Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências da Saúde: Ginecologia e Obstetrícia

# CÂNCER DE MAMA TRIPLO NEGATIVO E SUA RELAÇÃO COM O SISTEMA IMUNE

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Carcinoma

Carcinoma Ductal

Carga Tumoral

Citocinas

Células Dendríticas

Expressão Gênica

Fatores Imunológicos

Genes

Imunidade

Imuno-Histoquímica

Imunossupressão

Linfócitos

Medicina de Precisão

Metástase Neoplásica

Microambiente Tumoral

Moduladores Seletivos de Receptor Estrogênico

Neoplasias da Mama

Neoplasias de Mama Triplo Negativas

Progressão da Doença

Recidiva

Sistema Imunitário

Taxa de Sobrevida

Terapia Neoadjuvante

Trastuzumab

United States Food and Drug Administration

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**SUMÁRIO**

**LISTA DE ABREVEATURAS**

TIL - Infiltrado linfocítico tumoral

TNBC - Tumor de mama triplo negativo

HCPA - Hospital de clínicas de porto alegre

TCLE - Termo de Consentimento Livre e Esclarecido

CDI - carcinoma ductal invasor

P - Índice de Significância Estatística

OR - Odds Ratio (razão de probabilidades)

pCR - resposta patológica completa

FDA - Food and Drug Administration

SERM - Selective estrogen receptor modulators

BL1 - basal-like 1

BL2 - basal-like 2

M - Mesenquimal

LAR - receptor androgênico luminal

HER 2 - Human Epidermal growth factor Receptor-type 2

TDM1 - Trastuzumab emtansine

IL2 - interleucina 2

PD-L1 - Programmed death-ligand 1

QN – Quimioterapia neoadjuvante

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**RESUMO**

INTRODUÇÃO

O câncer de mama triplo negativo consiste em uma doença agressiva e heterogênea com prognóstico reservado e sem terapia alvo estabelecida. Evidências mostram que o microambiente tumoral exerce papel fundamental no desenvolvimento e progressão da doença e que o infiltrado linfocítico tumoral (TILs) seria capaz de predizer resposta à quimioterapia neoadjuvante. Através deste estudo, buscamos validar esta relação e compreender melhor o comportamento deste tumor.

MATERIAL E MÉTODOS

Foram avaliadas as pacientes com diagnóstico histológico de carcinoma mamário invasor (CMI) submetidas à quimioterapia neoadjuvante (QN) no Hospital de Clínicas de Porto Alegre no período de 2007 a 2019. Foram selecionadas aquelas classificadas por imuno-histoquímica como triplo-negativo. A análise dos TILS foi realizada através da leitura de lâminas em hematoxilina-eosina (HE) de core biópsia prévia ao tratamento, por dois patologistas independentes. Os TILs foram classificados entre baixo (0-10%), intermediário (10,1-40%) e alto (>40%) através das diretrizes do International Working Group. O índice de TILS foi correlacionado com as características clínico-patológicas e com a resposta tumoral à QN.

RESULTADOS

Foram avaliadas 406 pacientes submetidas a quimioterapia neoadjuvante, onde 79 eram triplo-negativos. Após revisão de prontuário, 50 pacientes foram selecionadas para leitura de TILs. A média de idade no diagnóstico foi de 49.4 anos (±1.3), sendo a maior parte das pacientes em pré-menopausa. Mais da metade encontrava-se em estadiamento clínico avançado do tumor (EC III) e recebeu quimioterapia contendo antraciclina. Vinte e uma (26.6%) pacientes apresentaram resposta patológica completa (pCR). A pCR esteve associada a menor risco de óbito e recidiva ($p \leq 0.05$), sendo a

sobrevida global de 34 meses e a sobrevida livre de doença de 27 meses. O TILs era baixo em 21 (26.6%) e 31 (39.2%) das pacientes e alto em 4 (5.1%) e 9 (11,4%), havendo concordância entre as avaliações por dois patologistas independentes. Foi identificada correlação inversa entre o status menopausal e a quantidade de TILs, embora a relação entre TILs e pCR não tenha apresentado significância estatística

CONCLUSÃO

A busca por um tratamento específico para tumores triplo negativos permanece um desafio à ciência. Através desta coorte transversal, foi analisado o perfil clínico das pacientes e o follow-up por período de doze anos. Nossos resultados, demonstraram que a pCR está relacionada com melhores taxas de sobrevida global e livre de doença. Além disso, demonstramos que os TILs estão associados a pacientes mais jovens, em estadio pré-menopausal. Não foi encontrada relação significativa entre TILs e pCR o que pode se justificar pela amostragem reduzida, mas também pela hipótese de redução no número de TILs em estadiamento avançado da doença. Embora mais estudos sejam necessários para demonstrar o real impacto dos TILs na resposta aos tratamentos, eles constituem um exemplo da relação entre defesa natural e carcinogênese e representam um pequeno retrato do cenário tumoral, facilmente replicável no dia a dia.

*Palavras chave: câncer de mama triplo negativo, infiltrado linfocitico tumoral, resposta patológica completa, pré-menopausa, quimioterapia neoadjuvante*

**ABSTRACT**

Triple negative breast cancer (TNBC) is an heterogeneous disease with poor prognostic and lack of target therapy. Evidence showed that the immune system plays a fundamental role in the development and progression of the disease. As a response of the immune system, tumor infiltrating lymphocytes (TIL) would be able to predict neoadjuvant chemotherapy response. This leads to new treatment strategies with immune checkpoint inhibitors. In this cross-sectional study we aim to validate this relation. ***Methods*** We analyzed 406 patients submitted to neoadjuvant chemotherapy and selected 76 with triple negative breast cancer. TILs were performed in 50 pre therapeutic TNBC core biopsies between 2007 and 2019. TILs were classified in low (0-10%), intermediate (11-39%) and high (40-100%) according to the International TIL working group. TILs were analyzed as a continuous parameter. Univariable and multivariable statistical models were used to assess the association between TILs concentration and pathological complete response (pCR), disease free survival (DFS) and overall survival (OS). ***Results*** pCR were achieved in 21 patients (26.6%) and associated with better DFS and OS. TILs did not have direct significant association with pCR, nodal involvement or tumor size. ***Conclusion*** Tumor microenvironment is involved in prognosis and response to therapy but to define a biomarker remains a challenge in clinical practice. Further research is warranted to define the real role of TILS in response to neoadjuvant chemotherapy.

