UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

MAHIRA DE OLIVEIRA LOPES DA ROSA

IMPACTO DA EXPRESSÃO DE LINFÓCITOS INFILTRANTES TUMORAIS E DO STATUS DE HER2-LOW EM UMA COORTE DE PACIENTES COM CÂNCER DE MAMA HER2-NEGATIVO TRATADAS COM QUIMIOTERAPIA NEOADJUVANTE

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Dissertação apresentada ao Programa de Pós-Graduação em Medicina: Ciências Médicas da Faculdade de Medicina da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção de Mestre em Medicina: Ciências Médicas.

Orientadora: Prof. Dra. Marcia Silveira Graudenz.

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RESUMO

Base Teórica: O tratamento do câncer de mama está em constante mudança. A doença pode ser categorizada como HER2 positivo, triplo negativo (TN) ou luminal (receptor de estrogênio positivo) HER2 negativo. O termo "HER2-negativo" inclui tumores sem expressão de HER2 (HER2-zero) e tumores com baixa expressão do receptor (HER2-low). Além das características intrínsecas das células tumorais, o microambiente tumoral (TME) também desempenha um papel importante no processo de carcinogênese. Vários estudos avaliam a presença de linfócitos infiltrantes de tumor (TIL) como papel prognóstico e preditivo no câncer de mama. O cenário neoadjuvante é a escolha para avaliar o impacto dos biomarcadores na resposta tumoral. Atualmente, a integração de novos biomarcadores em decisões de tratamento personalizadas continua a ser uma necessidade não atendida. Nesse contexto, este estudo se propõe a avaliar a correlação entre a infiltração linfocitária e as características clínicas na população HER2-negativa, com foco no "subtipo" HER2-low.

Objetivos: O objetivo principal foi descrever a porcentagem de TIL em tumores HER2-low e compará-la com tumores HER2-zero. Os objetivos secundários foram avaliar a densidade de TILs em relação ao perfil hormonal, grau tumoral e taxa de resposta patológica. Além disso, avaliar a concordância entre 2 patologistas ao analisar TILs.

Métodos: Este é um estudo retrospectivo que envolveu pacientes com câncer de mama invasivo HER2-negativo submetidas a quimioterapia neoadjuvante (NACT), seguida de cirurgia de mama. Amostras tumorais foram avaliadas quanto a densidade dos TILs. O TIL alto foi categorizado usando dois limites distintos (>= 20% e >=50%). A resposta patológica foi baseada na carga residual de câncer (RCB).

Resultados: Foram analisados dados de 119 pacientes. Dentre eles, 34 tinham HER2-zero (28,5%) e 85 com HER2-baixo (71,4%). Não houve diferença significativa na expressão de TIL (p=0,1367) ou resposta patológica (p=0,1168) relacionada ao status de HER2 (zero vs. baixo). A mediana do TIL foi de 10% na população em geral, bem como na população com HER2-low, enquanto na população com HER2-zero a mediana foi de 15%. A maioria dos tumores luminais apresentava TILs baixo (<20%), enquanto os TN apresentavam predominantemente TILs elevado, independentemente do status de HER2. A resposta patológica completa (pCR - RCB zero) teve mediana de TILs de 37,5%, enquanto na RCB I, II e III as medianas foram de 15%, 10% e 5%, respectivamente. Diferença significativa (p = 0,0033) foi observada usando um limiar de 20% para TILs, com 75% dos pacientes que alcançaram pCR exibindo TILs alto, enquanto 82,9% dos pacientes com pobre resposta ao tratamento (RCB III) demonstraram TILs baixo. Encontramos boa concordância entre os dois patologistas, sendo de 84,4% quando o limiar do TILs foi de 20%, e de 96,9% quando usamos um limiar de 50%, com coeficiente Kappa de 0,6825 e 0,7838, respectivamente.

Conclusão: Nesta coorte de pacientes, não foi identificada diferença significativa na distribuição das características clínico-patológicas e na densidade de TILs entre os subgrupos HER2-low e HER2-zero. A baixa expressão de HER2 isoladamente não conferiu um papel preditivo na resposta à NACT. A presença e alta densidade de TILs foram correlacionadas com melhor resposta patológica e houve uma boa taxa de concordância entre patologistas treinados na avaliação de TILs. São necessárias análises pré-planejadas e prospectivas para melhor compreender o papel dos TILs na população com HER2-low.

Palavras-chave: câncer de mama, tratamento neoadjuvante, linfócitos infiltrantes tumorais, HER2-low

ABSTRACT

Background: The treatment of breast cancer is constantly changing. The disease can be categorized as HER2 positive, triple negative (TN) or luminal (estrogen receptor positive) HER2 negative. The "HER2-negative" term includes tumors with no HER2 expression (HER2-zero) and tumors with low expression of the receptor (HER2-low). In addition to the intrinsic characteristics of tumor cells, the tumor microenvironment (TME) also plays an important role in the process of carcinogenesis. Several studies evaluate the presence of stromal tumor-infiltrating lymphocytes (TIL) as a prognostic and predictive role in breast cancer. The neoadjuvant setting is the choice for evaluating the impact of biomarkers on tumor response. Currently, the integration of novel biomarkers into personalized treatment decisions remains an unmet need. In this context, this study proposes to assess the correlation between lymphocyte infiltration and clinical characteristics in the HER2-negative population, focusing on the HER2-low "subtype".
Objectives: The primary objective was to describe the percentage of TIL in HER2-low tumors and compare it with HER2-zero tumors. The secondary objectives were to assess the TILs density regarding the hormonal profile, tumor grade and pathological response rate. Furthermore, to assess agreement between 2 pathologists when analyzing TILs.
Methods: This is a retrospective study that enrolled HER2-negative invasive breast cancer patients submitted to neoadjuvant chemotherapy (NACT) followed by breast surgery. Tumor samples were evaluated to assess the TILs density. High TIL was categorized using two distinct thresholds (>=20% and >=50%). The pathological response was based on residual cancer burden (RCB).
Results: Data from 119 patients were analyzed. Among them, 34 had HER2-zero (28.5%), and 85 with HER2-low (71.4%). There was no significant difference in TIL expression (p=0.1367) or pathological response (p=0.1168) related to HER2 status (zero vs. low). The median TIL was 10% in the overall population as well as in the HER2-low population, while in the HER2-zero population, the median was 15%. Most luminal tumors had low TILs (<20%), while TN predominantly had high TILs, regardless of HER2 status. Pathological complete response (pCR - RCB zero) had median TILs of 37.5%, while in RCB I, II, and III the medians were 15%, 10%, and 5%, respectively. A significant difference (p=0.0033) was observed using a 20% threshold, with 75% of patients that achieved pCR exhibiting high TILs whereas 82.9% of patients with poor response to treatment (RCB III) demonstrated low TILs. We found good agreement among both pathologists, 84.4% when the threshold of TILS was 20%, and 96.9% when we used a threshold of 50%, with a Kappa coefficient of 0.6825 and 0.7838, respectively.
Conclusion: In this cohort of patients, no significant difference was identified in the distribution of clinicopathological characteristics and density of TILs between the HER2-low and HER2-zero subgroups. Low HER2 expression alone does not confer a predictive role in response to NACT. The presence and high density of TILs were correlated with better pathological response and there was a good agreement rate among pathologists trained in the assessment of TILs. More well-designed and prospective analyses are needed to better understand the role of TILs in HER2-low population.

Key-words: breast cancer, neoadjuvant treatment, tumor-infiltrating lymphocytes, HER2-low

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ADC	Anticorpo conjugado à droga
ASCO	*American Society of Clinical Oncology*
BCFI	Intervalo livre câncer de mama
CAP	*College of American Pathologists*
CEP17	Centrômero do cromossomo 17
CM	Câncer de mama
CMTN	Câncer de mama triplo negativo
CTL	Linfócitos T citotóxicos
DDFS	Sobrevida livre de doença a distância
DFS	Sobrevida livre de doença
DRFI	Intervalo livre de recorrência a distancia
DRFS	Sobrevida livre de recorrência a distância
ERBB2	Gene que codifica a proteína HER2
ESMO	Sociedade Europeia de Medicina Oncológica
FDG	Fluorodeoxyglucose
FISH	Hibridização por imunofluorescência in situ
FOXP3	Célula Forkhead box P3
HER2	Receptor do fator de crescimento epidérmico humano-2
IC	Intervalo de confiança
ICI	Inibidor de checkpoint imune
IDFS	Sobrevida livre de doença invasiva
IHQ	Imuno-histoquímica
INCA	Instituto Nacional de câncer
ISH	Hibridização in situ
LPBC	Câncer de mama predominantemente linfocitário
KI67	Índice de Proliferação Celular

MFS	Sobrevida livre de metástase
NACT	Quimioterapia neoadjuvante
OR	Razão de Odds
pCR	Resposta patológica completa
PET/CT	Tomografia computadorizada com emissão de pósitrons
PDL1	Ligante de morte programada 1
PPGCM	Programa de Pós-Graduação em Ciências Médicas
RCB	Carga tumoral residual (*residual cancer burden)*
RE	Receptor de estrogênio
RH	Receptor hormonal
RNA	Ácido ribonucleico
SG	Sobrevida global
SLP	Sobrevida livre de progressão
TIL	Infiltrado linfocitário tumoral
TME	Microambiente tumoral
TN	Triplo negativo
Treg	Linfócito T regulador
UFRGS	Universidade Federal do Rio Grande do Sul

SUMÁRIO

1. INTRODUÇÃO

O câncer de mama é o tumor maligno mais comum e a principal causa de morte por câncer entre as mulheres em todo o mundo (Sung *et al.*, 2021). Esta é uma doença muito heterogênea e seu tratamento está em constante transformação. A doença pode ser categorizada em 3 grandes subtipos (HER2 positivo, triplo negativo, e luminal HER2 negativo) baseado na análise da expressão proteica no exame de imuno-histoquímica (Parise *et al.*, 2009). O conhecimento profundo da fisiopatologia de cada subtipo de câncer de mama e a determinação de biomarcadores é fundamental para o desenvolvimento de novas estratégias terapêuticas.

Dentre os subtipos de câncer de mama, o subtipo HER2 negativo inclui tumores com ausência de expressão HER2 (HER2 zero) e tumores com expressão baixa do receptor (HER2-low). Estes tumores podem ser triplo negativo ou luminais (com receptores hormonais positivos). Os tumores HER2-low correspondem a cerca de 50% dos tumores considerados HER2 negativos (Tarantino *et al.*, 2020) e o termo HER2-low ganhou destaque nos últimos anos em decorrência do surgimento de novo tratamento com anticorpo droga conjugado (ADC) bastante ativo neste subgrupo (Modi *et al.*, 2022).

Além das características intrínsecas das células tumorais, o microambiente tumoral também apresenta papel importante no processo da carcinogênese (Bhowmick; Moses, 2005). As células imunes infiltrantes podem modular o crescimento tumoral determinando a resposta e progressão (Kim; Stein; O’Hare, 2005). Neste contexto, diversos estudos avaliam a presença de linfócito infiltrante tumoral (TIL) como um marcador imunobiológico com importante papel prognóstico e preditivo no câncer de mama (Wang *et al.*, 2016). A padronização da avaliação quantitativa de TILs, determinada por grupo de estudo internacional (Salgado *et al.*, 2015), permite o uso deste parâmetro histológico como um biomarcador adicional ao manejo do câncer de mama.

O cenário de tratamento neoadjuvante é considerado o ideal para avaliar o impacto de biomarcadores na resposta e evolução tumoral. As evidências são consistentes correlacionando a presença de TILs como fator prognóstico favorável e como preditor de resposta a quimioterapia na doença inicial no câncer de mama triplo negativo e HER2 positivo. Embora existam dados sobre a heterogeneidade da expressão de HER2 (Pernas;

Tolaney, 2020), os dados na literatura explorando da relação da presença de TILs e desfecho no subgrupo de pacientes HER2-low são escassos. A análise histológica retrospectiva de amostras tumorais HER2-negativo submetidas a tratamento neoadjuvante permite a avaliação do infiltrado linfocitário tumoral e sua correlação com características clínicas e com resposta tumoral na população HER2-low.

2. REVISÃO SISTEMÁTICA

2.1 REVISÃO DA LITERATURA

A revisão da literatura foi realizada por meio da base de dados do Pubmed e atualizada em outubro de 2023. A busca inicial concentrou-se nos termos câncer de mama (*"Breast Neoplasm" [MeSH Terms]),* infiltrado inflamatório tumoral *("lymphocytes, tumor infiltrating" [MeSH Terms]*), tratamento neoadjuvante *("neoadjuvant therapy"[MeSH Terms]*), e a seguir adicionado o termo HER2low com estratégias distintas de busca.

