (or instance, predominantly or preferentially anti-inflammatory like Treg) in the FI is associated with fewer CD3+ cells, owing to the decrease of the number of CD8+ cells in FI.

![Graphical representation of the averages of absolute values for CD68, CD8, CD3, and CD4 markings vs the 4 analyzed areas (FI, PT, IT, N)](image)  

**Fig. 88** Graphic representation of the averages of absolute values for CD68, CD8, CD3, and CD4 markings vs the 4 analyzed areas (FI, PT, IT, N)

Descriptive statistics indicate an approximately 3-fold increase of the average of absolute values for the CD20 marking in the analyzed tumor areas, compared to the N area. B cells seem to have a suppressing action on the growth of cancer cells, associated with a favorable prognostic (Mahmoud et al., 2012), possibly by dampening the inflammatory reaction. The CD20 marker was positively and significantly correlated for all the analyzed areas of interest, but not with the other markers (CD3, CD8, CD4, CD68, CD45, CD44) as regards absolute values.

For the CD44 marker, the same negative correlations were established between tumor areas and the N area, but these correlations were not statistically relevant (p > 0.05). On the other hand, no statistical correlations resulted between the absolute values obtained for the CD44 marking versus the absolute values for other markings within these researches, for none of the interest areas.
CD44 was extensively used in combinations with other markers to isolate stem cells from solid tumors (Slomiany et al., 2009; Lee et al., 2010). CD44 is seen as a potential marker for stem cells in most cancers, and more frequently in mammary, prostate, pancreas, ovary and colorectal cancers (Du et al., 2008; Bapat, 2010). CD44 is expressed on the surface of cancer cells and assists the hematogenic metastasizing by the interaction with P-selectins and S-selectins (Napier et al., 2007). Some studies demonstrated that tumor stem cells are involved in the tumor genesis, metastasizing, and treatment resistance (Gupta et al., 2009).

The CD45RO marker in the FI for absolute values was positively correlated with CD45 in the PT ($r = 0.907, p = 0.001$) and IT ($p = 0.561, p = 0.037$). Lymphocytes expressing the marking for CD4 (common leukocyte antigen) include CD4+ T lymphocytes, CD20+ B lymphocytes, and many cells in the myeloid line, including tumor-associated macrophages often identified by immune marking with CD68 (DeNardo et al., 2009). Increased leukocyte infiltration is associated with the higher survival rate of patients under 40 years of age (Ménard et al., 2007) and with a favorable prognostic for patients with tumors with increased tumor-associated macrophage infiltration (Pupa et al., 1996). Other researches claimed the presence of CD68+ cells in the tumor tissue is correlated with an unfavorable evolution (Bingle et al., 2002; Mukhtar et al., 2011; DeNardo DG et al., 2011); with an

![Graphic representation of the averages of absolute values for CD20, CD45RO and CD44 markings vs the 4 analyzed areas (FI, PT, IT, N)](image-url)

**Fig. 89** Graphic representation of the averages of absolute values for CD20, CD45RO and CD44 markings vs the 4 analyzed areas (FI, PT, IT, N)
increase of the tumor grade (Esserman et al. 2006; Volodko et al., 1998; Lee et al., 1997); with increased angiogenesis (Uzzan et al., 2004; Tsutsui et al., 2005; Bolat et al., 2006; Chen et al., 2005); with decrease of the disease-free survival (Leek et al., 1996; Campbell et al., 2010); and with increased risk of metastasizing (Robinson et al., 2009).

CD45RO, a shorter isoform of CD45 detected in our markings, is a marker for memory lymphocytes present both in normal and tumor tissues, with increased density in mammary cancer. CD45RA+ regulatory T cells are converted to the CD45RO+ phenotype after activation. Nevertheless, the patterns of gene expression for CD45RO+ and CD45RA+ Treg are very distinct, which indicates they might be different cell species (for instance, as idioype specific) in different stages of differentiation, but rather functionally distinct lineages. CD45RO+ and CD45RA+ Treg are preferentially located in different tissues owing to the expression of different receptors for tissue-specific chemokines (Nicola et al., 2010).