*Key words: Triple negative breast cancer, tumor infiltrating lymphocytes, neoadjuvant chemotherapy, pathological complete response*

## INTRODUÇÃO

### ENTENDER O PASSADO PARA MOLDAR O FUTURO

Descrito pela primeira vez no Egito antigo, o câncer de mama é uma patologia há longo tempo reconhecida e profundamente estudada [1]. À medida que desvendamos sua grande heterogeneidade, observamos a complexa relação que o tumor mantém com o indivíduo e o microambiente ao seu redor. Compreender porquê pacientes com fenótipos e tumores semelhantes apresentem comportamentos e desfechos distintos permanece um grande desafio, o qual impulsiona a busca por uma medicina de precisão e personalizada.

Nos últimos 50 anos houveram alguns marcos históricos para o tratamento do câncer de mama que mudaram a vida de muitas mulheres. Dentre estes, podemos citar a aprovação do Tamoxifeno pelo FDA em 1977 [2]. A relação entre estrogênio e câncer de mama era conhecida desde o século 19, quando Dr George Thomas Beatson (universidade de Edimburgo) observou que as mamas de coelhos deixavam de produzir leite quando seus ovários eram removidos. Paralelamente, submeteu pacientes com câncer de mama avançado à ooforectomia bilateral, constatando melhora clínica em parte delas[3]. Em 1960 então Jensen et colleagues detectaram receptores estrogênicos na superfície das células neoplásicas mamárias, explicando o princípio do tratamento com privação ou bloqueio hormonal. Desta forma, os moduladores seletivos de receptor de estrogênio (SERMs) foram o primeiro passo para terapia alvo. A próxima grande quebra de paradigma dar-se-ia em 1998 quando o FDA aprovou o tratamento anti-Her2 com Trastuzumabe. O gene Her-2 codifica um receptor de tirosina quinase que participa da sinalização de funções em células normais e malignas do tecido mamário. 20 a 25% dos tumores de mama apresentam o gene superexpresso, associados a fenótipos clínico agressivos com tumores de alto grau e alta taxa de proliferação. A introdução do tratamento com anticorpo monoclonal anti-Her2 associado a quimioterapia reduziu em

aproximadamente 50% a taxa de recorrência a distância e aumentou em 30% a taxa de sobrevida destas pacientes[4,5]. Antes considerada uma doença de prognóstico reservado, hoje com o desenvolvimento de novas drogas como pertuzumabe, TDM1, tucatinibe, deruxtecan, entre outras, as pacientes com doença Her2 positivo apresentam boa resposta a terapia alvo inclusive no cenário metastático.

O incremento nos tratamentos direcionados do câncer de mama só foi possível através do avanço nos estudos moleculares, o que permitiu a identificação e classificação da doença. A partir de técnicas de imuno-histoquímica e microarray Perou [6,7], em 2000, descreveu quatro subtipos de câncer de mama com diferentes padrões de expressão gênica: dois subtipos positivos para receptores hormonais (luminal A e B) e dois subtipos com baixa expressão de receptores hormonais, um deles HER2 enriquecido e outro "basal like". Nesta dissertação, daremos foco a este último subtipo, mais especificamente os tumores triplo negativos, uma vez que apresentam prognóstico desfavorável por ser uma doença de natureza agressiva e heterogênea e diferente dos demais, não possui terapia alvo definida.

## TUMORES DE MAMA TRIPLO NEGATIVO (TNBC)

O subtipo de câncer de mama basal like é caracterizado pela baixa expressão de HER2 e receptores hormonais, apresenta alta proliferação celular e alta expressão de um conjunto de genes exclusivos chamados "conjunto basal". Estudos mostram que o subtipo basal like difere molecularmente do subtipo luminal e é mais parecido com tumores oriundos da camada basal da epiderme como carcinoma escamoso de pulmão e carcinoma epitelial de ovário [8]. Prevalente em mulheres jovens e afro-americanas, ele está também associado a mutações do gene BRCA1, no qual as portadoras apresentam risco maior que 50% de desenvolver câncer de mama, sendo 80% do subtipo basal-like[9].

Tumores basal-like são muitas vezes chamados erroneamente de "triplo-negativos": 75% dos tumores triplo negativos são de fato do subtipo basal-like, porém outros 25%

expressam algum tipo de receptor hormonal. TNBC representam 15-20% dos tumores de mama e estão associados a alta taxa de recorrência e pior sobrevida: pacientes com doença residual após quimioterapia neoadjuvante apresentam 6 vezes mais risco de recorrência e 12 vezes mais risco de morte por doença metastática[10, 11].

Na busca por compreender esta que parece ser uma entidade completamente diferente do câncer de mama, Lehmann classificou o TNBC em sete subtipos através de expressão gênica de *microarray* [12]. Em 2016, refinou sua pesquisa e definiu 4 subtipos: basal-like 1, basal-like 2, mesenquimal e receptor androgênico luminal. Os subtipos moleculares diferem entre si em grau, risco de recidiva, padrão de metástase e são, principalmente, preditivos de resposta à quimioterapia neoadjuvante, sendo BL1 o que apresenta maior taxa de resposta patológica completa (52%). Além da classificação molecular, Lehmann avaliou o microambiente tumoral, identificando aproximadamente 20% dos TNBCs como imunomoduladores, ou seja, tumores enriquecidos em células e cheque-point imunes[13].