A seleção dos trabalhos teve por objetivo focar em artigos de revisão e estudos clínicos envolvendo abordagem do microambiente tumoral, por meio da análise de infiltrado inflamatório tumoral, em população de pacientes com câncer de mama HER2 negativo. Alguns artigos de revisão foram selecionados a fim de compor a base teórica conceitual sobre o tema. A seleção deu-se por meio leitura criteriosa de títulos e resumos. Foram excluídos artigos focados exclusivamente em câncer de mama HER2 positivo, com envolvimento de populações ou subtipos específicos ou doença metastática.

TERMO PESQUISADO	Artigos encontrados	Artigos selecionados*
"Breast Neoplasm" [MeSH Terms]	504.069	-
"neoadjuvant therapy"[MeSH Terms]	53.623	-
"lymphocytes, tumor infiltrating" [MeSH Terms]	18.113	-
" HER2low" **	7.750	-
"Breast Neoplasm" [MeSH Terms] AND "neoadjuvant therapy"[MeSH Terms] AND "lymphocytes, tumor infiltrating" [MeSH Terms]	485	95
"Breast Neoplasm" [MeSH Terms] AND "neoadjuvant therapy"[MeSH Terms] AND "lymphocytes, tumor infiltrating" [MeSH Terms] AND "HER2low"**	67	25
"Breast Neoplasm" [MeSH Terms] AND "AND "lymphocytes, tumor infiltrate ng" [MeSH Terms] AND "HER2low"***	8	5

Tabela 1: Resultados da pesquisa de revisão da literatura

*Artigos selecionados após revisão criteriosa de títulos e resumos.

** Para HER2low a estratégia de busca incluiu os termos: *"HER2low"[All Fields] OR (("receptor, erbb 2"[MeSH Terms] OR ("receptor"[All Fields] AND "erbb 2"[All Fields]) OR "erbb-2 receptor"[All Fields] OR "her 2"[All Fields] OR "genes, erbb 2"[MeSH Terms] OR ("genes"[All Fields] AND "erbb 2"[All Fields]) OR "erbb-2 genes"[All Fields]) AND "low"[All Fields]) OR "HER2-low"[All Fields] OR ("HER2"[All Fields] AND "low"[All Fields]) OR (("receptor, erbb 2"[MeSH Terms] OR ("receptor"[All Fields] AND "erbb 2"[All Fields]) OR "erbb-2 receptor"[All Fields] OR "her 2"[All Fields] OR "genes, erbb 2"[MeSH Terms] OR ("genes"[All Fields] AND "erbb 2"[All Fields]) OR "erbb-2 genes"[All Fields]) AND "low"[All Fields]) OR (("receptor, erbb 2"[MeSH Terms] OR ("receptor"[All Fields] AND*

"erbb 2"[All Fields]) OR "erbb-2 receptor"[All Fields] OR "her 2"[All Fields] OR "genes, erbb 2"[MeSH Terms] OR ("genes"[All Fields] AND "erbb 2"[All Fields]) OR "erbb-2 genes"[All Fields]) AND "low"[All Fields])
*** Para esta busca os termos utilizados foram: ("HER2low") OR ("HER 2 low")) OR ("HER2-low")) OR ("HER2 – low")) OR ("HER 2 – low")) OR ("HER-2 low")

A busca {"Breast Neoplasm" [MeSH Terms] AND "neoadjuvant therapy" [MeSH Terms] AND "lymphocytes, tumor infiltrating" [MeSH Terms]} resultou em 485 trabalhos científicos publicados, e destes foram selecionados para revisão 95 artigos. Quando adicionado o termo HER2low (com 2 diferentes estratégias de busca descritas acima), 73 artigos foram publicados e destes, 30 foram revisados integralmente. No total 125 manuscritos foram selecionados para compor a base teórica e discussão neste trabalho.

2.2 CONCEITOS E REVISÃO TEÓRICA

EPIDEMIOLOGIA CÂNCER DE MAMA

O câncer de mama (CM) é o tumor maligno mais comum e a principal causa de morte por câncer em mulheres (Sung *et al.,* 2021). No mundo, estimou-se cerca de 2,2 milhões de novos casos de CM em 2020, sendo responsável por cerca 685 mil óbitos para o mesmo ano. No Brasil o número estimado de casos novos de câncer de mama, para o triênio de 2023 a 2025, é de 73.610 casos, correspondendo a um risco estimado de 66,54 casos novos a cada 100 mil mulhere (Santos *et al.,* 2023) s. O diagnóstico em estágios iniciais ou localmente avançado é fundamental para o adequado controle e cura da doença. Dados do Instituto Nacional de Câncer (INCA) revelam que no Brasil, aproximadamente 68% das pacientes são diagnosticadas com doença inicial (Santos *et al.,* 2023). O tratamento curativo deve ser multidisciplinar e individualizado considerando características tumorais e do paciente.

SUBTIPOS CÂNCER DE MAMA

O câncer de mama é uma doença heterogênea causada por várias alterações genéticas nas células epiteliais da mama, com diferentes manifestações clínicas e desfechos (Lüönd; Tiede; Christofori, 2021). Do ponto de vista histológico, 70 a 80% dos carcinomas invasivos são classificados como subtipo 'não-especial', anteriormente

denominados carcinoma ductal invasivo. Dentre os subtipos 'especiais', os mais frequentes são o carcinoma lobular invasivo (5 a 15%) e o carcinoma tubular puro (2 a 4%) (Kim *et al.*, 2020). Outro dado avaliado na histologia é o grau de diferenciação tumoral determinado pela classificação de Nottingham, que avalia padrão arquitetural, grau nuclear e índice mitótico, para estratificar os tumores em grau 1 (bem diferenciado), grau 2 (moderada diferenciação) e grau 3 (mais agressivo, com pobre diferenciação) (ELSTON; ELLIS, 1991).

Estudos de perfil de expressão gênica identificaram cinco categorias de câncer de mama (Sørlie *et al.*, 2001), são elas: luminal A, luminal B, receptor 2 do fator de crescimento epidérmico humano (HER2) enriquecido, basalóide e normal-símile (Perou *et al.*, 2000). Essas categorias moleculares correlacionam-se com marcadores da imuno-histoquímica (IHQ) rotineiramente realizados em biópsias mamárias para a determinação do subtipo tumoral e definição terapêutica. A IHQ é uma técnica patológica que identifica, por meio da coloração de lâminas e testes de anticorpos, a expressão proteica no tecido amostrado. Além da IHQ, por vezes se faz necessário teste de hibridização in situ (ISH) que utiliza técnica molecular para identificação de amplificação gênica (Wolff *et al.*, 2018). O painel básico em oncologia mamária inclui receptores hormonais (RH), receptores de estrógeno (RE) e de progesterona, e receptor do fator de crescimento epidérmico humano tipo 2 (HER2), além de índice de Ki67 que fornece dados sobre proliferação celular. Por meio destes testes é possível identificar tumores luminais (RH+/HER2-), HER2-positivo (HER2+) e triplo negativo (RH-/HER2-) (Parise *et al.*, 2009).

Os tumores luminais, aqueles com expressão de RH positivos na IHQ, correspondem a cerca de 70% dos tumores da mama, estão relacionados a sensibilidade a terapia hormonal e apresentam melhor prognóstico (Yersal, 2014). Tumores HER2 positivos representam cerca de 15-20% das neoplasias da mama. São tumores mais agressivos, entretanto apresentam boa resposta a terapia alvo anti-HER2, melhorando a sobrevida das pacientes (Rosa *et al.*, 2020). O subtipo de câncer de mama triplo-negativo (CMTN) responde por 11 a 20% e apresenta comportamento biologicamente agressivo, tendem a ser maiores, mais indiferenciados e com envolvimento linfonodal mais frequente ao diagnóstico (Loizides; Constantinidou, 2023). Esse perfil está associado a maiores taxas de recorrência e mortalidade (Oualla *et al.*, 2020).

CÂNCER DE MAMA HE2-LOW

O HER2 é um importante biomarcador prognóstico e preditivo no CM, e o uso de terapia direcionadas anti-HER2 é o padrão de tratamento quando consideramos o tumor HER2 positivo (Hudis, 2007). Tradicionalmente define-se HER2 positivo quando o teste de IHQ apresenta maior expressão de ErbB2 (3+ ou 2+ com ISH amplificado) e HER2-negativo quando avaliação IHQ demonstra pontuação 0, 1+, ou 2+ sem amplificação por ISH (Parise *et al.*, 2009). Os tumores HER2 negativos careciam de benefício clínico com uso de agentes tradicionais de bloqueio da via do HER2. Nos últimos anos, novos medicamentos (ADCs que envolvem terapia anti-HER2) demonstraram importante atividade antitumoral não apenas na população com alta expressão de HER2, mas também em tumores com baixa expressão, destacando o potencial alvo terapêutico neste subgrupo (Modi *et al.*, 2022). Em 2020, Tarantino et al. propôs pela primeira vez o conceito de "HER2-low" em CM, se referindo a baixa pontuação HER2 (IHQ de 1+ ou 2+/ISH negativo) (Tarantino *et al.*, 2020). A **figura 1** ilustra a mudança na interpretação do HER2 em CM, de uma análise dicotômica para três categorias (Zhang; Peng, 2022).

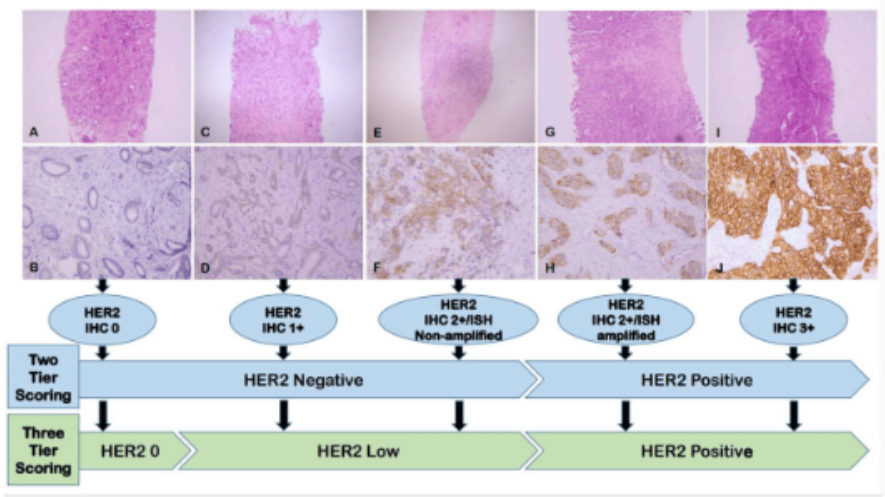

Figura 1: Mudança de pontuação de HER2 do sistema dicotômico para três categorias. *(A , C , E , G , I): Coloração com hematoxilina e eosina de câncer de mama, 40×; (B , D , F , H , J): HER2 IHC correspondente, ×200. Abreviaturas: HER2: receptor 2 do fator de crescimento epidérmico humano; IHQ: Imunohistoquímica; ISH: Hibridização in situ. (*Reproduzido Zhang et al., 2023).

Em relação ao comportamento biológico, os tumores HER2-low são em sua maioria luminais (3). O impacto prognóstico é controverso, e coortes retrospectivas demonstraram curvas de sobrevida sobreponíveis, sugerindo que tumores HER2-*low* não possuem um pior prognóstico ou comportamento biológico distinto quando comparados

aos HER2-zero (Denkert *et al.*, 2021; Tarantino *et al.*, 2020, 2022). Estes resultados não suportam a interpretação do HER2-low como um subtipo biológico distinto de CM (Zhang; Peng, 2022). Embora a nossa compreensão do HER2-low tenha avançado significativamente nos últimos anos, ainda são necessários esforços adicionais, incluindo investigação básica e translacional, para melhor definição do seu papel como biomarcador.

TRATAMENTO NEOADJUVANTE

Diz-se do tratamento neoadjuvante, aquele que é realizado previamente ao procedimento cirúrgico na doença localizada, a fim de possibilitar um procedimento cirúrgico menor (cirurgia conservadora no caso do câncer de mama) e o tratamento de micrometástases (25). Além disto, o uso de tratamento pré-operatório permite avaliar a sensibilidade tumoral à terapia utilizada, observando a resposta tumoral in vivo (Korde *et al.*, 2021). Por este motivo, a análise de tumores submetidos a neoadjuvância permite observar a interação da biologia tumoral com as drogas utilizadas e, desta forma, identificar possíveis biomarcadores para a individualização de tratamento.