A definite dynamic in the distribution of cell marking for molecules typical to leukocytes generally found in lesion tissue areas is thus also obvious for markers CD45RO and CD44. CD45RO typically highlights memory lymphocytes; CD44 is also representative for the traffic of memory T lymphocytes to mucous epithelial areas (Fig. 89). Without a references for values reflecting the normal tissue status in our lot, and moreover without the evolutionary dynamic image of lesions (only a static image of the diagnostic piece was available), we found that the whole mammary lesion area is systematically populated with lymphocytes (either resident or transiting), and heterogeneity of the various compartments is induced in the representation of these populations. We believe this leukocyte population can have temporal (dynamic) characteristics relevant for the tumor progression.

Overall, for all the determinations we analyzed, we underline that the basic goal was to establish the feasibility of the numerical analysis of immune marking images to explore the layout of the inflammatory infiltration in the paraffin-included mammary neoplastic lesion. As detailing the possible contribution of this layout to the determination of DFS and survival would have requires a much larger patient lot, we did not actually expect to get into the statistic relevance of cases to detect such effects. We thus point out that for many of the immune markings applied to the infiltration we have observed tendencies (as impact direction, but for p values without explicit statistical support) of influence on the evolution profile, generally in accordance with the ones mentioned in published works. Correlations at p < 0.05 between magnitude values for the C68 immune marking and survival revealed a
negative impact on the survival of patients with Her2- and a positive impact on patients with RE+, which indicates that different prognostics are possible for the immune system, based on the tumor subtype.

Chapter 15. Discussions

15.1 Considerations on the quantitative analysis technique

Many studies (such as Jochems, Schlom, 2011) have proved the connection between the immune infiltration, the prognostic and the response to treatment in several types of human carcinoma; although the idea is recurrent, it seemed not accepted until recently as a practical oncological approach, including therapy. Accordingly, going to bibliography reports on the exploration of the immune status of human patients within the context of mammary neoplasms reveals - beyond the discrepancies or even the contradictions possibly associated to the diverse technologies used or to the target cases particularities - the constant present of immune elements in primary lesions and in many metastatic disseminations. Therefore, the opportunity of a detailed description of the immune status, with all the difficulties associated to the structural complexity of this approach, has never been truly separated from diagnostic intentions. The inclination to computer-aided solution is exploited by several research groups, and the benefits seem promising as regards the definition of subtypes with distinct immunology, and possibly with different immunotherapy indications.

Within this context, the opportunity of addressing a technical solution (computer-aided immunohistochemical analysis) and revealing the possible impact in the evolutionary determination of mammary cancer for inflammatory infiltration always seemingly common in the diagnostic balance is the center of the scientific goals of this research.

Several technical solutions are already commercially available for the automated analysis of histological imagery. On the molecular medicine interdisciplinary platform, we had access to one of these solutions, namely the TissueFAX system, which’s flexibility in the configuration of the image analysis parameters seemed compatible with the diversity of cell phenotype forms present in human mammary lesions; thus we assayed the necessary elements for the formulation of an image analysis algorithm for the inflammatory reaction associated to neoplasia. Using digital solutions for the analysis of infiltrations is a direction already promoted in recent studies, more or less similar with our work, and retrospectively we were able to identify at others our own work practices and findings.
The HistoFAXS system used for our research is semiautomatic; the quantitative analysis requires the operator to set several parameters resulting in a correct cell delimitation; the system then automatically generates statistical data based on the cell masks set up in accordance with the established parameters.

During the first stage of our research, we followed the traditional approach to the learning of new techniques, going through a validation analysis of the results of computer-aided technique by a morphopathologist experienced in this area, working on a wide range of microscopic fields and immunohistochemical markings. We mention that in both cases, human versus automated analysis, the various histological objects were grouped in categories (of arbitrary diversity, such as small nuclei cells versus large nuclei cells, clear cytoplasm cells vs chromogen-stained cytoplasm); the benefits of automation consist in the possibility of indefinitely extending the number of analyzed cells beyond the usual 30/60/100 or 200 elements of classic histological scoring and in the accuracy of quantifications and the elimination of subjective factors from the definition of optical object categories. Specifying numerical values for the various set of parameters we exploited was the premise of the reproducibility of analyses and of the possibility of comparing images with different lesion sources and markings.

Primary diagnostic parts in our cases were standard-processed paraffin-included blocks. We have assayed the optimal conditions for immune marking (concentration, time, sequence, types of kits, antibody species, viewing conditions) that could help convenient and automated viewing, and also visual examination (Zeiss Z1 microscope, 20X lens) of histopathological specimens. Overall, the quality of our immunohistopathology proceedings was in accordance with specialty guides.