## O MICROAMBIENTE E O CÂNCER DE MAMA

O conceito de *immunosurveillance* [14] no qual o sistema imune frequentemente reprime carcinomas potenciais foi proposto pela primeira vez em 1957 por Macfarlane Burnet e Lewis Thomas, mas pela dificuldade de demonstrar a existência de sua hipótese ele ficou esquecido até a década de 90, quando foram descobertas as células natural killers (NK) e a produção endógena de interferon y (INF-y). A predição lógica do conceito de *immunosurveillance* é que o paciente imunossuprimido apresentaria maior incidência de câncer. Isto ficou claro para tumores de origem viral como linfoma não-Hodgkin, sarcoma de Kaposi e carcinomas de gênito-urinário e ano genital associados à infecção por Epstein Barr, herpes vírus e papiloma vírus. Porém, dados epidemiológicos de pacientes transplantados mostram também aumento na incidência de neoplasia de pulmão, cólon, pâncreas e melanoma[15]. E por que o câncer ocorreria em indivíduos imunocompetentes? De certa forma, o próprio sistema imune realiza seleção

darwiniana de variantes tumorais capazes de sobreviver em um ambiente imunogênico – *tumor sculpting.*

Assim nasceu o novo conceito de *Immunoediting*[16,17] tumoral descrito por Dunn et al em 2004, que consiste na hipótese de um duplo papel do sistema imune sobre o processo dinâmico de controle tumoral, promovendo tanto a destruição quanto a progressão do mesmo. Este processo é divido em três fases: eliminação, equilíbrio e escape. As células tumorais que conseguem sobreviver à resposta imune, entram em estado de dormência e, após, regulam seu caminho para sobrevivência e expressam moléculas que promovem imunossupressão e angiogênese[18].

Alvo de debate nas últimas décadas, o papel do sistema imune no câncer de mama tem se revelado de extrema importância, principalmente em cenário de doença agressiva. Embora as condutas hoje sejam baseadas na avaliação das células tumorais e suas expressões gênicas, os componentes do estroma tumoral, tipicamente células imunes inatas e adaptativas, parecem impactar no prognóstico e prever resposta a terapias específicas[19]. O infiltrado linfocítico tumoral (TILs) observado em maior quantidade no câncer de mama quando comparado ao tecido normal atingiu recentemente nível de evidência I como fator prognóstico em tumores triplo negativos e Her2 positivos. Este infiltrado é expresso principalmente por linfócitos CD4 T-helper e CD8 T-citotóxico, responsáveis por resposta inflamatória tipo I necessária para eliminar o câncer[20,21,22, 23]. O cenário neoadjuvante é considerado o melhor modelo para avaliar a interação entre drogas antitumorais, o microambiente e a resposta do paciente. Já existem evidências de que o sistema imune contribui com os efeitos antitumorais de regimes citotóxicos e terapia monoclonal. Agentes quimioterápicos como antraciclina e taxano estimulam a resposta imune através da redução da carga tumoral e modificação do microambiente com, por exemplo, liberação de citocinas como IL2 e interferon. Assim, a intensidade da resposta imune irá influenciar a efetividade da terapia instituída e está relacionada a melhores resultados [24,25].

Tumores triplo negativos não possuem terapia alvo e são basicamente tratados com quimioterapia, a qual em modelo neoadjuvante constitui um teste de sensibilidade "in vivo" de resposta ao tratamento. Vinte a 30% das pacientes com tumores TNBC alcançam resposta patológica completa com quimioterapia neoadjuvante, o que por sua vez está associado a melhores taxas de sobrevida [26,27,28]. No entanto, o fator que determina a sensibilidade à terapia nestes tumores permanece incerto.

INFILTRADO LINFOCITICO TUMOTAL (TILs)

Tendo em vista o papel do sistema imune e sua relação com quimioterapia, deu-se início a busca por um marcador que pudesse predizer quais pacientes teriam melhor resposta ao tratamento.

A associação entre Infiltrado Linfocítico Tumoral e desfechos em câncer de mama havia sido observada pela primeira vez em 1992 por Aaltomaa e col. Os TILs consistem na mistura de células imunes pró-inflamatórias com predomínio de linfócitos CD8 T citotóxicos e CD4 T *helper,* além de células dendríticas e natural killers e assim como células com ação supressora imune incluindo células B e T-reg, encontrados tanto no tumor como no microambiente ao seu redor[29,30]. Eles são detectados através de microscopia em lâminas de hematoxilina e eosina e sua quantidade é mensurada utilizando a área ocupada como uma porcentagem da área total de estroma, seguindo os *guidelines* desenvolvidos pelo *International Immuno-Oncology Biomarker Working Group* [31]. A medida do TILS estromal é preferida em relação ao TILS intratumoral uma vez que é mais fácil de ser realizada e reproduzida pelos patologistas.

Nos últimos anos, muitos estudos buscaram comprovar a relação entre TILs e resposta patológica dentro de grandes ensaios que avaliaram drogas quimioterápicas em adjuvância ou neoadjuvância, como GeparDuo, GeparTrio, Geparsixto, BIG 02-98, NeoALLTO, ECOG 2197 e 119, e FINHer [32, 33, 34 ,35]. Em 2018, Denker publicou no Lancet a análise de 3771 paciente tratadas com quimioterapia neoadjuvante constatando uma relação positiva entre o aumento de TILs e resposta patológica completa em tumores

triplo negativos, onde, a cada 10% de incremento de TILs, há um aumento no odds ratio para pCR[36]. Tumores com mais de 60% de TILs são considerados linfócitos predominantes, apresentam taxa de pCR de 41,7% e corresponde a 20% dos TNBC. As evidências mais robustas na relevância clínica de TILs estão associadas a tumores em estádio inicial onde a sobrevida livre de doença e sobrevida global reduzem 14% e 17% respectivamente a cada 10% do aumento de TILs [37]. Todavia, com o decorrer natural da doença, em estadios mais avançados, os TILs parecem perder poder significativo[38].