Como resultado da neoadjuvância podemos ter progressão de doença (onde há aumento do tamanho tumoral após o tratamento), resposta parcial (quando há redução da doença, mas ainda existem células tumorais viáveis) e resposta patológica completa (pCR), quando há ausência de células neoplásicas no leito tumoral do tecido mamário e linfonodos axilares (26). A carga residual tumoral (RCB) é avaliada patologicamente na peça cirúrgica e baseia-se numa pontuação que leva em consideração dimensão do leito tumoral, porcentagem de células invasivas e in situ, diâmetro da maior metástase nodal e o número de linfonodos comprometidos (**Figura 2**). O RCB, que é validado como importante fator prognóstico (Yau *et al.*, 2022), é classificado como zero, I, II ou III, sendo RCB 0 correspondente a pCR, com melhor resposta tumoral, e RCB III representando piores respostas, com maior carga de doença residual (Symmans *et al.*, 2007). A definição de pCR inclui a ausência total de carcinoma invasor residual, independente da presença de carcinoma in situ (ypT0N0 ou ypTisN0) (Yau *et al.*, 2022).

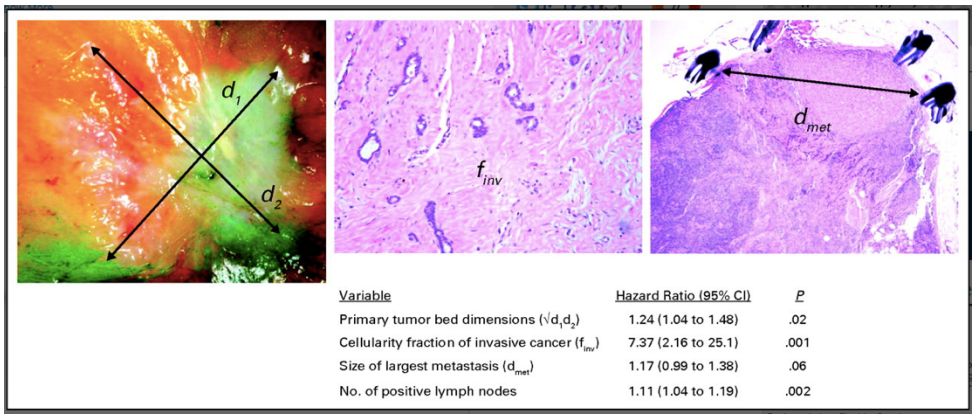

Figura 2: Modelo de avaliação RCB (reproduzida Symmans et al., 2007).

Pacientes com câncer de mama que atingem pCR apresentam melhor sobrevida, embora análise não tenha conseguido validar a pCR como desfecho substitutivo para sobrevida livre de progressão (SLP) e sobrevida global (SG) (28). O impacto prognóstico é maior nos subtipos mais agressivo, triplo negativos e HER2 + (Cortazar *et al.*, 2014). Existem poucos dados publicados até o momento avaliando o impacto do HER2-low na taxa de resposta completa. Aparentemente na população luminal, as pacientes com doença HER2-low tem menores taxas de pCR em relação a população HER2 zero. Enquanto a população triplo negativa não demonstrou essa diferença (Denkert *et al.*, 2021). A distinção de pacientes, respondedores daqueles não respondedores, por meio biomarcadores preditores de pCR pode auxiliar na melhor definição e personalização terapêutica. A decisão terapêutica atual é baseada apenas em achados patológicos tradicionais das células tumorais, como achados anatomopatológicos e de expressão proteica, sem considerar o componente do microambiente tumoral, que envolve células infiltrantes não tumorais.

CÂNCER DE MAMA E MICROAMBIENTE TUMORAL

O sistema imunológico desempenha um papel essencial na iniciação e progressão do câncer (Oualla *et al.*, 2020). A intensidade da resposta imune do tumor influencia na eficácia da terapia do câncer e no prognóstico (García-Teijido *et al.*, 2016). Nesse contexto, a imunoterapia, com uso de inibidores de checkpoints imunes (ICIs), tem emergido como uma estratégia promissora de tratamento. Atualmente, é prática clínica padrão no cenário neoadjuvante em combinação com quimioterapia, bem como

tratamento de primeira linha em pacientes selecionados com doença metastática, nos tumores triplo negativos (Bao *et al.*, 2019; Gennari *et al.*, 2021; Mediratta *et al.*, 2020; Schmid, Peter *et al.*, 2020). Apesar desses avanços recentes, ainda carecemos de biomarcadores que auxiliem na personalização do tratamento da CM em geral, e no subtipo TN em particular. Em comparação com outros subtipos, ele é considerado o mais imunogênico e está frequentemente associado a níveis mais altos de infiltração de células imunes, particularmente linfócitos infiltrantes de tumor (TILs) (Kudelova *et al.*, 2022). A presença de TILs no câncer de mama triplo negativo (CMTN) parece ser um biomarcador prognóstico independente, podendo ser potencialmente utilizado como preditor de resposta a terapias sistêmicas, como quimioterapia e imunoterapia (García-Teijido *et al.*, 2016). Este papel preditivo para estratégias de escalonamento ou descalonamento ainda precisa ser melhor estabelecido.

A microambiente tumoral tem papel fundamental no desenvolvimento e progressão tumoral. As células do câncer interagem ativamente com células não malignas, como células do sistema imunológico, vasculatura linfática, fibroblastos e pericitos (Balkwill; Capasso; Hagemann, 2012) (**Figura 3**). A regulação da resposta imune resulta da interação com diferentes classes de células imunológicas (Li; Tsang; Tse, 2021).

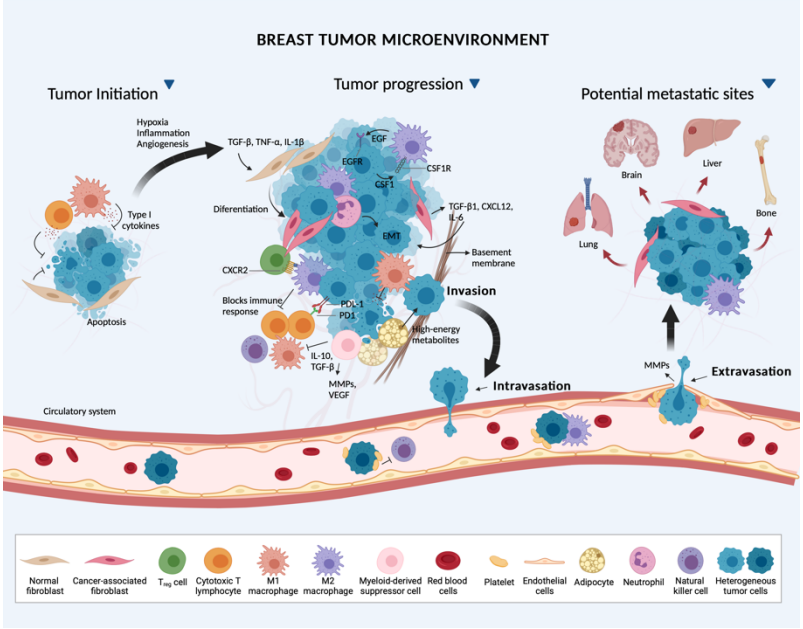

Figura 3: Microambiente tumoral no câncer de mama (reproduzida: Terceiro et al., 2021)

O sistema imunológico pode reconhecer e eliminar células tumorais. Ainda assim, os tumores podem escapar do sistema imunológico e criar um ambiente imunossupressor, favorecendo o desenvolvimento e a progressão da doença (Gonzalez; Hagerling; Werb, 2018). Durante a imunoedição do câncer, o sistema imunológico do hospedeiro molda a evolução tumoral em três fases por meio da ativação de mecanismos imunes inatos e adaptativos (Mittal *et al.*, 2014; Ravelli *et al.*, 2017). Na primeira fase ocorre a eliminação das células cancerosas que destruídas por um sistema imunológico competente (Mittal *et al.*, 2014). Algumas células tumorais conseguem esporadicamente sobreviver à destruição imunológica entrando na segunda fase, de equilíbrio, onde as células apresentam resistência. A fase de escape representa a terceira e última fase do processo, onde células tumorais crescem progressivamente, tornam-se clinicamente evidentes e se estabelece um ambiente imunossupressor (Dunn; Old; Schreiber, 2004; Mittal *et al.*, 2014).

O microambiente influencia significativamente o comportamento maligno e o crescimento do tumor e das células adjacentes. Ele tem a capacidade de reprogramar células vizinhas, podendo neutralizar a progressão de células cancerígenas, e definir a sinalização de vias celulares, impactando os resultados das terapias (42). Portanto, as características do microambiente definem a interação entre sistema imunológico do hospedeiro e células tumorais e, consequentemente, interfere na resposta às terapias (El Bairi *et al.*, 2021). Enquanto respostas específicas e reações inatas podem ser aproveitadas para controlar o desenvolvimento do CM (impedindo a iniciação, progressão e metástase), as células imunossupressoras podem facilitar a evasão imune. O envolvimento do microambiente no desenvolvimento e evolução tumoral está diretamente relacionado com supressão do sistema imunológico, evasão da detecção imunológica e resistência a drogas (Fan; He, 2022). O infiltrado linfocitário é constituído por todas as células de natureza linfocítica que infiltram os tecidos tumorais (Zagami; Carey, 2022). Baseado neste infiltrado inflamatório, três categorias foram definidas para caracterizar os tipos de tumores: deserto imunológico, compreendendo tumores desprovidos de linfócitos; imune excluído, em que os linfócitos estão presentes apenas no estroma peritumoral; e inflamado ("quente"), com alta infiltração de células T (El Bairi *et al.*, 2021). A compreensão da interação tumor-célula imune permite o desenvolvimento de estratégias para regulação imunológica levando a bloqueio de sinais inibitórios e a resposta antitumoral mais eficaz.

Apesar do câncer de mama não ser um tumor essencialmente imunogênico, devido a baixa carga mutacional (Alexandrov *et al.*, 2013), estudos determinam que a presença de infiltrado tumoral linfocitário pode ser um marcador imunobiológico com importante papel prognóstico e preditivo no câncer de mama. Uma das primeiras publicações correlacionando a prevalência de TIL com desfecho favorável de sobrevida data de 1992 (Aaltomaa *et al.*, 1992). A partir daquele momento, diversos estudos exploraram a relação entre maior porcentagem de TIL e melhor prognóstico.

LINFÓCITOS INFILTRANTES NO TUMOR (TIL)

Por definição, TILs são células imunes mononucleares que saem do sangue e ocupam o microambiente tumoral, compreendendo uma mistura de células T citotóxicas e auxiliares, células B, macrófagos, células natural killer e células dendríticas (Balkwill; Capasso; Hagemann, 2012) (**Figura 4**). Os linfócitos T são responsáveis por cerca de 75% dos TILs (Ahn *et al.*, 2015a), e as células CD8+ são abundantes no microambiente.

A presença de células T CD3+ é frequentemente observada representando a população de células T maduras com antígenos co-diferenciados em sua superfície, servindo como marcadores para linfócitos T totais dentro do tecido. A caracterização adicional da população de células infiltrantes revela subconjuntos distintos, incluindo linfócitos T CD8+, linfócitos T auxiliares CD4+ e Tregs CD4+. Os linfócitos T auxiliares CD4+ podem ser categorizados em subtipos Th1 e Th2 com base na secreção de citocinas e participam da imunidade celular e humoral, respectivamente. Os linfócitos T auxiliares CD4+ auxiliam na morte celular mediada por linfócitos T CD8+, contribuindo ativamente para a resposta imune tumoral (Fan; He, 2022). Por outro lado, as Tregs, que constituem 10% de todos os linfócitos T CD4+ no sangue periférico de indivíduos saudáveis e podem corresponder a 30-50% deles nas lesões tumorais, inibem a ativação de linfócitos T CD8+ e CD4+, desempenhando um papel crucial na imunossupressão e angiogênese e potencialmente dificultando a resposta imune antitumoral do organismo. Este acúmulo significativo de subconjuntos Treg imunossupressores pode incluir alta infiltração de células Forkhead box P3 (FOXP3+) (Fan; He, 2022). Essas células de Tregs FOXP3+ podem suprimir respostas imunes contra autoantígenos, dificultar a imunidade antitumoral e são indicadores prognósticos de mau resultado (Lee *et al.*, 2013).

Vários estudos estabeleceram uma correlação entre TILs e prognóstico no CMTN, indicando que o aumento da expressão de linfócitos T CD8+ está associado a melhores desfechos clínicos (Mahmoud *et al.*, 2011; Yazaki *et al.*, 2023). Dados referentes ao câncer de mama luminal são discordantes, tendendo a não mostrar impacto significativo do infiltrado inflamatório em desfechos nesta população (Liang *et al.*, 2018).

Dentro do microambiente tumoral, os TILs devem ser avaliados analisando a presença, densidade e distribuição de células imunes dentro do ambiente do tumor. Isso pode ser feito por meio de várias técnicas, incluindo exame histológico, imunohistoquímica, citometria de fluxo e perfil de expressão gênica (Hendry *et al.*, 2017).

