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The Value of Tumor Infiltrating Lymphocytes (TIL) for Prediction of Response to Neoadjuvant Chemotherapy (NAC) in Breast Cancer according to the Molecular Subtype

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Abstract: *Introduction:* Antitumor host immune response is an important factor, but its role is not fully established. The role of tumor infiltrating lymphocytes (TIL) in breast cancer as an immunological biomarker has been significantly explored over the past years. The number of patients treated with neoadjuvant chemotherapy (NAC) increased and identification of a biomarker to predict the probability of pCR (pathological complete response) is a high priority. *Materials and methods:* We evaluated 334 cases of BC treated with NAC followed by surgical resection from 2020-2022 in Ist Clinic of Oncological Surgery, Oncological Institute "Prof Dr I Chiricuta" Cluj Napoca. Of the above, 122 cases were available for histological evaluation both in pre-NAC biopsy and post-NAC resection tissue. Evaluation of biopsy fragments and resection parts using hematoxylin eosin (H&E). The TIL evaluation took place according to the recommendations of the International TIL Working Group (ITILWG). *Results:* There was a strong association between elevated levels of pre-NAC TIL. At the same time, there is a statistically significant correlation between stromal TIL and tumor grade, the number of lymph node metastases, the molecular subtype, and the number of mitoses ($p < 0.005$). Intratumoral TIL showed a significant correlation with tumor size, distant metastasis, molecular subtype, number of mitosis, stage and lymph node metastasis ($p<0.005$). We also demonstrated that high pre-NAC TILs represent a strong predictive marker for pCR. *Conclusion:* This study reveals the role of TIL as a predictive biomarker in breast cancer not only for the well-established TNBC (triple negative breast cancer) and HER2+ (Her2 overexpressed) subtypes but also in Luminal A and B molecular subtypes. In this scenario, the evaluation of TILs as novel predictive and therapy-predicting factors should become a routinely performed analysis that could guide clinicians into the choice of the most appropriate therapy.

Keywords: breast cancer; TIL; modified radical mastectomy; breast conservative surgery; predictive biomarker

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1. Introduction

Breast cancer represents the 2nd cause of mortality among women with a major psycho-social impact worldwide [1–4]. Referring to the role of the immune system as a modulatory system within these tumor types, there are numerous controversies in the literature with the addition of numerous research studies [5,6]. The body's antitumor response is based on the lymphocyte population (TIL-tumor infiltrating lymphocytes), an aspect intensively researched in the last decade [7]. Since an increasing number of breast cancer patients benefit from neoadjuvant therapy (NAC), it is crucial to standardize a biomarker for predicting the tumor response rate [8–14]. The response rate to neoadjuvant therapy is standardized in 4 types: complete response (pCR), partial response (PR), stable pathology (SD) respectively progressive pathology in evolution (PD) [15–18]. TIL represent mononuclear cells belonging to the immune system, which have the property of transition from the blood circulation level to the tumor level, where they initiate the immunomodulatory mechanism [19]. This cell group presents a particular heterogeneity, being made up of cytotoxic T cells, T helper cells, B cells, macrophages, natural killer cells and dendritic cells, all these cell subspecies are part of the tumor microenvironment [20–22].

2. Materials and Methods

We evaluated 334 cases of BC treated with NAC followed by surgical resection from 2020-2022 in Ist Clinic of Oncological Surgery, Oncological Institute "Prof Dr I Chiricuta" Cluj Napoca. Ethical clearance was obtained from the institutional ethics committee. Out of these, 122 cases were available for histological evaluation both in pre-NAC biopsy and post-NAC resection tissue. The surgical specimens were obtained by core needle biopsy and excisional biopsy of primary breast tumor. Four-micrometer-thick tissue sections from the surgical specimens fixed in 10% formalin and embedded in paraffin were reviewed, and representative tissue blocks were selected. There are two types of TIL: stromal and intratumoral. Stromal TIL are dispersed in the stroma and have no direct contact with carcinoma cells while intratumoral TIL are defined as lymphocytes in direct contact with tumor cells. Evaluation of biopsy fragments and resection parts using hematoxylin eosin (H&E). The TIL evaluation took place according to the recommendations of the International TIL Working Group (ITILWG).