Embora pouco provável que um único biomarcador seja responsável por predizer respostas aos tratamentos, os TILs expressam a relação do tumor com sistema imune de maneira simples e praticável. A introdução dele à rotina do patologista é mais um passo em busca da medicina de precisão [39].

## REVISÃO DA LITERATURA

### ESTRATÉGIAS PARA LOCALIZAR INFORMAÇÕES

A revisão da literatura foi realizada utilizando artigos científicos publicados na base de dados eletrônica Pubmed, Scielo e Embase. A pesquisa foi realizada em inglês, português e espanhol. Uma busca manual foi executada na lista de referência dos artigos de interesse, não identificados pela pesquisa eletrônica, para selecionar estudos adicionais relevantes à questão principal da pesquisa. Foram incluídas também webpage do International Immuno-Oncology Biomarker Working Group on Breast Cancer

### PALAVRAS-CHAVE

Tumor infiltrating lymphocytes AND breast cancer

breast cancer AND TIL

breast cancer treatment history

target therapy and breast cancer

gene expression

Precision medicine

Target therapy

Triple negative breast cancer

TILs AND triple negative breast cancer

Immune infiltrates in breast cancer

Tumor infiltrating lymphocytes AND biomarker

Immunotherapy AND breast cancer

Immune targeting

Checkpoints AND breast cancer

Neoadjuvant Chemotherapy AND triple negative breast cancer

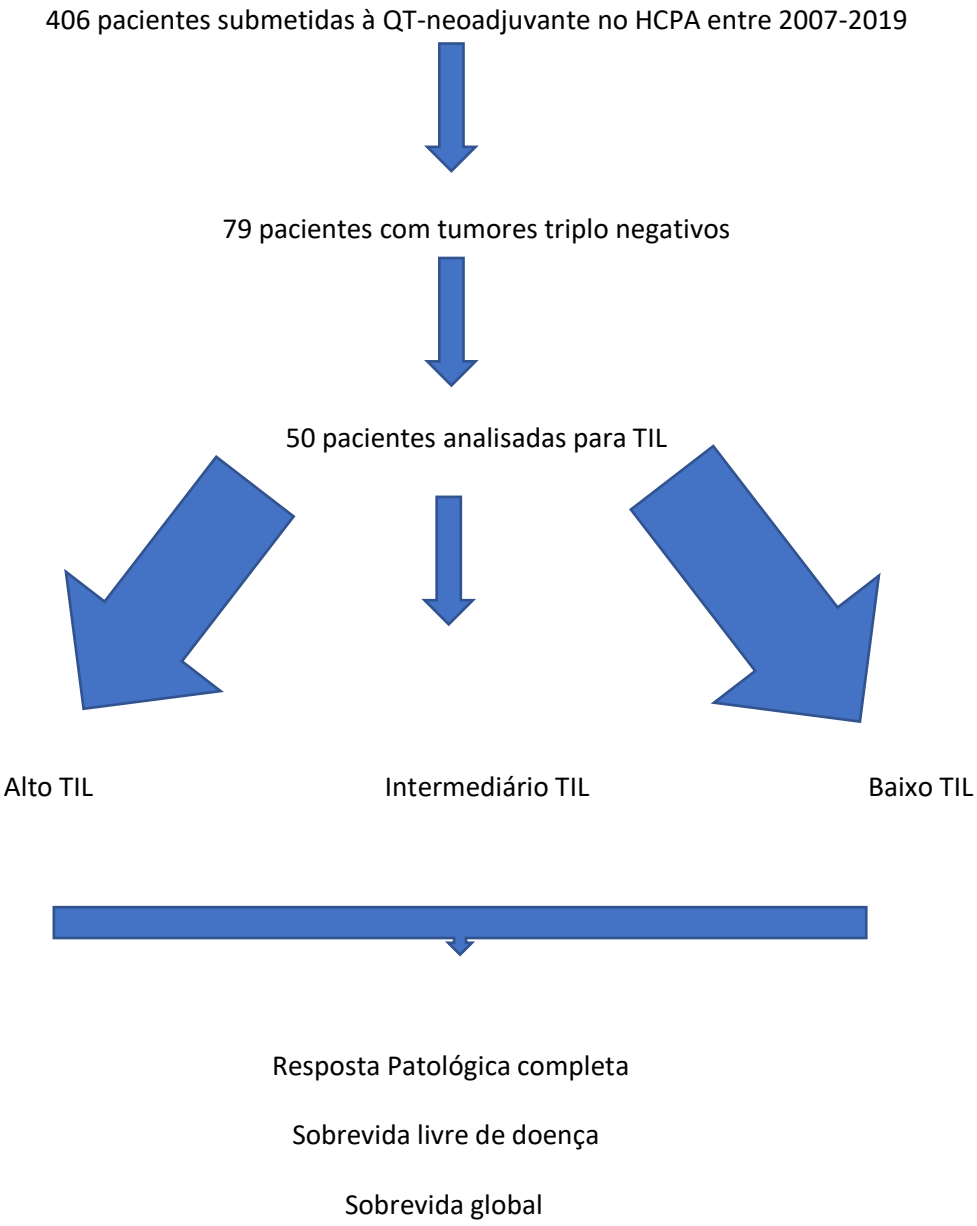
Tumor infiltrating lymphocytes AND pathological complete response

Immune system

Immune microenvironment

Immune checkpoints

**MAPA CONCEITUAL**



**JUSTIFICATIVA**

O câncer de mama triplo negativo constitui doença agressiva, acometendo muitas pacientes jovens. Apresenta altas taxas de recorrência e prognóstico reservado. Diferente de outros subtipos, não possui terapia alvo, estando seu tratamento restrito à cirurgia e à quimioterapia convencional. Seu aspecto imunogênico, avaliado a partir do infiltrado linfocítico tumoral, representa perspectiva importante na busca de terapias alternativas e na identificação das pacientes com maior benefício da mesma.