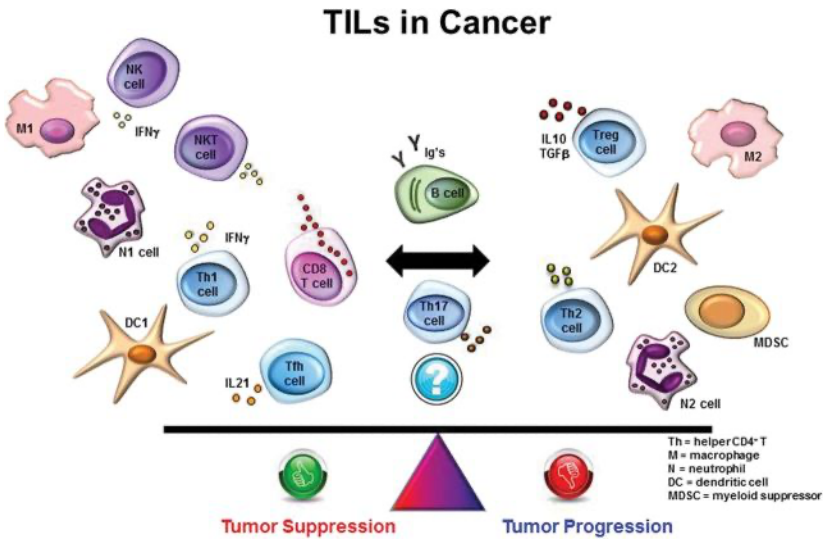

Figura 4: TILs em câncer (reproduzida: Salgado et al., 2015)

O *TILs working group* é um grupo cooperativo de pesquisadores que desenvolveu diretrizes para padronizar e permitir maior reprodutibilidade da avaliação de TILs. Em resumo, as regras gerais para avaliação dos TILs vão desde orientações pré-analíticas, como preparo e fixação da lâmina (a espessura ideal da lâmina deve ser de 4 a 5 µm de tecido fixado em formalina e embutido em parafina) até questões analíticas conforme descrito na **figura 5** (Salgado *et al.*, 2015). Os TILs devem ser avaliados dentro dos limites do componente invasivo de um tumor. No entanto, apenas o componente estromal deve ser considerado, pois não são influenciados pela densidade celular ou por padrões de crescimento tumoral, permitindo maior acurácia na avaliação. As áreas ocupadas por células de carcinoma não devem ser incluídas na área total avaliada. TILs em áreas com artefatos de esmagamento, hialinização regressiva e necrose, e aqueles em um local de biópsia prévia, devem ser excluídos da consideração. A porcentagem de TILs presentes

em um determinado tumor deve ser calculada dividindo-se a área ocupada por células inflamatórias mononucleares pela área total do estroma tumoral. O valor deve ser dado como uma porcentagem e é uma variável contínua. Se o percentual de TILs for incerto, o caso deve ser discutido com um segundo patologista (Salgado *et al.*, 2015). A padronização da avaliação permite maior reprodutibilidade e concordância (Denkert *et al.*, 2016) e, portanto, facilita o desenvolvimento de estudos avaliando TILs como potencial biomarcador de impacto na prática clínica.

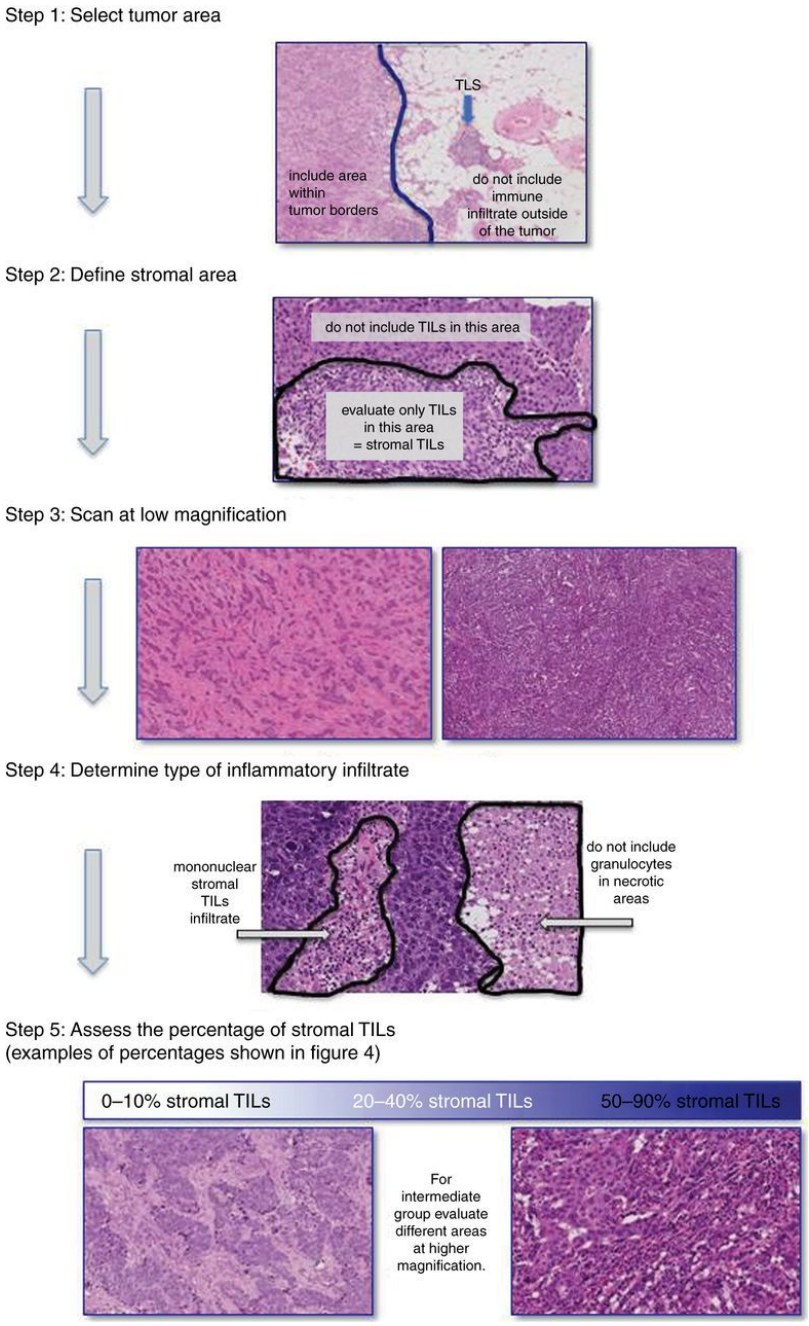

Figura 5: Cinco passos para avaliação de TIL estromal no câncer de mama (reproduzida Salgado et al., 2015)

PREVALÊNCIA DE TILs NO CÂNCER DE MAMA

O subtipo molecular do CM afeta a interação com o sistema imunológico. CMTN é mais frequentemente infiltrado por TILs do que tumores luminais (El Bairi *et al.*, 2021). Embora devamos valorizar as recomendações do *International TILs Working Group*, não há consenso sobre o ponto de corte ideal para determinar TIL elevado ou baixo (Salgado *et al.*, 2015). Um grupo alemão realizou um estudo avaliando o valor preditivo e prognóstico das TILs e definiu três grupos: TILs baixo (0-10%), intermediário (11-59%) e alto (≥ 60%) (El Bairi *et al.*, 2021). Determinar um ponto de corte específico é uma questão complexa, pois o impacto da expressão de TILs, tanto no prognóstico quanto na importância preditiva desse biomarcador, parece ser linear.

Alguns estudos usam o termo câncer de mama predominantemente linfocítico (LPBC) para definir tumores que têm uma densidade de TILs maior ou igual a 50-60% (Adams *et al.*, 2014a; Denkert *et al.*, 2010, 2018; Gao *et al.*, 2020; Pruneri, Giancarlo *et al.*, 2016; Tian *et al.*, 2016), considerados como tendo "TILs alto". Outros estudos utilizaram diferentes pontos de corte para avaliar o impacto dos TILs. É importante enfatizar que, embora o CMTN seja considerado o mais imunogênico entre todos subtipos, a maioria tem uma densidade baixa ou intermediária de TILs (Sukumar *et al.*, 2021). A **Tabela 2** resume alguns estudos recentes que avaliaram a prevalência de TILs estromais (sTILs) em CMTN inicial (subtipo que apresentam maior número de evidências com impacto em desfechos). Nem todos os estudos citados seguiram as regras de avaliação definidas pelo grupo de trabalho de TILs, o que dificulta análises comparativas. Apesar disso, os estudos concordam quanto à prevalência de sTILs nas populações estudadas**.**

Autor	**Número de pacientes CMTN (n)**	**Limiar definidor "TILs alto"**	**Prevalência**
Kimura et al., 2023	54	50%	Alt 27.80% Baixo: 62.20%
Agarwal et al., 2023	108	60%	Alto: 15.7% Interm (11-59%): 29.9% Baixo (≤ 10%): 34.4%

Candelaria et al., 2022	284	20%	Alto: 32% Baixo: 68%
Jong et al., 2022	441	75%	Alto: 21% Interm (31-74%): 27% Baixo (≤ 30%): 52%
Stecklein et al., 2023	110	20%	Alto: 51% Baixo: 49%
Sharma et al., 2022	117	30%	Alto: 47% Baixo: 53%
Gluz et al., 2022	336	60%	Alto: 13% > 10%: 33,8% ≤ 10%: 66,2%
Loibl et al., 2019	174	60%	Alto: 14% Interm (11-59%): 48% Baixo (≤ 10%): 38%
Denkert et al., 2018	906	60%	LPBC*: 30%
Galvez et al., 2018	86	50%	Alto: 50%
O'Loughlin et al., 2018	75	50%	LPBC*: 12%
Adams et al., 2014	481	50%	LPBC* (≥ 50%): 5% Interm (11-49%): 75% Baixo (≤ 10%): 20%
Pruneri et al., 2016	897	50%	LPBC*: 21,9%
Pruneri et al., 2016	647	50%	LPBC*: 18%
Tian et al., 2016	425	50%	LPBC*: 3,5%
Denkert et al., 2015	314	60%	LPBC*: 28,3%
Denkert C et al., 2010	1058	60%	Alto: 61%

*LPBC: câncer de mama predominantemente linfocitário

Tabela 2 – Prevalência de TILs em CMTN

TILs COMO FATOR PROGNÓSTICO

Fator prognóstico está relacionado com característica que determina impacto positivo ou negativo na evolução clinica da doença independentemente da estratégia terapêutica utilizada. Dentre os marcadores prognósticos relacionados ao câncer de mama, a idade ao diagnóstico inferior a 35 anos e características patológicas, como maior extensão tumoral, alto grau histológico, invasão linfovascular, receptores hormonais negativos e amplificação de HER2, estão associados a pior desfecho de sobrevida (Nappi; Carrillo, 2008).

Neste contexto, a importância da resposta imune e o papel do microambiente tumoral no desenvolvimento e controle tumoral destacam o uso de TILs como biomarcador prognóstico. Uma metanálise de aproximadamente 13.100 pacientes demonstrou associação entre alta densidade de TILs e melhor prognóstico no CM, incluindo melhor SLP e SG (Wang *et al.*, 2016). Diversos estudos demonstram melhora da SLP e da SG tanto entre pacientes com TNBC, quanto tumores HER2+ com o aumento da infiltração linfocitária (Schlotter *et al.*, 2017), apesar de existirem estudos que não encontram diferença estatística na população HER2+ (Hida *et al.*, 2016). Na doença RH positivo alguns estudos exploram o tema e tem resultados conflitantes, sendo em sua maioria negativos para correlação TILs com desfecho de resposta ao tratamento ou sobrevida nesta população de pacientes (Liang *et al.*, 2018). Contrariando os achados relatados acima, Watanabe et al, sugere que TILs baixo na doença residual em pacientes RH positivo foi relacionado com melhor sobrevida livre de recorrência, podendo ser considerado marcador prognóstico (Watanabe *et al.*, 2018). Outro estudo demonstrou interessantemente que o aumento de cada 10% de TILs em pacientes com tumores luminais foi associado a risco 10% maior de morte (Schlotter *et al.*, 2017). Os dados publicados até o momento destacam o impacto do TILs no subtipo triplo negativo de forma consistente, no entanto nos demais subtipos (HER2 positivo e luminal HER2 negativo) o impacto permanece controverso.