15.2 Considerations on the myelolymphoid infiltration and the immune scores

The set of markers used in our study describes the major lineages of leukocytes possibly involved in human mammary tumor lesions. Since our main goal was to develop the methodology sustaining consistent (and possibly wide) use of inflammatory infiltration, on a larger scale than reflected in typical diagnostics bulletins for clinical use, we structured our reagents kit and the size of the studied cases lot accordingly, obviously also taking into account the available resources, reasoning that the fine demonstrations of evolution
The impact of inflammatory subcomponents could be approached later based on this study.

Prognostics in mammary cancer is influenced by classical factors concerning the tumor and the host, such as the patient’s age, the histological type and grade, the size of the tumor, the ganglion status, the estrogen and progesterone receptors, and the human epidermal growth factor receptor 2 (HER-2) (Lal et al., 2005). There is now convincing evidence that cancer development and progression depend on the complex interaction between the tumor and the host’s inflammatory response (Vakkila, Lotze, 2004; DeNardo, Coussens, 2007; Gottfried et al, 2008; Colotta et al, 2009; Hanahan, Weinberg, 2011). The role of the tumor inflammatory cell infiltration in the prognosis of survival in patients with operable mammary cancer was the subject of the review of specialized publications. (Mohammed et al, 2012). Such works concluded that despite the many researches and considerable efforts over a period of more than 90 years, the relation between the various features of the tumor inflammatory cell infiltration and the survival of patients with mammary cancer after primary operations remained unclear. Studies analyzing the relation between the inflammatory infiltration at the invasive edge and survival produced contradictory data, namely: out of a total of 24 studies, 13 reported an association between the inflammatory infiltrate and the improved survival, 4 reported no associations, and 7 reported associations between the inflammatory infiltration and decreased survival. This was explained by the absence of methodological validation, by the small size of samples, by the heterogeneity of the cancer subtype, and by insufficient follow-up (Mohammed et al, 2012).

The phenotyping resolution level (in terms of number of markers for viewing the cell species and functions) could allow illustrating a wide range of phenotypes and imagery presentations (appearance) of inflammatory cell species, and refining the individual contributions of functionally distinct immune cell species.

We have started from a basic list of markers including reagents (monoclonal antibodies) targeting the classic major markers for B and T lymphocytes and myeloid cells (also accepted for diagnostics in cases such as lymphoma): CD3, CD8, CD20, CD68. To maintain the compatibility with the diagnostics routine, we only used single staining (and not simultaneous multiple marking - also a more difficult technique). We must mention this as the reactivity of our antibodies extends the marking to other cell species, besides the main ones. Indeed, CD3 is a single marker for T lymphocytes, both αβ and γδ lineages and both dominant αβ subsets, respectively: Th (typically CD4+) and Tc
(typically CD8+). CD4 is a marker found (although in smaller concentrations) in super macrophage monocytes, subsets of dendritic cells, besides Th. Few solutions are available for revealing CD4 in paraffin, but we were finally compelled to use one of them. Similarly, CD8 is a marker potentially present in subsets of dendritic cells. The immune marking on CD68 can also result in ambiguity, as it can be expressed in subsets of dendritic cells, not only in macrophage sets; CD20 is possibly a similar case. To all these considerations, we must add the possibility of marker caption by the already documented transfer of exosome vesicles between cells, or the random phenomenon of illegitimate marker expression.

All these considerations belong to the fine details of the marker definition and selection strategy, extremely important for creating so-called investigation panels; they reflect more than intrinsic methodological limits, i.e. mandatory nuances in the explanation of the histological, physiological and pathological vision for understanding the status diagnostics of neoplastic inflammatory infiltration. We maintain that the cell phenotype entities are clearly and conveniently characterized in flow cytometry and hematology; the description of their tissue variation could still produce new information and surprise.

Therefore, we considered that we should limit the list of additional markers to three: CD45RO (typical for memory lymphocytes), CD4 (to explore apparently contradictory spatial results in the relative representation of the Tc subset within the general T population) and CD44 (typical for memory cell varieties and possible for epithelial stem cells).

Overall, we found that based on these markers we could provide a reasonably complete explanation of the structure of the inflammatory infiltration of different lesion spots; we also highlighted differences in the representations of major subsets apparently structured according to their relative position from the tumor, and possibly according to their molecular histological subtype. Indeed, we noted within the invasive fronts a systematically oversized CD3+ CD8- T cell population - most probably due to the Th subset (based on the analysis of the representation of the CD8, CD3, CD4 immune marking), in accordance with other reports.