3. Immunohistochemical analysis

The evaluation of the TILs was performed according to the guidelines of the "International Working Group for TILs in Breast Cancer—2014". In detail, a section of 4–5 μm at a magnification of 200–400x was evaluated for each patient. The evaluation of the TILs was performed by a percentage count of the stromal areas occupied by the lymphocyte and plasma cellular infiltrate, instead excluding the areas occupied by tumor cells. This evaluation considered only the mononuclear infiltrate within the borders of the invasive tumors. Large areas of central necrosis or fibrosis are not included in the evaluation.The stratification of the general study group by groups in relation to the sTIL value is distributed as follows (Tab. 6):

Group A – includes 17 patients with a value of TILs between 0-10%
Group B – includes 41 patients with a value of TILs between 10-40%
Group C – includes 62 patients with a value of TILs between 40-90%
Group D – includes 2 patients with a value of TILs over 90%

4. Statistical considerations

Statistical analysis was performed using SPSS 23.0 for windows (SPSS, Inc., Chicago, IL, USA) was used for data analysis. The associations between TILs, ITIL, and clinicopathological variables were examined using $\chi 2$ tests. Multivariable analysis of pCR was carried out using a binary logistic regression model. Normally distributed continuous data were expressed as means (SD) and were assessed using the analysis of variance (ANOVA), independent-sample t-test or paired t-test.

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Nonparametric data were analyzed using the Mann-Whitney and Wilcoxon tests. Two-sided tests were performed to declare statistical significance at $p < 0.05$.

5. Ethical consent

All processes approached during the study with the inclusion of human subjects benefited from the approval of the ethics commission according to national and international standards in direct relation to the Helsinki declaration of 1964. This article does not include studies on laboratory animals. The consent mentioned above was received from and approved by each participant in the study (The Ethics Commission for Research and Development Activities and for Quality Assurance of Clinical Trials of the "Prof. Dr. Ion Chiricuță" Oncological Institute in Cluj Napoca, appointed by decision of the manager (IOCN no. 189 -03.06.2021- Application no. 10442).

6. Evaluation of TIL

We evaluated 334 cases of BC treated with NAC (the majority of NAC regimens contained anthracycline and taxane. trastuzumab or lapatinib were typically used in HER2 positive patients) then followed by surgical resection from 2020-2022 in Ist Clinic of Oncological Surgery, Oncological Institute " Prof Dr I Chiricuta" Cluj Napoca. Ethical clearance was obtained from the institutional ethics committee. Of the above, 122 cases were available for histological evaluation both in pre-NAC biopsy and post-NAC resection tissue. There are two types of TIL: stromal and intratumoral. Stromal TIL are dispersed in the stroma and have no direct contact with carcinoma cells while intratumoral TIL are defined as lymphocytes in direct contact with tumor cells. Evaluation of biopsy fragments and resection parts using hematoxylin eosin (H&E). The percentage of stromal as well as intratumoral TILs (iTILs) was evaluated separately. iTILs was defined as the percentage of mononuclear cells within the epithelium of the invasive tumor cell nests. Stromal TILs (sTILs) was defined as the percentage of tumor stroma area that contains a lymphocytic infiltrate without direct contact to tumor cells. TIL-assessment in the residual disease setting should be done within the borders of the residual tumor bed, as defined by the presence of the residual tumor cells, in analogy with the definition of the residual tumor bed of the Residual Cancer Burden (RCB)-index [21]. The entire largest cross-sectional area of the residual tumor bed should be used for histologic TIL-assessment. One section (4–5 μm) per patient can be considered to be sufficient for practical purposes. However, if the residual tumor bed is large than 2 cm more slides need to be assessed, with one slide for each cm of tumor bed as a minimum. For example, if the largest diameter is>5 cm, then at least 5 representative slides from the largest cross-sectional area should be considered. If the residual tumor bed is thus only 2 cm one slide is considered enough. Assessing numerous slides for each case should thus be possible mentioning the number of assessed slides specifically in the study protocol [23].

3. Results

The stratification of the study group was done according to the molecular subtype based on the St Gallen classification. In accordance with this, a percentage of 37.7% (n=46 cases) were included in Luminal A, followed by TNBC (triple negative breast cancer) cases with 25.9% (n=31) respectively Her2 overexpressed with 20.49% (n=25). A more limited number of cases were included in the Luminal B group (Her2- approximately 9.01% respectively Her2+ 7.37%). (Table 1)

Table 1. Stratification of the study lot according to the St Gallen classification.