A população celular no microambiente tumoral é impactada pelo tratamento sistêmico, tornando-se mais ou menos imunogênico. Neste contexto é importante ressaltar a característica dinâmica da densidade de TILs na amostra tumoral durante a evolução da doença. O estudo NeoTRIP, que avaliou o uso de imunoterapia no tratamento neoadjuvante do CMTN, demonstrou aumento de TILs após apenas 1 ciclo de terapia sistêmica neoadjuvante em amostras pareadas (Bianchini *et al.*, 2022). O valor prognóstico foi avaliado tanto em doenças iniciais quanto residuais após terapia neoadjuvante. A presença e maior densidade de TIL na doença residual foi associada a melhor prognóstico em pacientes com CMTN (Dieci *et al.*, 2014; Pons *et al.*, 2023), enquanto a baixa densidade de TILs na doença residual foi identificada como fator de pior desfecho, estando associado a menor SLP na população TN (Ochi *et al.*, 2019). O benefício de sobrevida com aumento dos níveis de infiltração após o uso de quimioterapia neoadjuvante (NACT) foi demonstrado em uma meta-análise que agrupou estudos que

realizaram análises pareadas da densidade de TILs antes e depois da terapia neoadjuvante (ZHOU Q.; WANG, B.Y.; ZHAO, S.D.; YANG, J., 2021).

Conforme descrito na **Tabela 3**, vários estudos avaliaram o papel prognóstico da presença e densidade de TILs nos desfechos de sobrevida de pacientes com CMTN em estágio inicial. Embora alguns estudos tenham sido realizados antes da padronização da avaliação das TILs, a correlação positiva entre TILs e melhor prognóstico tem sido consistente neste subtipo de pacientes. Os achados confirmaram o comportamento linear onde para cada 10% de aumento na densidade de TILs estromais, há uma redução no risco do evento (recidiva ou morte) na ordem de 5 a 20%. Dois estudos (de Jong *et al.*, 2022; Pruneri, Giancarlo *et al.*, 2016) descrevem excelentes resultados de sobrevida, com taxas de SG em 10 anos de cerca de 95% em pacientes com CMTN inicial e TILs alto. O impacto foi confirmado em análise conjunta de 2148 pacientes submetidos a tratamento adjuvante para CMTN (Loi *et al.*, 2013) e mostrou que os pacientes com TILs alto estágio II tiveram melhor prognóstico e melhor SG em comparação com pacientes no estágio clínico I com menor infiltração de TILs (Loi *et al.*, 2022). Esse resultado reforça o conceito de que as características biológicas devem ser melhor compreendidas e avaliadas para a classificação de risco dos pacientes com CM. Eles sugerem que os TILs podem ser superiores ao sistema de estadiamento anatômico usado na oncologia mamária por décadas.

Autor	n	Desfechos	Resultados
Agarwal et al., 2023	108	DFS* e SG+	sTIL alto associado a melhor DFS* e SG+
Jong et al., 2022	441	SG+ e DRFS‡	A cada 10% sTIL, redução do risco de evento em 19% (HR 0.81)
Jong et al., 2020	481	SG+ e DRFS‡	A cada 10% sTIL, redução do risco de evento em 17% (HR 0.83)
Loi et al., 2022	2148	IDFS§, DDFS¶ e SG+	A cada 10% sTIL, redução do risco de evento em 13 to 17% (IDFS§ HR 0.87; DDFS¶ HR 0.83; SG+ HR 0.84)
Gluz et al., 2022	336	IDFS§, DDFS¶ e SG+	sTIL alto associado com melhor IDFS§, DDFS¶ e SG+
Gao et al., 2020	18170	DFS* e SG+	sTIL alto associado com melhor DFS* (HR 0.907) e SG+ (HR 0.869)
Park et al., 2019	476	IDFS§, DDFS¶ e SG+	sTIL alto Estadio I: 5 anos IDFS§ 91%; 5 anos DDFS¶ 97%; 5 anos SG+ 98%

Denkert et al., 2018	906	DFS* e SG+	A cada 10% sTIL redução do risco de evento em 7 to 8% (DFS* HR 0.93; SG+ HR 0.92)
Leon-Ferre et al., 2018	605	IDFS§ e SG+	Menor TIL associado com pior IDFS§ e SG+
Pruneri et al., 2016	897	DFS*, DDFS¶ e SG+	A cada10% TIL, melhora na DFS*, DDFS e SG+
Pruneri et al., 2016	647	BCFIΩ, DFS*, DRFI♠ e SG+	A cada 10% TILs, redução do risco de evento em 11 to 17% (BCFIΩ HR 0.87; DFS* HR 0.89; DRFI♠ HR 0.84; SG+ HR 0.83)
Tian et al., 2016	425	DFS*, DDFS¶ e SG+	A cada 10% sTIL, redução do risco de evento em 5% (recorrência ou morte)
Dieci et al., 2015	199	SG+	SG 10anos: TIL alto 89%, TIL baixo 68%
Adams et al., 2014	481	DFS*, SG+ e DRFI♠	A cada 10% sTIL, redução do risco de evento em 14%
Dieci et al, 2014	278	MFSτ e SG+	A cada 10% sTIL, redução do risco de evento em 21% (metástase ou morte)
Loi et al., 2013	256	DFS* e SG+	A cada 10% sTIL, redução do risco de evento em 15-17%

Tabela 3: TILs como biomarcador prognóstico em CMTN

*DFS: sobrevida livre de doença; +SG: Sobrevida global; ‡DRFS: sobrevida livre de recorrência a distancia; §IDFS: sobrevida livre de doença invasiva; ¶DDFS: sobrevida livre de doença a distância; ΩBCFI: intervalo livre câncer de mama; ♠DRFI: intervalo livre de recorrência a distancia; τMFS: sobrevida livre de metástase. sTIL: TIL estromal

A correlação prognóstica permaneceu evidente em uma coorte de pacientes que não realizaram tratamento quimioterápico. Park et al., avaliaram aproximadamente 480 pacientes com doença precoce, submetidos exclusivamente a tratamento local e não expostos a nenhuma terapia sistêmica, e mostraram uma impressionante SG a longo prazo naqueles pacientes com alta porcentagem de sTIL (≥ 30%) (Park *et al.*, 2019). No mesmo contexto, uma coorte holandesa avaliou mais de 400 pacientes com idade < 40 anos e portadores de doença negativa para linfonodos, não submetidos a nenhum tratamento sistêmico. Pacientes com TILs altos (mais de 75%) tiveram melhor prognóstico e um risco reduzido de recorrência à distância e morte do que aqueles com TILs baixos (De Jong *et al.*, 2020). A incidência de metástases ou morte em 15 anos de foi de apenas 2,1%, e comparada favoravelmente aos 38% observados na coorte TIL baixa (aqueles com menos de 30%) (de Jong *et al.*, 2022). Esses dados levantam a hipótese do uso de TILs na prática clínica para orientar estratégias de descalonamento para pacientes com doença precoce e "TILs alto".

Além da análise quantitativa de TILs, a avaliação do subtipo de células T presente no microambiente é de extrema relevância e tem sido abordada em diversos estudos. Como mencionado, diferentes subtipos de células T podem desempenhar um papel ativador ou supressor na carcinogênese, progressão e resposta ao tratamento, a depender da subpopulação celular identificada (Liu *et al.*, 2011). Um microambiente com infiltração predominantemente de células T CD8+ e CD4+ tem sido correlacionada com bom prognóstico em vários tipos de câncer (Ahn *et al.*, 2015b; Gu-Trantien *et al.*, 2013; Mahmoud *et al.*, 2011). Em contraste, uma maior infiltração com Tregs (associada à imunossupressão) determina pior prognóstico e associa-se a tumores mais agressivos, características clínico patológicas desfavoráveis, recidivas tardias e pior sobrevida (Bates *et al.*, 2006; Kim *et al.*, 2014; Liu *et al.*, 2014; Ravelli *et al.*, 2017). A correlação entre Treg infiltrante e linfócitos T CD8+ demonstrou ter significado prognóstico e preditivo, dependendo da localização e densidade de cada subpopulação (Ravelli *et al.*, 2017).

A análise molecular da atividade das células imunes dentro do microambiente tumoral também foi avaliada em diversos estudos. Um estudo com 279 paciente com câncer de mama HER2 negativo encontrou correlação entre TILs e índice de atividade celular imune por meio expressão gênica por sequenciamento de RNA. Neste estudo TILs resultou em melhor papel preditivo de resposta, enquanto o índice teve melhor papel preditivo de sobrevida (Fasching *et al.*, 2023). No mesmo contexto, análise de perfil de expressão gênica avaliou superexpressão de 22 genes associado a TILs alto determinando uma assinatura genômica que foi associada com TILs estromais e intratumorais avaliados por patologia. O impacto prognóstico e preditivo não foi encontrado, sugerindo a necessidade de mais estudos para validação e estratificação da assinatura genômica (Kochi *et al.*, 2018).

TILs COMO FATOR PREDITIVO

Fator preditivo em oncologia é definido por característica tumoral que determina o tipo resposta a terapêutica instituída. A abordagem neoadjuvante é considerada um cenário ideal para avaliar a interação entre drogas antineoplásicas e resposta tumoral (Ravelli *et al.*, 2017). Evidências mostram que níveis mais altos de TILs dentro do microambiente determinam melhor resposta imune e estão associados a uma maior probabilidade de atingir pRC após uso de NACT (Ono *et al.*, 2012) e pRC parece ser

relacionada com melhores desfechos em longo prazo e taxas de sobrevida como descrito acima.

O estudo TILGen confirmou papel preditor de resposta em tumores TN e HER2+ com LPBC, que denotaram taxa de pCR de 66,7% comparado a 32,8% no grupo com baixo TILs (Würfel *et al.*, 2018). Valor preditivo de TILs baixo em 'não respondedores' a NACT foi avaliado em coorte de 991. Densidades de TILs >= 10% e >=17,5% foram consideradas fator preditor independente de baixa taxa de 'não respondedores' em tumores luminais e TN, respectivamente (Qian *et al.*, 2023). Em metanálise com 3215 pacientes, TILs foi preditor de pCR em CMTN (Odds ratio - OR = 2,49, IC 95%: 1,61-3,83), HER2 positivo (OR = 5,05, IC 95%: 2,86-8,92), mas não em tumores luminais (OR = 6,21, IC 95%: 0,86-45,15) (Mao *et al.*, 2014). Benefício do uso de antraciclina na terapia neoadjuvante alcançou taxa de resposta de até 74% em paciente com TILs alto (West *et al.*, 2011). A adição do uso de platina no regime neoadjuvante também foi avaliada. O aumento de TILs nos subtipos TN e HER2+ foi preditor de benefício da adição de carboplatina a neoadjuvância (Schlotter *et al.*, 2017). O estudo GeparSixto, que incluiu pacientes com CMTN, demonstrou associação positiva para pCR em pacientes com predomínio linfocitário. A chance de pCR foi de 3,71 vezes superior em relação à população 'não LPBC'. Neste estudo, os pacientes CMTN com LPBC tratados com sal de platina tiveram uma taxa de pCR de 75% (Denkert *et al.*, 2015). Outro estudo demonstrou pCR numericamente mais alta, mas sem diferença estatística, entre regimes baseados ou não em platina no subgrupo TIL alto (Abdullaeva *et al.*, 2023). Embora o CMTN tenha maior número de evidências quanto ao impacto de TILs na evolução da doença, alguns estudos demonstraram impacto positivo também na população luminal (RH positivo). Estudo gerador de hipótese analisou 111 casos de câncer de mama luminais submetidos a neoadjuvância. Neste estudo, a densidade de sTILs pareceu influenciar na resposta molecular com redução de KI67 (Dieci *et al.*, 2017). Outro estudo avaliou o microambiente imunológico em tumores luminais por meio de análises pareadas pré e pós quimioterapia. A análise demonstrou queda na expressão de sTIL e CD8+ pós tratamento e níveis elevados de marcadores teciduais de linfócitos e macrófagos se correlacionaram com resposta favorável ao tratamento nesta população, sem impacto prognóstico (Waks *et al.*, 2019). Diversos estudos têm demonstrado uma relação linear entre o nível de TILs e as taxas de resposta clínica e patológica especialmente em pacientes com CMTN submetidos ao neoadjuvância, conforme resumido na **Tabela 4**.

Percebemos que em alguns estudos há importante discrepância entre as taxas de pCR quando comparadas populações de TILs alto e baixo, conforme critérios de cada análise.