## HIPÓTESES

HIPÓTESE NULA

O infiltrado linfocítico tumoral não tem influência como preditor de resposta patológica à quimioterapia neoadjuvante e não influencia a sobrevida livre de doença e sobrevida global em pacientes com câncer de mama triplo negativo.

HIPÓTESE ALTERNATIVA

O infiltrado linfocítico tumoral tem influência como preditor de resposta patológica à quimioterapia neoadjuvante, influenciando a sobrevida livre de doença e sobrevida global em pacientes com câncer de mama triplo negativo

**OBJETIVOS**

PRINCIPAL

Avaliar a presença de TIL em tumores de mama triplo negativos e sua correlação com a resposta patológica após quimioterapia neoadjuvante.

SECUNDÁRIO

Correlacionar a presença de TIL com:

1. Sobrevida livre de doença e sobrevida global
2. Idade
3. Estado menopausal
4. Tipo histológico
5. Grau histológico
6. Estadiamento clínico inicial
7. Invasão angiolinfática

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ARTIGO

# TRIPLE NEGATIVE BREAST CANCER AND THE IMMUNE SYSTEM

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## INTRODUCTION

Triple-negative breast cancer (TNBC) accounts for 15-20% of all breast cancers and is characterized by aggressive clinical behavior and lack of target therapy [1]. Neoadjuvant chemotherapy (NACT) is largely used in TNBC with rates of pathological complete response (pCR) commonly higher than other breast tumor types. However, more than half of TNBC patients do not achieve a PCR and have a very poor prognosis [2,3]. The routine use of neoadjuvant anthracycline/taxane combinations in TNBC is nowadays being supplemented by ongoing investigations of other types of substances as immune checkpoint inhibitor therapies [4].

The development and progression of malignant tumors result from the interaction of the cells in the microenvironment including infiltrating immune cells. The immune system plays a dual role in promoting the protection of cancer and facilitating tumor escape and response to chemotherapy is partly dependent on that immunological reaction [5,6].

Recent studies have demonstrated improved progression free survival with the combination of atezolizumab/pembrolizumab and chemotherapy in programmed death-ligand 1 (PD-L1) positive metastatic TNBC [7,8]. PD-L1 has an important role in regulating our immune system, preventing overactivation of T cells and promoting the differentiation of regulatory T cells. Although PD-L1 is currently the only approved biomarker it remains controversial given the complexities of its clinical use due to variability in assay performance of the PD-L1 IHC antibodies, spatial and temporal heterogeneity, absence of a unified scoring system, and concerns about inter-reader reproducibility for scoring PD-L1 on immune cell [9].

Tumor-Infiltrating Lymphocytes (TIL) has also been proposed as a marker of the adaptive immune response as treatment reaction and outcome vary with its levels and is associated with pCR as a predictor of neoadjuvant response [10,11,12]. TIL can be assessed on a simple hematoxylin and eosin slide with reliable reproducibility among pathologists. A good biomarker should be analytically valid, robust, reproducible,

clinically useful, affordable, and accessible to pathologists especially considering the public health system [13].

The aim of this study was to establish the relationship between TILS and pCR in TNBC

## METHODS

### SELECTION AND DESCRIPTION OF PARTICIPANTS

All patients with breast cancer who underwent NACT between 2007 and 2019 at Hospital de Clinicas de Porto Alegre were included. Patients with TNBC were selected.

Male patients, patients with metastatic disease, and patients not submitted to surgery treatment were excluded.

This study was approved by the institutional ethics and research committee of Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil (registration number, CAAE: 02529018.5.0000.5327)

### PATHOLOGIC ASSESSMENT

Histopathologic analysis was performed using hematoxylin and eosin (H&E) stained sections of core biopsies obtained before the start of neoadjuvant chemotherapy. TILs were assessed by two independent blinded pathologists based on the guidelines of the international TIL working group [14]. Stromal TILs (sTils) were measured as percentage of immune cells in the stromal compartment within the borders of the invasive tumor. The number of TILs was analyzed as a continuous measurement and divided in 3 categories: Low (0-10%), Intermediate (11-39%) and High (40-100%). We did not evaluate intratumoral Tils. (iTils)

Clinicopathological features, such as age, menopausal status, tumor grade, lymphovascular invasion, tumor size, axillary involvement, percentage of Ki67, type of

treatment, disease-free survival, overall survival and rate of response to NAC were correlated with TILs percentage. Pathologic complete response (pCR) was defined as the absence of residual invasive carcinoma.

**STATISTICAL ANALYSIS**

Statistical analysis was performed using SPSS version 18.0 [SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.].

Descriptive analysis was expressed by measures of central tendency and dispersion: for quantitative variables by means ± standard errors of means (±SEM) or by medians (md) and interquartile ranges (IQR, 25th and 75th percentiles), according to the Shapiro-Wilk normality test; for qualitative variables by absolute (n) and relative (%) frequencies.

Comparisons of quantitative variables were performed using the Mann-Whitney test. Kaplan-Meier estimates for selected outcomes (e.g. death, relapse) were calculated, and the difference between participants with and without pathological complete response was compared using the Log-Rank test. Cox proportional hazard models were conducted to assess the effect of pathological complete response on the outcomes analyzed.

Agreement (Kappa index) and reliability (Cronbach's α) analyzes of the pathological outcomes were estimated between two pathologists (Examiner 1 and Examiner 2).