Parameters	N	%
Luminal A	46	37.7
Luminal B Her2 -	11	9.01
Luminal B Her2 +	9	7.37

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Her2+	25	20.49
TNBC	31	25.9

Referring to the types of interventions performed within the study group, it can be observed that an important percentage 48.36% (59 cases) benefited from breast conservative surgery (BCS) and 22.95% (28 cases) from oncoplastic surgery (OBCS) a fact that reinforces the idea of modulating the surgical therapeutic strategy depending on the tumor molecular subtype and the response rate to neoadjuvant therapy. A relatively small percentage of approximately 28.66% (35 cases) benefited from Madden-Auchincloss MRM (Modified Radical Mastectomy). Current modern trends regarding oncological surgery of the mammary gland are related to the adoption of oncoplastic procedures, conservative procedures of the mammary gland without compromising oncological principles. (Table 2)

Table 2. Distribution of the batch according to tumor location and type of surgical intervention.

Parameters	**n**	**%**
Unifocal	81	66.39
Multifocal	26	21.31
Multicentre	15	12.29
The type of intervention		
MRM	35	28.66
OBCS	28	22.95
BCS	59	48.36

The modulation of the surgical therapeutic strategy according to the molecular parameters and the response rate to the neoadjuvant therapy is a desideratum that should not be missing from the logistics of the medical-surgical team. The evaluation of some molecular, cellular and general parameters that can represent primary prognostic factors regarding the rate of therapeutic response and at the same time the establishment of surgical management is a leading topic worldwide.

Table 3. General clinical-paraclinical characteristics.

Parameters	**n**	**%**
Location		
External superior quadrant (ESQ)	76	62.29
Internal superior quadrant (ISQ)	12	9.83
External lower quadrant (ELQ)	21	17.21
Internal lower quadrant (ILQ)	7	5.73
Central quadrant (CQ)	6	4.91

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Post-NAC tumor bed cellularity (RTC)		
below 30%	26	21.31
30-60%	77	63.11
over 60%	19	15.57
Breast		
Right	48	39.35
Left	74	60.65

From the point of view of the tumor topography, the majority of tumors (62.29%) were located at the ESQ level, respectively Spence's axillary extension, followed by the ELQ (17.21%). A relatively small number of cases presented tumors located at the ISQ, ILQ, and CQ level (approximately 20%). The impact of tumor topography on surgical procedures is a major one, favorable aesthetic results are defined by tumor location. 60.65% of the diagnosed tumors were located in the left breast, respectively 39.35% in the right breast (Tab. 3)

The microscopic analysis of the resection pieces shows the quantity and quality of cells at the level of the tumor bed, thus a percentage of 63.11% presented a moderate threshold of cellularity at the level of the tumor bed (between 30-60%), respectively 15.57% presented a high degree of cellularity at the level of the tumor bed (over 60%). A percentage of 21.31% of all cases presented a low degree of cellularity at the level of the tumor bed.Rajan R. et al published a study in 2004 that aims to analyze the cellularity in the tumor bed after NAC. It highlights a significant decrease in cellularity in the context of neoadjuvant chemotherapy. Damiano Gentile et al. publishes in 2023 a study that includes 495 patients which analyzes the tumor response after NAC and the cellularity in the tumor bed as a prognostic factor in the case of breast cancer patients. This study concretizes the fact that an RTC value below 40% is associated with a longer disease-free interval (DFS) respectively with an improvement in long-term survival. Another study published by Ahn S. et al concludes that there is no statistically significant correlation between cellularity in the post-NAC tumor bed (RTC) and long-term survival.

Referring to the characteristics of the study group, we can see that 58.19% (71 cases) of the cases are younger than 50 years old, respectively 41.8% (51 cases) are older than 50. Regarding the hormonal status, 59.01% (72 cases) are represented by fertile or reproductive age patients, respectively premenopausal, and approximately 40.99% (50 cases) are represented by women with a postmenopausal status.