Autor	N CMTN	Limiar para "TILs alto"	pCR TILs alto	pCR TILs baixo
Adbullaeva et al., 2023	132	40%	63.3%	46.1%
Agarwal et al., 2023	108	60%	52.90%	21.10%
Sharma et al., 2022	117	30%	78%	45%
Gluz et al., 2022	336	60%	59.30%	29%
Bianchini et al., 2020	260	40%	71%	-
Schmid et al., 2020	60	40%	74-78%	
Denkert et al., 2018	906	60%	50%	31%
Herrero-Vicent et al., 2017	164	40%	88%	9%
Tomioka et al., 2017	32	30%	30%	21%
Hida et al., 2016	48	50%	63%	17%
Denkert et al., 2015	314	60%	75%	-
Denkert et al., 2010	1058	60%	40%	7%

Tabela 4: TILs como biomarcador preditivo no CMTN

Heterogeneidade nos resultados obtidos sugerem que as subpopulações de células T envolvidas no infiltrado inflamatório no microambiente podem ter impacto na resposta ao tratamento. Isso está de acordo com o racional da função imunomoduladora do infiltrado imune e com o impacto prognóstico discutido anteriormente. Avaliação de escore imune com a determinação de distribuição espacial de densidade de células CD3 e CD8 demonstrou papel prognóstico significativo relacionado a pCR pós NACT (Rapoport *et al.*, 2022). A razão TIL CD8+/FOXP3+ pode ser um biomarcador útil para prever a resposta ao tratamento à terapia neoadjuvante em subtipos agressivos de câncer de mama, como CMTN e HER2 (Asano *et al.*, 2016; Miyashita *et al.*, 2015), bem como altos níveis de TILs CD4+ e CD8+ estão associados a uma maior probabilidade de obter pCR em CMTN (Rao *et al.*, 2017; Ravelli *et al.*, 2017). Análise de subtipos de células T envolvidas foi consistente com outros estudos demonstrando fator preditivo para maior taxa de pCR com aumento de infiltrado CD8 e menor taxa de pCR com maior infiltrado FOXP3+ (Mao *et al.*, 2014).

A análise da relação de TILs com preditor de resposta também foi avaliada por meio da exploração de método de imagem correlacionado a achados patológicos. Nível de TIL intemerdiário (10 a 50%) e alto (>50%) parece estar relacionado com maior

captação de fluorodeoxyglucose (FDG) no positron-emission tomography / computed tomography (PET/CT) em tumores triplo negativos e HER2 positivos quando comparado a tumores com TILs <10% (Murakami *et al.*, 2020). Foi desenvolvido um score de TIL baseado na captação de FDG no PET/CT. A taxa de pCR foi de 20% e 44,2% nos grupos de baixo e alto PET-TIL score, respectivamente (SASADA *et al.*, 2020).

Focando na população triplo negativa, o impacto dos TILs também foi avaliado em tumores tratados com imunoterapia em combinação com quimioterapia. Uma correlação entre TILs estromais e pCR foi demonstrada, sugerindo um valor preditivo para TILs em resposta à ICI. Esses dados devem ser considerados com cautela, pois muitas vezes resultam de análises exploratórias (Valenza *et al.*, 2023). Estudos avaliando o uso de Pembrolizumabe no contexto da terapia neoadjuvante nos estudos KN-173 (Schmid, P. *et al.*, 2020), I-SPY (Campbell *et al.*, 2019) e NeoPACT (Sharma *et al.*, 2022), exploraram a associação de TILs alto e pCR. Uma análise do estudo NeoPACT hipotetiza que mecanismos de resposta dependentes de linfócitos dominam a resposta terapêutica em tumores sTIL-altos. Enquanto, em tumores sTIL-low, a resposta pode estar mais relacionada ao índice de proliferação, identificado por assinaturas de expressão gênica de proliferação, sem impacto na população de TILs (Stecklein *et al.*, 2023). Estudos avaliando outros ICIs, como Atezolizumabe e Durvalumabe, também demonstraram uma associação positiva entre TILs e taxas de pRC. O estudo NeoTRIP encontrou uma taxa de pCR de 74,1% na população com TILs > 40% antes do ciclo 2 (Bianchini *et al.*, 2020). O estudo NeoMONO avaliou biomarcadores que pudessem predizer resposta precoce ou resistência à imunoterapia em uma coorte de 101 pacientes. Dados preliminares mostraram que TILs alto no início ou TILs aumentados após 2 semanas de monoterapia com Atezolizumabe foram associados a maior probabilidade de pRC, com taxas em torno de 85% (Erber *et al.*, 2023). O estudo GeparNuevo avaliou a combinação de Durvalumab ou placebo com quimioterapia e a densidade TIL foi critério de estratificação. A população com TILs altos (>60%) apresentou maiores taxas de pCR em ambos os grupos (Loibl *et al.*, 2021).

Em resumo, os TILs estão emergindo como um valioso biomarcador prognóstico e potencialmente preditivo em CMTN em estágio inicial, fornecendo informações importantes sobre o cenário imunológico do tumor e a resposta à terapia. Alguns consensos internacionais, como ESMO e St. Gallen (Burstein *et al.*, 2021; Cardoso *et al.*, 2019), endossam a relevância prognóstica dos TILs e defendem a inclusão rotineira da

contagem de TILs no relatório patológico. No entanto, falta validação e dados prospectivos para apoiar o uso desse biomarcador para otimizar e individualizar nossas estratégias terapêuticas atuais. Embora o impacto prognóstico seja evidente e consistente em vários estudos, até o momento não há suporte na literatura para o uso dos TILs como biomarcador para omitir ou escalar o tratamento sistêmico (Yazaki *et al.,* 2023).

TILs EM CÂNCER DE MAMA HER2 LOW

O impacto de TILs, especialmente nos tumores triplo negativo, é evidente e consistente em diversos estudos. Alguns estudos também demonstram o mesmo beneficio de maior infiltração linfocitária em desfecho clínico favorável na população HER2 positivo. Já o impacto na população luminal é bastante controverso conforme discutido previamente. No entanto raros são os dados de TILs na população HER2-low. Visando uma terapêutica individualizada e focando na seleção de pacientes, a combinação de biomarcadores (HER2-low e TIL, por exemplo) pode representar ponto fundamental na tomada de decisão. A revisão da literatura resultou em apenas 5 estudos avaliando o papel da infiltração de células linfocitárias no câncer de mama HER2-low, todos estudos foram publicados no último ano. Os estudos focaram nas características clínicas da população e distribuição de TILs avaliando seu impacto prognóstico. Análise retrospectiva brasileira avaliou 198 pacientes com câncer de mama inicial HER2 negativa não submetidos a tratamento sistêmico e não encontrou diferença significativa na densidade de TILs (Fernandes *et al.,* 2023). Outra coorte de 973 pacientes chineses, onde 63,2% eram tumores de HER2-low, também não demonstrou diferença na densidade de TILs entre a população HER2-zero versus low, e ambos subgrupos foram diferentes em relação ao HER2 positivo. Incrementos de 10% de TILs estromais na amostra foram relacionados a desfecho de sobrevida favorável na população HER2-low (Lu *et al.,* 2023). Apenas uma coorte de 529 pacientes, enriquecida por população luminal (57,6%), encontrou diferença significativa com menor densidade de TILs na população HER2-low. Neste estudo a população HER2-low também apresentou perfil de expressão gênica relacionado a pior prognóstico e depleção da imunidade, quando comparado a população HER2 zero. O estudo sugere menor resposta imune em tumores HER2-low quando comparados a HER2-zero (van den Ende *et al.,* 2022). Apesar da maior infiltração de TILs estar relacionado a características de maior risco (tumores maiores de 2cm, ki67 > 25%, maior

estagio patológico), análise determinou que a doença HER2-low com TILs alto, considerado densidade > 10%, apresentava redução do risco de recorrência ou metástase em coorte de 1763 pacientes, especialmente na população RH positivo (Sun *et al.*, 2023). E por fim, destacando a escassez de estudos e necessidade maior compreensão do impacto deste "biomarcador", uma análise de 293 pacientes não encontrou impacto prognóstico relacionado ao status HER2-low ou a expressão de TILs (Yue *et al.*, 2023).

A prevalência e intensidade de TILs nas populações HER2 zero e Low estão resumidas na Tabela 5. A maioria dos pacientes tem TILs abaixo de 10% com achados numericamente semelhantes entre HER2 zero e low.

Estudo	**País**	**n total**	**Estratificação TILs**	**HER2-zero**	**HER2-low**	**HER2-positivo**
Fernandes et al., 2023	Brasil	198	Baixo <= 10%	**86,20%**	**70,50%**	43,50%
			Intermediário 11 a 40%	11,30%	25,50%	47,80%
			Alto > 40%	2,40%	3,90%	8,70%
Lu et al., 2023	China	973	Baixo <= 10%	**63,50%**	**65,50%**	43,50%
			Intermediário 11 a 50%	27%	29,10%	41,80%
			Alto > 50%	9,50%	5,40%	14,70%
van den Ende et al., 2022	Holanda	529	Baixo <= 10%	**70,30%**	**66,90%**	0
			Intermediário 11 a 60%	20,20%	5,90%	0
			Alto > 60%	3,20%	3,40%	0
			Desconhecido	6,30%	23,70%	0
Sun et al., 2023	China	1763	Baixo <= 10%	**51,30%**	**39,90%**	27,70%
			Alto > 10%	48,70%	60,10%	72,30%

Tabela 5: Prevalência e intensidade de TILs nas populações HER2 zero e Low

Baseado nos estudos citados é possível concluir que os tumores HER2-low tem mais semelhanças com tumores HER2 zero, do que com tumores HER2 positivos. A similaridade entre os tumores HER2 negativo (zero ou low) é consistente nos diferentes estudos, e há diferenças significativas nas características clínicas em relação a população HER2 positivo. Da mesma forma, o papel prognóstico dos TILs nesta população parece estar relacionado a outros fatores (como status de receptor hormonal, por exemplo) e não ao status HER2. A baixa expressão de HER2 atualmente é um alvo terapêutico aos novos ADCs e até o momento nenhum estudo demonstrou que a expressão de HER2-low isoladamente seja fator prognóstico ou preditivo. A revisão da literatura não encontrou estudos avaliando a relação de TILs como preditor de resposta a quimioterapia neoadjuvante nesta subpopulação de pacientes.

3 MARCO CONCEITUAL

O conceito de câncer de mama HER2 negativo atualmente inclui tumores HER2-zero e HER2-low. O subtipo HER2-low surge como potencial alvo terapêutico frente ao advento de terapias ativas em tumores com baixa expressão de HER2. A estimativa do microambiente tumoral imune, por meio da avaliação do infiltrado linfocitário tumoral (TIL), tem demonstrado importante papel como biomarcador preditor de resposta a neoadjuvância e prognóstico nos subtipos triplo negativo e HER2 positivo. Existem poucos dados avaliando TIL na população HER2-low, que é o objetivo do presente estudo.

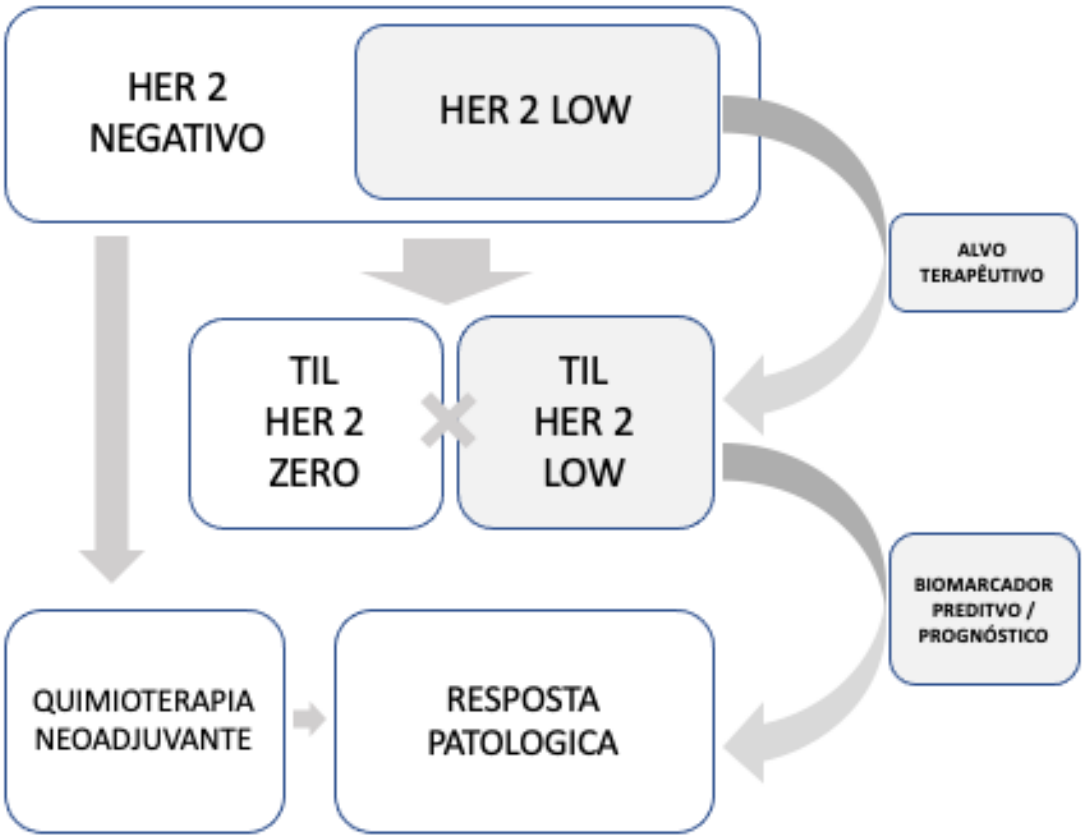

Figura 6 – Marco conceitual

4 JUSTIFICATIVA

A biologia do câncer de mama envolve características histopatológicas específicas das células tumorais e também das células infiltrantes. Sabe-se que o ambiente estromal tem papel significativo no crescimento do tumor, desenvolvimento de metástases e resistência à terapia. Desta forma, a compreensão deste microambiente, incluindo células imunes, permite identificação de biomarcadores com impacto na prática clínica.