Although analytical manipulation in combinations and associations of factors did not directly lead to identifying immune scores (like those defined in larger studies, like Galon et al.), our research still allowed the statistical detection of the unfavorable impact of macrophage super infiltration in lesions (CD69+) on the evolution of global variables such as the DFS and the survival - as did many published reports.
Among the particular elements of our research, we mention the image suggested by the comparison between all the immune markings we used is dominated by the tendency of infiltrations to develop into a gradient-type format, i.e. from the invasive front towards the intratumoral compartment (unlike the reporting method preferred in the published works of authors practically excluding the intratumoral area from their analyses); also, the apparent tendency of positive correlation between the proportions of the T and B sub-populations (both as percentages and densities) and the score reflecting the tumor grading (found in relatively few similar published reports).

The comparative analysis of the differences created in the structure and magnitude of inflammatory infiltrations in different areas and in different histological and molecular subtypes (such as RE- vs RE+, amplified vs. not amplified Her2) reveals the kind of reverse correlations also reported in published works, at least as trends (given the number of cases in our research).

A retrospective look to the reflection of the basic ideas of our research in the published works, in the context of our final results suggest that the topic of the exact definition of the structure of the local inflammatory cell array in the primary mammary tumor is seen as a promising source of bio-markers, still incompletely exploited, and not necessarily easy to investigate using classic methodology.

**Conclusions:**

- The numerical analysis of immunohistochemical imagery is an exploration technique equally feasible, extensive/multidimensional (in terms of markers) and opportune for the diagnostics evaluation for clinical use and for translational purposes;
- The infrastructure compatible with such techniques is nearly mature, and the learning of the appropriate analytical approach is easy for the various categories of medical staff;
- Knowing the types of errors and the limits of the employed techniques for the quantitative evaluation of the myelolymphoid infiltration in mammary cancer results in increased objectivity of the results;
- The quantitative analysis of the myelolymphoid infiltration is influenced by the intensity of tissue section staining; the optimal processing of imagery using the HistoQuest technique is obtained with intense staining for the markers used;
- The automatic use of cut off is preferable, but with a parallel qualitative evaluation of the analyzed images;
The use of the HistoQuest software is objective and reproducible, and useful in immunohistochemical evaluations for scientific and diagnostic documentation;
Implementing analyses based on the computer-aided imagery eliminates the subjectivity of the interobserver and intraobserver variability;
This technique is fit for the study of the tumor microenvironment, with its myelolymphoid infiltration component;
The numerical analysis of immune markings reveals a massive involvement of the inflammatory infiltration in the mammary neoplastic lesion, which disagrees with the scarce mentions in the anatomic pathology bulletins; Either as a passive actor or a partner in oncogenesis, the inflammatory infiltration is systematically ignored in clinics as a predictive and prognostic parameter;
The inflammatory infiltration is markedly heterogeneous in the diagnostics lesion both structurally (major cell lineages) and territorially (histoarchitecture areas); This modulation of the distribution could be documented exhaustively through the automatic imagery analysis, and it could be considered an interesting bio-marker itself;
The myelolymphoid infiltration mostly accumulates at the invasive front, followed by the peritumoral and intratumoral areas, with variable representation in the normal area, depending on the immunohistochemical marking used;
The spatial gradient of the distribution of the myelolymphoid infiltration supports the idea of chemoattraction at tumor level;
The accumulation of T lymphocytes (as overall population) and of cytotoxic T lymphocytes follows the gradient in the four interest areas, i.e. the decrease from the invasive front towards the peritumoral, intratumoral and normal areas.
The maximum difference between the CD3+ common T lymphocyte and the CD8+ cytotoxic T lymphocyte populations (interpreted as the population of CD4+ T helper cells) was found in the invasive front, which suggests its important role in the tumor development;
The CD68+ phenotype had a negative impact on the survival of Her2neu negative patients, and a positive impact for the patients with positive estrogen receptors, which suggests that the immune system could play an individualized prognostic role based on the tumor subtype;
Fine details of the functions of various inflammatory cells in various places - currently available owing to the conventional immunohistochemical markers, \textit{in situ} hybridization, or non conventional topographic proteomics techniques - are the very logical extension of our results, and the first step towards the possible specification of an exploration markers kit (diagnostics panel) for the exploitation of knowledge about the reactive infiltration in individualized anti-neoplastic therapy.

**Selective bibliography:**


**The list of publications**