Microscopic analysis of tumor resection pieces highlights a preponderance of tumors with a G2 grading (mBloom-Richardson classification) in 44.26% of cases, respectively a G1 and G3 in approximately 27.04% and 28.68% respectively. It was analyzed at the level of the study group and the rate of mitotic proliferation at the level of the tumor bed, resulting in a percentage of 50% of cases with mitotic proliferation over 11 at the level of the tumor bed, a fact that can highlight an aggressive tumor profile. From the point of view of the dimensions, tumors 59.83% (73 cases) have tumor sizes between 2-5 cm, 26.22% (32 cases) with tumor sizes over 5 cm and a percentage of 13.93% (17 cases) with tumor sizes below 2 cm.

Analyzing tumoral lymphovascular invasion shows that 66.39% (81 cases) do not present lymphovascular invasion, respectively 33.66% (41 cases) with present lymphovascular invasion. Regarding the presence of distant metastases in 78.68% (96 cases) they were absent, respectively in 21.31% (26 cases) they were present. (Table 4)

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Table 4. Clinical-paraclinical characteristics of the study group.

Parameters	**n**	**%**
Age		
<50	71	58.19
≥50	51	41.8
Menopausal status		
Premenopausal	72	59.01
Postmenopausal	50	40.99
Grading (mBloom Richardson)		
1	33	27.04
2	54	44.26
3	35	28.68
Mitotic Count		
0-5	25	20.49
6-10	36	29.50
≥11	61	50
Tumor size		
<2	17	13.93
2-5	73	59.83
>5	32	26.22
Lymphovascular invasion		
Present	41	33.66
Absent	81	66.39
Distant metastases		
Present	26	21.31
Absent	96	78.68
TNM		
I	17	13.93
II	73	59.83
III	24	19.67
IV	8	6.55

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Table 5. Anatopathological characteristics of the batch.

Parameters	**n**	**%**
NAC response		
pCR[i]	33	27.04
PR.[ii]*(over 30%)*	42	34.42
SD[iii]	29	23.77
PD[iv]*(over 20%)*	18	14.75
Histological type		
Infiltrative Ductal Carcinoma	89	72.95
Lobular	17	13.93
Mucinous	4	3.27
Medullary	9	7.37
Metaplastic	3	2.45

[i] pCR=complete response to NAC (neoadjuvant therapy – based on anthracyclines and taxanes +/- targeted therapy – Transtuzumab or Lapatinib); [ii] PR = partial response to NAC (dimensional reduction of the tumor over 30%); [iii] SD=no response to NAC, stable disease; [iv] PD=progressive disease below NAC with tumor size increase (over 20%).

The analysis of the histological types within the study group is presented in the above table. A predominance of infiltrative ductal carcinoma can be observed with a percentage of 72.95% (89 cases), followed by lobular carcinoma in a proportion of 13.93% (17 cases). Histological types such as mucinous, medullary or metaplastic carcinoma were identified in a significantly lower number (Mucinous 3.27% vs Medullary 7.37% respectively Metaplastic 2.45%). An important analysis within the study group was represented by the evaluation of tumor response to neoadjuvant therapy. The evaluation of tumor response to neoadjuvant therapy can be quantified considering several classifications (Therasse, Miller-Payne, Chevallier, Sataloff classification). In our group, we adopted the Therasse classification, that is Miller-Payne, in order to assess the degree of tumor response to neoadjuvant therapy. Thus, 27.04% (33 cases) of the cases presented a complete pathological response to NAC therapy (pCR), respectively 34.42% (42 cases) presented a partial response. 23.77% (29 cases) show no response to NAC therapy (SD) without the dimensional change of tumor formations, respectively 14.75% (18 cases) show dimensional changes of post-NAC formations, with an increase of over 20% compared to the initial size (Tab. 5). The stratification of the general study group by groups in relation to the sTIL value is distributed as follows (Tab. 6): Group A – includes 17 patients with a TILs value between 0-10%; Group B – includes 41 patients with a value of TILs between 10-40%; Group C – includes 62 patients with a value of TILs between 40-90%; Group D – includes 2 patients with a value of TILs over 90%.

Table 6. Stratification of the batch into groups according to the value of TILs.

GROUP	**No. of cases**	**%**
A	17	13.93
B	41	33.6
C	62	50.81
D	2	1.63

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Analyzing the response rate after NAC, we highlighted the fact that approximately 61.46% of patients presented a favorable response rate after NAC, whether we are talking about pCR or PR. In order to highlight the predictive value of TILs through statistical analysis, we established a cut-off value regarding the potential response to NAC superimposed on each molecular subtype. Thus, for patients included in the molecular framework of Luminal A, a cut-off value of TILs above 20% has a predictive potential regarding the favorable response rate to NAC (p=0.05). Regarding the patients included in Luminal B, a cut-off value of TILs of over 20% shows a predictive potential in terms of the response rate, whether we are talking about pCR or PR (>30%) (p=0.093).; 95% CI, 0.89–0.92).