Com o aparecimento de novas moléculas com atividade em tumores com baixa expressão de HER2, previamente considerados HER2 negativos, surgiu o conceito de HER2-low para representar este subgrupo. O status de HER-low atualmente é terapêutico, e determinar a biologia tumoral desta subpopulação é de fundamental importância. O estudo de amostras submetidas a tratamento neoadjuvante possibilita análise tumoral previamente a exposição a qualquer tratamento e também dados objetivos de resposta, podendo assim avaliar tanto impacto prognóstico quanto preditivo dos achados.

Portanto, a análise quantitativa da porcentagem de infiltrado linfocitário tumoral em tumores de mama HER2-negativos (zero e low) foi planejada para trazer novos achados patológicos que pudessem contribuir para a compreensão do comportamento do microambiente imune do câncer de mama neste subgrupo de pacientes e auxiliar na individualização das decisões terapêuticas.

5 OBJETIVOS

5.1 Objetivo Principal

Descrever a porcentagem de linfócito infiltrante tumoral (TIL) em tumores HER2-low em pacientes submetidas a quimioterapia neoadjuvante e comparar com a porcentagem nos tumores HER2-zero.

5.2 Objetivos Secundários

5.2.1 Avaliar a porcentagem de TILs quanto ao receptor hormonal;

5.2.2 Correlacionar a porcentagem de TILs com grau tumoral;

5.2.3 Correlacionar a porcentagem de TILs com taxa de resposta patológica após a quimioterapia neoadjuvante;

- Avaliar o impacto da densidade TIL <20% e >=20% como fator preditivo de resposta patológica tumoral;
- Avaliar o impacto da densidade de TIL <50% e >=50% como fator preditivo de resposta patológica tumoral;

5.2.4 Avaliar a concordância entre 2 patologistas na análise de TILs.

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7 ARTIGOS

7.1 ARTIGO - RESULTADOS

Impact of tumor-infiltrating lymphocytes expression and HER2-low status in a cohort of HER2-negative breast cancer patients treated with neoadjuvant chemotherapy

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ABSTRACT

Background: Breast cancer can be categorized as HER2 positive, triple negative (TN) or luminal (with estrogen receptor-positive). The “HER2-negative” term includes tumors with no expression (HER2-zero) and tumors with low expression of the receptor (HER2-low). Actually, tumor cell characteristics define the treatment of breast cancer. In addition to the intrinsic features, the tumor microenvironment also plays an important role in the process of carcinogenesis. The presence of stromal tumor-infiltrating lymphocytes (TIL) was identified as a prognostic and predictive factor in breast cancer. The integration of novel biomarkers into personalized decisions remains an unmet need. In this context, this study proposes to assess the correlation between lymphocyte infiltration and clinical characteristics in the HER2-negative population, focusing on the HER2-low “subtype”.

Methods: Retrospective study that enrolled HER2-negative invasive breast cancer patients submitted to neoadjuvant chemotherapy. Tumor samples were required to assess the TIL density. High TIL was categorized using two distinct thresholds (>= 20% and >=50%) arbitrarily. The pathological response was based on residual cancer burden (RCB). The main objective was to describe the percentage of TIL in HER2-low tumors and compare it with HER2-zero tumors. And then assess the TIL density regarding the hormonal profile, tumor grade and pathological response rate. Furthermore, to assess agreement between 2 pathologists when analyzing TILs.

Results: Data from 119 patients were analyzed. Among them, 34 had HER2-zero (28,5%), and 85 with HER2-low (71,4%). There was no significant difference in TIL expression (p=0,1367) or pathological response (p=0,1168) related to HER2 status (zero vs. low). The median TIL was 10% in all samples as well as in the HER2-low population, while in the HER2-zero population, the median was 15%. Most luminal tumors had low TILs (<20%), while TN predominantly had high TILs, regardless of HER2 status. Pathological complete response (pCR - RCB zero) had median TILs of 37.5%, while in RCB I, II, and III the medians were 15%, 10%, and 5%, respectively. Notably, a significant distinction (p=0.0033) was observed using a 20% threshold, with 75% of patients that achieved pCR exhibiting high TILs whereas 82.9% of patients with poor response to treatment (RCB III) demonstrated low TILs. We found good agreement among both pathologists, being 84.4% when the threshold of TILS was 20%, and 96.9% when we used a threshold of 50%, with a Kappa coefficient of 0.6825 and 0.7838, respectively.

Conclusion: No significant difference was identified in the distribution of clinicopathological characteristics and density of TILs between the HER2-low and HER2-zero subgroups. Low HER2 expression alone did not confer a predictive role in response to neoadjuvant chemotherapy. The presence and high density of TILs were correlated with better pathological response and there was a good agreement rate among pathologists trained in the assessment of TILs.

INTRODUCTION

Breast cancer is the most common malignant tumor and the leading cause of cancer death among women worldwide (1). This is a very heterogeneous disease and its treatment is constantly changing. The disease can be categorized into 3 major subtypes (HER2 positive, triple negative and luminal HER2 negative) based on analysis of protein expression in immunohistochemistry (IHC) examination (2). In-depth knowledge of the pathophysiology of each subtype of breast cancer and the determination of biomarkers is essential for the development of new therapeutic strategies.

Among the subtypes of breast cancer, the HER2 negative subtype includes both tumors with no HER2 expression (HER2-zero) and tumors with low expression of the receptor (HER2-low). These tumors are triple negative or luminous (with hormone receptors positive). HER2-low tumors resulted in around 50% of tumors being considered HER2-negative (3) and the term HER2-low has gained prominence in recent years due to the emergence of new treatment with antibody-drug conjugate (ADC) quite active in this subgroup (4).

In addition to the intrinsic characteristics of tumor cells, the tumor microenvironment also plays an important role in the process of carcinogenesis (5). Infiltrating immune cells can modulate growth and determine tumor response and progression (6). In this context, several studies evaluate the presence of tumor-infiltrating lymphocytes (TIL) as an immunobiological marker with an important prognostic and predictive role in breast cancer (7). The standardization of the quantitative assessment of TILs, determined by an international study group (8), allows the use of this histological parameter as an additional biomarker to the arsenal in breast cancer.

The neoadjuvant treatment setting is considered ideal for evaluating the impact of biomarkers on tumor response and evolution. The evidence consistently correlates the presence of TILs as a favorable prognostic factor and as a predictor of response to chemotherapy in early disease in triple-negative and HER2-positive breast cancer. Although there is data exploring the heterogeneity of HER2 expression (9), data in the literature lack to explore the relationship between the presence of TILs and outcomes in the subgroup of HER2-low patients. Currently, the integration of novel biomarkers into personalized treatment decisions remains an unmet need. The impact of HER2-low status remains poorly understood in the neoadjuvant setting. The retrospective histological analysis of HER2-negative tumor samples submitted to neoadjuvant treatment, the objective of this study, allows the evaluation of the impact of TILs of these samples, and their correlation with clinical characteristics and tumor response in HER2-low population.

METHODS

This is a retrospective cohort study that enrolled HER2-negative invasive breast cancer patients from Centro de Atenção Integral à Saúde da Mulher (CAISM/UNICAMP) in Campinas, Brazil. The inclusion criteria were women, > 18 years old, diagnosed with stage I to III HER2 negative invasive breast cancer according to AJCC 7th edition, submitted to neoadjuvant chemotherapy (NACT) followed by breast surgery, with HER2 score performed on breast biopsy specimen before initiation of systemic treatment (to determine HER2 status) and availability of slides and/or tumor blocks from the initial biopsy. Patients with bilateral disease, status HER2 indeterminate or incomplete clinicopathological data were excluded. All included patients completed neoadjuvant treatment before the start of this study, as recommended by the attending physician under current guidelines, without any inference. The study includes translational research through a review of tissue samples for biomarker analysis, without any intervention. Tumor samples were required to assess the TIL density.

The database was created from a review of medical records and anatomopathological reports. As part of the clinical routine, the samples from the diagnostic biopsy (performed before the start of NACT) and the surgical specimen (obtained from breast surgery) were both processed by the CAISM/UNICAMP pathology laboratory before the start of this study. The baseline clinicopathological characteristics considered in this analysis were age at diagnosis, menopausal status, histological grade and subtype, clinical staging (EC), estrogen receptor (ER) status, HER2 status and residual cancer burden (RCB). Tumor grade was carried out according to the Nottingham

criteria (10). A standardized IHC panel including the expression of ER and HER2 was performed. HER2 expression was evaluated using the Dako 0485 antibody (Agilent, Santa Clara, CA, USA), and results were interpreted according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines (11). HER2-low was defined as IHC score of +1 or +2 with in situ hybridization (ISH) non-amplified. After breast surgery, the RCB was calculated to determine pathological response (12). Pathological complete response (pCR) was defined as no residual invasive tumor in the primary tumor bed and ipsilateral axillary lymph nodes (ypT0/Tis, N0).

All samples from each case were evaluated for TIL density determination, and part of these samples were reviewed by a second pathologist to asses agreement in the analysis. To determine the percentage of TILs in the stroma, the standardized evaluation of the inflammatory infiltrate was provided according criteria and recommendations of the International TILs Working Group (8). TILs were presented as a continuous parameter and the percentage refers to the stromal area occupied by mononuclear infiltrate. The description of the analysis carried out used the range of TILs every 10%, and in a dichotomous way (high and low TILs), we characterized the TILs using arbitrarily two different thresholds (>=20% and >=50%).

STATISTICAL ANALYSIS

Given the paucity of data on the prevalence of TILs in HER2-low and zero populations, there was no sample size calculation for the present study. A non-probabilistic convenience sample of 119 patients consecutively included in the biobank of the Centro de Atenção Integral à Saúde da Mulher CAISM/UNICAMP was included. The primary objective was to describe the percentage of tumor lymphocytic infiltrate (TIL) in HER2-low tumors in patients undergoing NACT and compare it with the percentage in HER2-zero tumors. The secondary objectives were to assess the percentage of TILs regarding the hormone receptor, tumor grade and pathological response rate after neoadjuvant chemotherapy. Furthermore, we aim to assess agreement between 2 pathologists when analyzing TILs.

Regarding the prevalence of HER2 subgroups (HER2 zero and HER2-low) and clinicopathological characteristics, categorical variables were described using frequencies and percentages, while continuous variables were described using means and standard deviations (or median), minimum and maximum. The relationships between the HER2 subgroups (HER2-zero and HER2-low) with the categorical variables were analyzed using Pearson's chi-square test and Fisher's exact test. When statistical significance was found, the adjusted residuals were used to identify statistically significant local associations. All tests adopted a significance level of 5%. The Kappa coefficient was used to measure inter-rater agreement for scoring TILs. Statistical analysis was performed using R® and Microsoft Corporation software, Microsoft Excel Version 16.

RESULTS

Data from 119 patients with early-stage HER2-negative breast cancer undergoing neoadjuvant treatment were analyzed. Among them, 34 had HER2-zero (28,5%), and 85 with HER2-low (71,4%). Basic clinicopathological characteristics were compared

according to HER2 status and are presented in Table 1. Among the included population, the mean age was 52 years (range 22 to 76 years). The majority of the population was postmenopausal (54.6%) and almost all had non-special subtype - ductal invasive (96.6%). A minority of patients had grade 1 disease (4.2%), with 50.4% having grade 2 and 45.4% having grade 3. Clinical staging I corresponded to only 5.1% of patients, while 58.8% had EC II and 36.1% had EC III disease. Overall, HER-low tumors shared similarities in features with HER2 zero tumors. None of the tests (age, menopausal status, subtype, staging, hormonal profile and RCB) showed significant differences between the HER2 zero and HER2-low groups. The population was enriched in luminal tumors (ER-positive), which represented 67.2% of the sample.