To evidence possible associations between qualitative variables of interest, the Chi-Square test was used with adjusted residual analyses. Furthermore, Spearman correlations were conducted between the studied variables. In all analyses, the level of significance used was set at 5%.

**RESULTS**

Seventy-nine out of 406 patients (19.4%) who underwent NACT meet the inclusion criteria. Technical feasibility to perform Til quantification was possible in 50 patients.

The mean age of diagnosis was 49.4 (±1.3) years and 38 (48.1%) of them were pre-menopausal status (table 1)

Histopathologic characteristics and interventions were shown in Table 2. The majority of tumors were classified as grade 3 – 46 (58.2%) and no special ductal type invasive carcinoma – 58 (73.4%). Stage III was the most prevalent: EIIIA 23 (29.1%), EIIIB 21 (26.6%) e EIIB 20 (25.3%). Half of the patients underwent radical mastectomy - 40 (50.6%)- and more than that received axillary lymphadenectomy - 52 (65.8%). The mean chemotherapy applied were cyclophosphamide-adriamycin followed by taxane - 70 (74,7%).

During a follow up of 12 years, disease free survival was 27.0 (15.0 – 60.0) months and overall survival 34.0 (18.0 – 61.5) months (table 3).

Pathological complete responses (pCR) were achieved in 21 patients (26.6%) and were associated with better prognosis as demonstrated in the overall survival curve (Figure 4A e 4B) and disease free survival curve (Figure 4C e 4D).

TILs quantification and distribution are described in Table 4. A substantial concordance (índice Kappa = 0.703) on TILs evaluation was revealed by the two independent blinded pathologists, with reliable consistency (α de Cronbach = 0.936). TILs average were similar (Mann-Whitney test, p=0.473). High TILs were a minority (11,4% e 5,1%) during a follow upo f 12 years.

By Spearman correlation analysis we identified an inverse relation between menopausal status and TIL quantification ($\rho$ =-0.326, $p \leq 0.05$). With complementary analysis TILS decrease in post menopausal women (Mann-Whitney test, p=0.029 and Qui-square test with residual adjusted analysis, p=0.060). There was no difference on tumor size/grade or nodal status as well as clinical stage by TILs.

## DISCUSSION

The study reassured the aggressive behavior of triple negative breast cancer in a follow up of more than 10 years demonstrating an overall survival of 34 months. We were able to demonstrate pCR as a surrogate of long-term survival after neoadjuvant chemotherapy, but did not establish a statistically significant relationship between TILs and pCR or prognosis.

Previous studies have shown that TILs can predict pCR to neoadjuvant therapy. For instance, Denker demonstrated that lymphocytes-predominant breast cancer had a 41.7% pCR rate, whereas tumors without any infiltrating lymphocytes had a 2.8% pCR rate from the Gepartrio trial [15]. Also rates of pCR increased sharply when levels of sTils were greater than 5% regardless of treatment group in the evaluation made by Salgado et al over patientes from the NeoALTTO study. [16]. Similar tendency has been seen by Ono, Dieci, Issa-Nummer and others [17,18,19].

Different from most of previous studies that evaluated patients in early stage of the disease, the majority of our patients [46 – 58.2%] were stage III and one possible explanation for the lack of results in the correlation between Tils and pCR is the suggestion that TIL infiltration tends to weaken throughout the natural history of breast cancer from the early to advanced stages, as observed in studies like PANACEA and KEYNOTE-086. Tils levels are also lower in multiple treatment patients as compared to those treated in the first line settings for metastatic disease [20,21,22].

When considering the correlation of Tils levels and clinical factors, there is no absolute conclusive evidence. Loi and colleagues have conducted a pooled individual patient analysis from 2,148 patients from nine studies to investigate the prognostic value of TILs in early-stage TNBC. In this study, they also compared TILs levels with clinicopathologic features. It was identified lower TILs quantities in older patients, low grade tumours, large tumours and those with higher burden of nodal disease [23]. In our multivariate analysis, we identified a significant inverse relationship between Tils and menopausal status, with higher quantities in premenopausal younger patients. That can be explained

by the fact that with aging the numbers of several immune cells can decrease, including NK cells, T lymphocytes, and B lymphocytes. The association between aging and the decreased adaptive immune efficacy has been subject of studies since the term “immunosenescence” was introduced into the literature in 1980 by Makinodan [24,25,26].

On the other hand, we were not able to demonstrate a correlation between levels of TILs and tumor size, grade, nodal involvement or lymphovascular invasion, but the same can be seen in the BIG02-98 trial [27] and in the Tamioka trial, this one in which the distribution of Tils were analyzed on clinicopathological factors in a total of 32 TNBC and detected no difference on tumour size or nodal involvement [28].

In other respects, the study revealed the good reproducibility and affordable aspects of the TIL assessment by showing reliable consistency between the two blinded pathologists in simple hematoxylin and eosin slides. A good biomarker is typically evaluated on a continuous scale and must be analytically valid. The international Immuno-oncology Biomarker Working Group was established in 2012 with the purpose of creating an internationally standardized approach for Til evaluation. Two multicenter international ring studies were conducted to provide data on interobserver variability of Tils. 120 pretherapeutic core biopsies from the neoadjuvant GeparSixto trial were evaluated, 60 in each study. The first study ring had 32 participants pathologists and the second study ring had 28, all from 27 different institutions in 9 countries. The primary endpoint for interobserver variance was the intraclass correlation coefficient (ICC) which is the proportion of total variance that is attributable to the biological variability among patients’ tumors. The first ring study did not reach its endpoint. For the second ring study there was a different digital slide system and feedback references images were provided. The pre specified endpoint was met in ring study 2 and there was a concordance rate of 92% [29, 30]. Therefore, trough guidelines and daily practice, Tils can be a strong tool to translate the immunological spectrum of breast cancer.