Table 7. Values of sTIL and iTIL in the context of various clinicopathological parameters.

PARAMETERS	N	STIL (MEAN ± SD)	*P VALUE*	ITIL	*P VALUE*
T GRADE					
1	33	7.01±3.9		21.71±11.03	
2	54	39.4±19.06	0.001	73.01±39.03	0.001
3	35	68.63±20.13		171.31±41.32	
TUMOR SIZE					
2	17	41.23±22.13		83.41±44.24	
2-5	73	57.28±29.17	0.002	101.38±33.17	0.002
≥5	32	69.11±20.19		153.31±42.19	
MOLECULAR SUBTYPE					
LUMINAL A	46	21.29±17.55		43.12±29.12	
LUMINAL B	20	33.19±19.23	0.001	68.47±38.12	0.002
HER2+	25	44.21±28.24		98.42±56.44	
TNBC	31	69.11±27.31		128.12±54.12	
DISTANT METASTASIS					
PRESENT	26	71.23±23.97	0.1931	162.23±46.32	0.005
ABSENT	96	44.23±27.28		95.21±61.12	
LYMPHOVASCULAR INVASION (LVI)					
PRESENT	41	68.12±28.67	0.001	152.21±50.12	0.05
ABSENT	81	39.19±27.12		79.12±54.18	
TNM					
I	17	31.23±21.83	0.068	62.46±54.12	0.01

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II	73	48.23±28.13	91.46±51.12	
III	24	68.23±27.13	138.18±46.23	
IV	8	61.17±23.97	141.12±44.23	

We obtained statistically significant correlations between TILs and tumor grade (0.152 [0.091,0.262]; z = 6.80; p < 0.05), tumor size (0.154 [0.085,0.198]; z = 5.72; p < 0.05), and molecular subtype (0.134 [0.090,0.264]; z = 4.80; p < 0.05). However, we did not find a statistically significant correlation between TILs and distant metastases (0.64 [0.78,0.122]; z = 5.44; p = 0.1931), nor between TILs and TNM stage (0.111 [0.76,0.101]; z = 2.80; p = 0.068). Statistically significant correlations were obtained between ITIL and tumor grade, tumor size, distant metastases, TNM and molecular subtype (0.167 [0.091,0.262]; z = 5.90; p < 0.05).

As can be seen (Tab. 7), we obtained different mean values depending on the molecular subtype, thus patients with tumors belonging to the non-luminal molecular subtype (HER2+ and TNBC) having higher values of TILs and ITIL compared to patients belonging to the molecular subtype luminal (Luminal A and B).

Regarding the two TIL subtypes (intratumoral and stromal), it can be observed that iTIL is in direct correlation with the tumor stage according to the TNM classification (p = 0.01), while in the case of sTIL we did not obtain a statistically significant coefficient. Comparing the values obtained, it can be stated that iTIL has a higher specificity in relation to the tumor stage compared to sTIL, which does not sublimate the predictive potential of sTIL.

7. Discussions

In recent years, immunogenic studies and targeted therapies have been found more and more frequently in the oncological therapeutic arsenal. Breast cancer is the best-studied neoplastic subtype from a histopathological and immunohistochemical point of view, in therapeutic dynamics there are many possibilities for therapeutic titration based on major predictive factors [24–29].

There have been numerous studies on the potentially predictive histopathological and immunohistochemical parameters regarding the response rate to neoadjuvant therapy in breast cancer [30,31]. The presence of TILs represents the expression of the antitumor immune response, and this marker could represent a major predictive factor regarding the therapeutic response after NAC [32–37]. Analyzing the specialized literature we note that there are two types of lymphocytic tumor infiltrates (TIL), one with stromal localization and one with intra-tumoral localization [38]. Referring to the predictive value of the 2 types of infiltrates regarding the therapeutic response to neoadjuvant therapy in the case of breast cancer, there are numerous controversies in the medical literature [39–41]. Most of the studies with an impact from a qualitative and quantitative point of view show favorable results in favor of TILs as a predictive marker in tumor response to neoadjuvant therapy compared to TILi [42–45]. In relation to the molecular subtype of the tumor, most studies of TIL as a predictive factor of response to NAC give us favorable results in the case of HER2+ and TNBC patients, and only a few gives us proactive results in the case of patients classified as Luminal A and B subtypes [46].

The West trial demonstrates the fact that high values of TILs increase the tumor response rate to neoadjuvant therapy and are associated with better long-term survival in the case of HER2 overexpression and TNBC patients [47–50]. The results from the literature show a particular heterogeneity regarding the predictive value of TIL in relation to the molecular tumor subtype [51,52]. The results obtained by us are in accordance with a study published by Denkert et al. which demonstrates the predictive value of TIL in relation to pCR in the case of all molecular subtypes of breast cancer [53]. We have obtained a statistically significant correlation between stromal TIL and tumor grade, tumor size, the number of distant metastases and molecular subtype (p <0.05). Intratumoral TIL showed a significant correlation with tumor grade, tumor size, distant metastasis, molecular subtype, stage and lymph node metastasis (p<0.05). We also demonstrated that high pre-NAC TILs represent a strong predictive marker for pCR. Shiqi Li et al. publishes in 2022 a systematic

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review that includes 29 publications demonstrating that high levels of TILs can predict the response rate to NAC in breast cancer patients with a HER2+ molecular profile (OR = 2.54 95% CI, 1.50–4.29) respectively in the case of patients with TNBC molecular profile (OR = 3.67, 95% CI, 1.93–6.97). Mukta Pujani et al. demonstrates the existence of a signification correlation between stromal TIL and tumor grade, lymph node metastasis, molecular subtype and mitosis. Intratumoral TIL showed a significant correlation with tumor size, mitosis, tumor grade, distant metastasis, stage and lymph node metastasis. [54]

Angelico, G. et al. shows in a prospective analysis the fact that there is a heterogeneity regarding the value of iTIL and sTIL in relation to the tumor stage. They demonstrate a statistically significant correlation between iTIL and sTIL values respectively stage I, II according to the TNM classification. [55]

In the case of patients with a positive hormonal profile, this systematic review does not obtain statistically significant results (OR = 1.68, 95 %CI, 0.67–4.25). In this systematic review, in the case of HER2+ and TNBC patients, a cut-off value of TILs was 20%, a value from which significant results were obtained regarding the tumor response rate to NAC therapy. Another meta-analysis published by Zhao-hua Gao et al. 2020 which included 33 profile studies (18170 patients) conclusion of which underlines the predictive value of TILs regarding the tumor response rate to NAC in the case of patients with TNBC and HER2+ molecular profile [56]. High values of the TILs are associated with a degree of favorable response to the NAC and with a longer-term survival (OS). Lin He et al. publishes in 2020 a meta-analysis that includes 22 clinical trials (15,676 patients) demonstrating that each 10% increase in TILs improves the long-term survival (OS) of patients with a HER2+ molecular profile (pooled Hazard ratio (HR), 0.92; 95% CI, 0.89–0.95) and TNBC (pooled HR, 0.90; 95% CI, 0.89–0.92). At the same time, this meta-analysis demonstrates that high levels of TILs are associated with a favorable response rate to NAC therapy (pCR) regardless of the molecular subtype of breast cancer [57,58]. An important remark of the meta-analysis, the neoplasias included in the HER2+ and TNBC molecular subtypes showed significantly higher values of TILs compared to the Luminal A and Luminal B subtypes (pooled HR, 1.06; 95% CI, 0.99–1.13)

8. Conclusions

Following our study, we can conclude that the TIL (iTIL and sTIL) value has a predictive potential regarding the response rate of breast tumors to neoadjuvant therapy (NAC) regardless of the molecular subtype. Different tumor response cut-off values were highlighted depending on the molecular subtype, highlighting the fact that non-luminal tumors (TNBC and HER2+) present higher average values compared to luminal tumors (Luminal A and Luminal B). We consider that the TIL (stromal and intratumoral) value represents a reliable biomarker for predicting the tumor response to NAC and requires routine investigation in the case of all patients with breast neoplasm and at the same time the establishment of cut-off values at the global level through the implementation of large-scale population studies.

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