After the 16th St Gallen International Breast Cancer Consensus Conference, WHO and ESMO 2019 Clinical Practice Guidelines Tils reach level 1b evidence as a prognostic marker in early TNBC and the routine quantification is endorsed and encouraged.

We agree there are several limitations in our investigation, especially by the small sample studied. We reassured the prospect that pCR after neoadjuvant therapy is an indicator of better prognosis in TNBC. However it remains unclear which patient will respond to treatment. There are a lot of aspects of the patients, the tumours and their interface that we do not know. Tils seems to bring information about what is happening in this microenvironment, giving us the chance to do something ahead, so we can guarantee that the patient will respond to treatment.

Trying to understand what makes someone more susceptible to respond to treatment, bringing up all the information and characteristics that we can, is one way to stand for precise medicine, the future medicine. Thus, more studies are warranted to better define the specific role of Tils in breast cancer.

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## CONSIDERAÇÕES FINAIS

O câncer de mama é a principal causa de morte por câncer entre as mulheres no mundo.

Dentre os fatores prognósticos da doença está o subtipo molecular, o qual prediz resposta ao tratamento indicado. Os tumores triplo negativos por sua vez, acometem pacientes jovens, em estágios avançados, apresentando pior sobrevida global e maior risco de recidiva local e sistêmica. O tratamento tradicional realizado com quimioterapia, cirurgia e radioterapia é diversas vezes insuficiente, levando as pacientes a quadro evolutivo desfavorável.

A compreensão de que o sistema imune tem influência sobre a resposta ao tratamento e comportamento deste tipo de tumor abriu portas para um novo cenário clínico, onde novas linhas de pesquisa e drogas ascenderam. A imunoterapia já vinha sendo utilizada em outras neoplasias, mas foi em 2020 que o FDA aprovou sua aplicação no tratamento do câncer de mama, inicialmente pelo Atezolizumabe em tumores triplo negativo avançados. Mais recentemente, em junho de 2021, Pembrolizumabe foi aprovado no tratamento da doença em estadio inicial.

Ainda muito recente, mas a imunoterapia traz a perspectiva de mudar a história natural dos subtipos mais agressivos do câncer de mama. Identificar o perfil da paciente com maior benefício a este tratamento, permanece um desafio. Ao que os estudos indicam, mais de um biomarcador será necessário para tal conclusão.

**PERSPECTIVAS**

A medicina de precisão é uma realidade cada vez mais presente para as pacientes com câncer de mama. São inúmeras as opções de tratamento, sempre que possível, personalizado, permitindo maiores benefícios com menores efeitos adversos. A imunoterapia vem para preencher uma lacuna que faltava nesta equação, o tratamento de tumores triplo negativos. ‘

**ANEXOS**

**Figure 1. Table 1.** Sample characterization.

| **Variable** | **Total (N=79)** |
|---|---|
| Age at diagnosis (years) – mean ± EPM | 49.4 ± 1.3 |
| (minimum - maximum) | (27.0 – 73.0) |
| Menopausa – n (%) | |
| No | 38 (48.1) |
| Yes | 36 (45.6) |
| INO | 5 (6.3) |

n – absolute frequency. n% – relative frequency. EPM – mean standard error. INO – information not obtained.

**Figure 2. Table 2.** Histopathological data and interventions.

| Variable | Total (N=79) |
|---|---|
| Grade – n (%) | |
| Grade 2 | 14 (17.7) |
| Grade 3 | 46 (58.2) |
| not rated /INO | 19 (24.1) |
| Histological type – n (%) | |
| Non-special type invasive ductal carcinoma | 58 (73.4) |
| Undifferentiated | 13 (16.5) |
| special type | 7 (8.9) |
| not rated /INO | 1 (1.3) |
| initial EC – n (%) | |
| IIA | 13 (16.5) |
| IIB | 20 (25.3) |
| IIIA | 23 (29.1) |
| IIIB | 21 (26.6) |
| IIIC | 2 (2.5) |
| Surgery – n (%) | |
| Conservative | 35 (44.3) |
| Radical | 40 (50.6) |
| did not operate /NSA | 4 (5.1) |
| axillary surgery – n (%) | |
| Axillary sentinel lymph node biopsy | 22 (27.8) |
| axillary lymphadenectomy | 52 (65.8) |
| INO | 5 (6.3) |
| chemotherapy treatment – n (%) | |
| 4AC + 4T | 42 (53.2) |
| 4AC + 12T | 14 (17.7) |
| 4AC | 11 (13.9) |
| 4AC + 12T + Carboplatin | 3 (3.8) |

| | | |
|---|---|---|
| | T alone | 2 (2.5) |
| | Others | 7 (8.9) |
| radiotherapy treatment – n (%) | | |
| | 25F | 25 (31.6) |
| | 30F | 35 (44.3) |
| | Outros | 7 (8.9) |
| | Did not undergo radiotherapy | 9 (11.4) |
| | INO | 3 (3.8) |
| lymphovascular invasion – n (%) | | |
| | No | 47 (59.5) |
| | Yes | 22 (27.8) |
| | NSA | 6 (6.7) |
| | INO | 4 (5.1) |

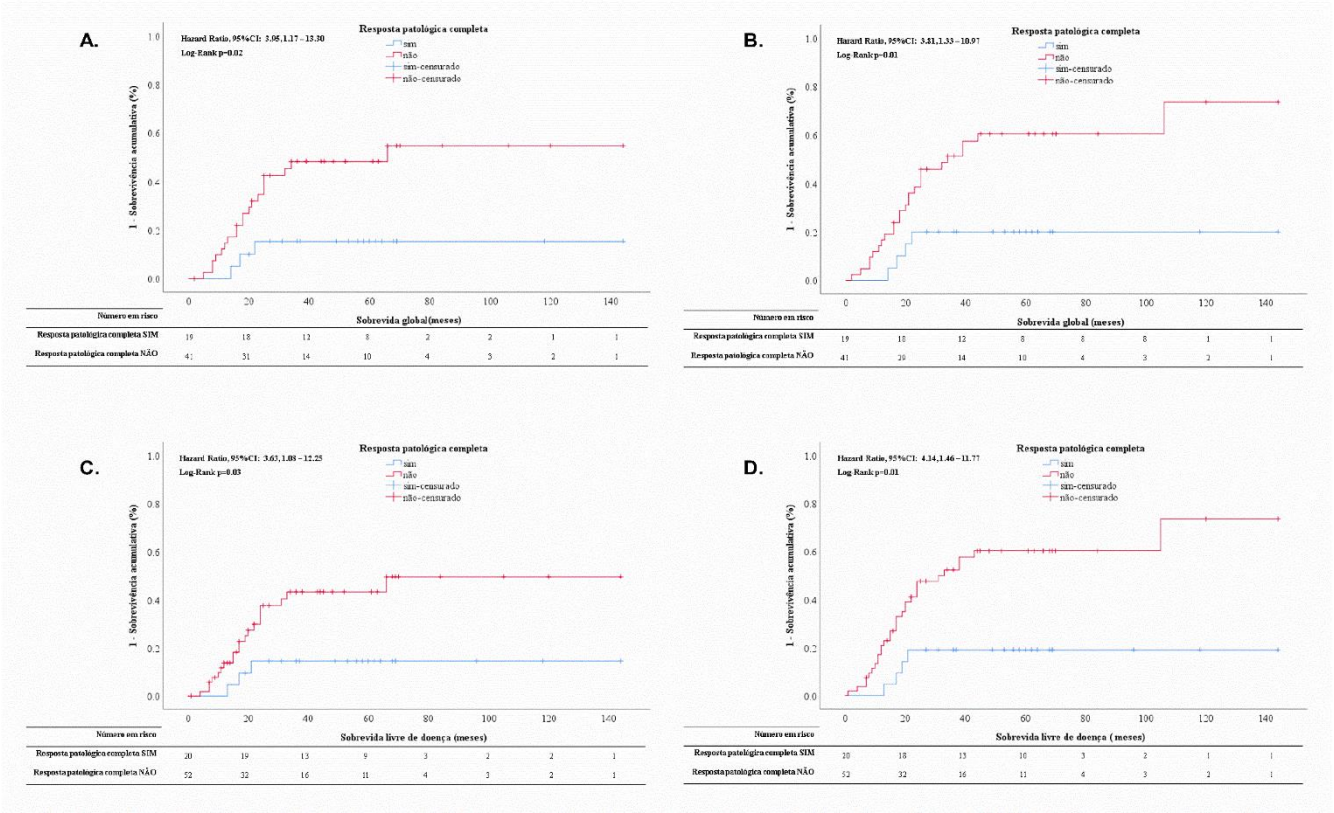
n – absolute frequency. n% – relative frequency. INO – information not obtained. NSA – not applicable. F – radiotherapy fraction. AC - adriamycin/cyclophosphamine. T- taxane.

**Figure 3. Table 3.** Post-intervention follow-up.

| Variable | | Total (N=79) |
|---|---|---|
| Relapse – n (%) | | |
| | No | 34 (43.0) |
| | Yes | 37 (46.8) |
| | NSA | 1 (1.3) |
| | INO | 7 (8.9) |
| Complete pathological response – n (%) | | |
| | No | 54 (68.4) |
| | Yes | 21 (26.6) |
| | INO | 4 (5.1) |
| Death – n (%) | | |
| | No | 39 (49.4) |
| | Yes | 26 (32.9) |
| | INO | 14 (17.7) |
| Overall Survival (months) – md (IQR) | | 34.0 (18.0 – 61.5) |
| | (minimum - maximum) | 2.0 – 144.0 |
| | NSA | 1 (1.3) |
| | INO | 14 (17.7) |
| Disease Free Survival (months) – md (IQR) | | 27.0 (15.0 – 60.0) |
| | (minimum - maximum) | 0.0 – 144.0 |
| | INO | 1 (1.3) |

n – absolute frequency. n% – relative frequency. md – median. IQR – interquartile range (percentiles 25 e 75). INO – information not obtained. NSA – not applicable.

**Figure 4.**




Survival curves. A. Overall survival (months) and death. B. Overall survival (months) and relapse. C. Disease-free survival (months) and death. D. Disease-free survival (months) and relapse. Kaplan-Meier Log-Rank and Cox Regression.

**Figure 5**. **Table 4.** Characterization of tumor infiltrating lymphocytes.

| Variable | | Evaluator 1 (N=79) | Evaluator 2 (N=79) | |
|---|---|---|---|---|
| Tumor infiltrating lymphocytes – n (%) | | | | **Kappa =** 0.703 |
| | Low | 21 (26.6) | 31 (39.2) | |
| | Intermediate | 20 (25.3) | 13 (16.5) | |
| | High | 9 (11.4) | 4 (5.1) | **α de Cronbach =** |
| | NSA | 10 (12.7) | 10 (12.7) | 0.936 |
| | INO | 19 (24.1) | 21 (26.6) | |

| | | Total (N=79) | *p-value |
|---|---|---|---|
| **Evaluat. 1** | Tumor infiltrating lymphocytes – md (IQR) | 72.0 (28.0 – 175.0) | 0.473 |
| | (minimum - maximum) | 1.0 – 794.0 | |
| **Evaluat. 2** | Tumor infiltrating lymphocytes – md (IQR) | 56.5 (26.0 – 173.5) | |
| | (minimum - maximum) | 1.0 – 725.0 | |

n – absolute frequency. n% – relative frequency. md – median. IQR – interquartile range (percentiles 25 e 75). INO – information not obtained. NSA – not applicable. *Mann-Whitney test. Significance fixed at 5% for all analyses.