Of 119 samples, 118 were able to be evaluated for TIL intensity according to standardized guidelines from the TILs working group. The median TIL was 10% in the general population as well as in the HER2-low population, while in the HER2-zero population, the median was 15%. Low TIL expression was predominant in the general population. The majority of patients had low TIL regardless of the cutoff point considered (64.4% with TIL <20% and 87.3%, <50%). For analyses regarding hormone receptors, tumor grade, and RCB, a cutoff point of 20% was considered to determine the significance of the impact of TILs.

Regarding ER expression, patients with triple-negative (ER-negative) tumors more frequently presented TILs >= 20% when compared to luminal tumors (61.5% and 22.8%, respectively), as shown in Figure 1A. Table 2A describes the distribution of TILs concerning ER and HER2 status. Luminal tumors continued to have low TILs in the majority, while triple negatives showed a predominance of high TILs, regardless of HER2 status. Figure 1B illustrates that the distribution of TILs >= 20% in ER-negative tumors was numerically higher in HER2-zero tumors compared to HER2-low (83.3% versus 51.9%, respectively). However, when evaluated separately, TN tumors did not show a statistical difference between the groups (p=0.0832) (Table 2B). In ER-positive patients, the distribution was similar between the groups and the statistical test confirmed the absence of association (p=0.5513) (Table 2C).

Tumor grade was not different regarding HER2 status (p=0.6316). Only 5 patients included in the sample had grade 1 disease and all of them had TILs values between 0 and 10%. Table 3A shows the relationship between the distribution of high TILs and tumor grade 3, both at the 20% (p <0.0001) and 50% (p <0.003) cutoff. Considering the 20% threshold, patients with grade 2 disease predominantly had low TILs (85%), while the majority of patients with grade 3 had high TILs (62.3%) in the general population. When analyzed taking HER2 status into account (Table 3B), the sample description suggested that grade 3 tumors had a higher frequency of high TILs in HER2 zero compared to HER2-low (76.5% versus 55.6%) (Figure 2A), however, there was no significant difference (p=0.2251).

Table 4 shows the distribution of TILs according to RCB. The pCR (RCB zero) had a median TIL of 37.5%, while in RCB I, II and III the medians were 15%, 10% and 5%, respectively. Notably, a significant difference (p = 0.0033) was observed using a 20% threshold. Considering the extremes of response, 75% of patients who achieved pCR exhibited high TILs, while 82.9% of patients with the worst response to treatment (RCB

III) demonstrated low TILs, this analysis was statistically significant (p-value 0.0005). The linear behavior of the impact of TILs is visible in figure 2B, which illustrates that the groups with the best pathological response had a higher proportion of patients with high TIL density. When considering HER2 status, similar to what occurred in grade 3, although numerically patients with RCB III present high TILs more frequently in HER2 zero in relation to HER2-low (37.5 % versus 11.1%), it is not possible to determine statistical significance. Adjusted residual analysis found an association of RCB 0 with high TILs and RCB III with low TILs in the HER2-low population (Table 4B).

To obtain data on inter-observer variability in TIL assessment, 32 hematoxylin-eosin (HeE) slides, corresponding to approximately 27% of the total sample, were randomly selected and evaluated by a second pathologist following the previously mentioned international criteria. Considering a cutoff point of 20% for the TIL, they agreed in 27 cases (84.4%) and the Kappa coefficient was equal to 0.6825. This agreement was statistically significant (p-value < 0.0001) (Table 5A). Likewise, when we considered the cutoff point of 50% for the TIL, the pathologists agreed in 31 cases (96.9%) and the Kappa coefficient was equal to 0.7838, again with statistical significance (Table 5B). We can conclude that the standardization of the evaluation of TILs when using previously determined criteria allows the reproducibility of the evaluation, contributing to the viability of using the inflammatory infiltrate as a biomarker.

DISCUSSION

The development of biomarkers continues to be a point of fundamental relevance for defining personalized therapy. Despite the growing amount of information on the impact of TILs on disease progression, and the standardization of assessment by the TILs working group (8), more clinical research and prospective translational studies are needed to understand the potential role of TILs as a biomarker. The HER2-low “subtype” has recently gained prominence given the high sensitivity to antiHER2 targeted therapy with the development of new ADCs.

The present study is part of a line of research that aims to evaluate the behavior of HER2-low disease and possible individualities that could classify it as a “new tumor subtype". The analysis evaluated the impact of TILs on the HER2-negative population with a focus on the role of the cellular infiltrate in the tumor microenvironment of HER2-low breast cancer. According to our results, patients with HER2-low disease share characteristics with HER2-zero cases. The median distribution of TILs was numerically lower in the HER2-low population versus HER2 zero (10 and 15%, respectively). Evaluating data presented in the literature, analysis of a cohort of 529 patients, enriched by the luminal population (57.6%), found a significant difference with lower density of TILs in the HER2-low population when compared to HER2-zero. Interestingly, even in this study, the HER2-low population also showed gene expression related to worse prognosis and depletion of immunity, suggesting that HER2-low tumors may have a lower immune response when compared to HER2-zero (13). Contrary to these findings, studies demonstrated a slightly higher percentage of TIL in HER2-low population when compared to HER2-zero (14) or similarity in clinicopathological characteristics,

including infiltration of stromal TILs, without differences in HER2 zero and low population (15).

Regarding the hormonal profile, in our study, the population with luminal disease showed less lymphocytic infiltration than TN tumors, both in HER2-low and HER2-zero disease. When evaluating the TN population separately, we noticed a trend towards a higher frequency of high TILs (>= 20%) in HER2-zero disease, compared with HER2-low, but this difference was not statistically significant. It is worth mentioning that our analysis was exploratory with a great imbalance between the groups. A discrepant result was found, in a previous published study, suggesting that luminal tumors had a higher TIL density in HER2-zero tumors, while in ER-negative tumors the density of TILs was lower in HER2-zero when compared to HER2-low (15).

We meet a positive correlation between high tumor grade and higher density of TILs. When analyzing the impact on pathological response, the median TILs were higher in those patients who achieved pCR, regardless of zero versus low HER2 status. Although we recognize the linear nature of the impact of TILs, both as a predictive and prognostic factor (7,16), we found a cutoff point of 20% with a significant impact on the outcome of pathological response.

Our study did not assess the potential prognostic impact. Lu et al., did not find an impact of TILs on survival specifically in ER-positive disease, but demonstrated an independent prognostic factor in the general HER2-low population (15). In contrast to this finding, Sun et al (17) evaluated 1763 patients and found a positive association of high levels of TILS and a significant impact on improved survival, especially in the HER2-low ER-positive subtype. While Yue et al. did not demonstrate a prognostic impact related to HER2-low status or the expression of TILs (18). We realize that the findings are discordant and the number of studies addressing the topic remains quite small.

A fundamental point for determining a biomarker is the adequate assessment of the variable. We found good agreement among pathologists, being 84.4% when the threshold was 20%, and 96.9% when we used a threshold of 50%, with a Kappa coefficient of 0.6825 and 0.7838, respectively. This data is consistent with what was reported by Denkert et al.(19). The study evaluated the agreement in TILS assessment on 120 samples among multiple pathologists in two rounds. The Kappa values at the end of the second round at the 20% and 50% cutoffs were 0.65 and 0.72, with agreement rates of 85% and 93%, respectively (19).

The main limitations of the present study include the small sample size, especially in the subgroup analysis, in addition to the retrospective nature and the imbalance of patients with different HER2 status. All analyses are exploratory, therefore, even in cases where a positive association was found in the statistical analysis, the credibility of the result is low. Among the strengths of the study, the methodology that allowed standardized centralized analysis of TILs as per international guidelines for all patients, and the originality stands out. Although there are currently publications evaluating TILs in the HER2-low population, none of them focus on pathological response as a related outcome in this population. The findings of the study and the review of articles published to date allow us to conclude that low HER2 expression alone could not confer a predictive

role for response to neoadjuvant chemotherapy and the impact of TILs in this specific population remains uncertain.

CONCLUSION

In this cohort of patients, no significant difference was identified in the distribution of clinicopathological characteristics and density of TILs between the HER2-low and HER2-zero subgroups. The presence and high density of TILs were correlated with better pathological response and there was a good agreement rate among trained pathologists in the assessment of TILs. Regarding the impact of TILs on breast cancer outcomes, there are several retrospective studies consistent with their favorable results, but prospective studies are still needed to determine the clinical applicability of this biomarker. As for the impact of HER2 low status, there is a growing number of studies demonstrating that low HER2 expression does not appear to be an independent factor, nor a prognosis, nor a predictive factor for response to conventional therapy. The data presented to date corroborate HER2-low status as a marker of sensitivity to targeted therapy. More well-designed and prospective analyses are needed to understand the role of TILs in HER2-low population.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Research Ethics Committees of UFRGS and UNICAMP under registration (No.: CAAE - 57159722.3.1001.534) and individual consent for this retrospective analysis was obtained (Biobank Informed Consent Form - CONEP B-056)

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FIGURES (RESULTS ARTICLE)

Figure 1: A - Distribution of TILs by hormone receptor; B – Distribution of TILS by hormone receptor in terms of HER2 status. (ER -: estrogen receptor-negative; ER +: ER-positive)

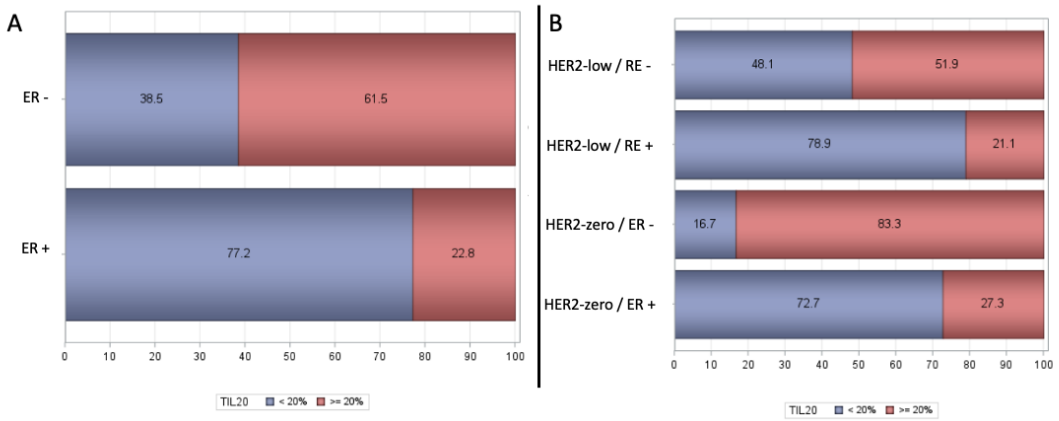
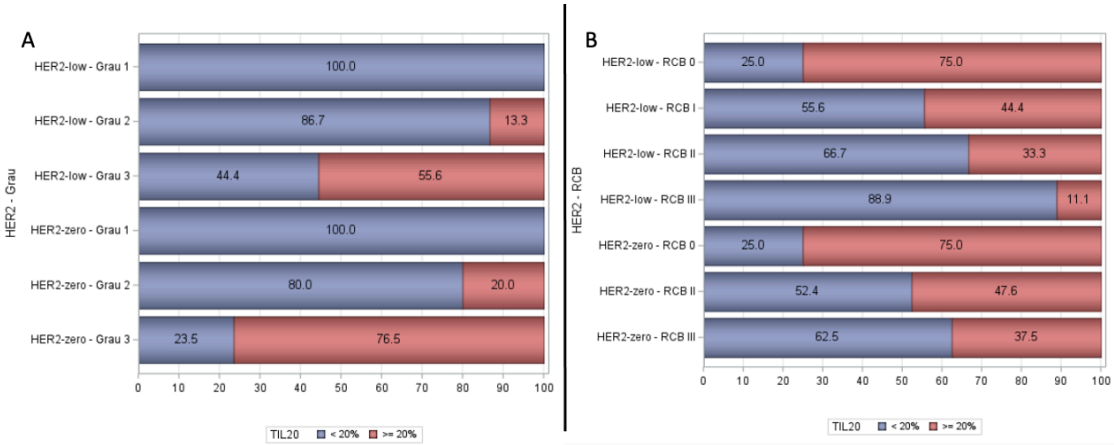


Figure 2: A - Distribution of TILs by tumor grade regarding HER2 status; B- Distribution of TILs by RCB regarding HER2 status



TABLES (RESULT ARTICLE)

Table 1: Study subjects and tumor characteristics

Table 1. Study subjects and tumor characteristics.

		HER2		Total	p-value
		HER2 zero (N=34)	HER2-low (N = 85)		
Age at diagnosis					
	N	33	85	118	
	Mean (Standard deviation)	54,2 (11,6)	51,6 (11,8)	52,3 (11,8)	0,2781 †††
	Minimum and Maximum	33,0 - 76,6	22,4 - 76,3	22,4 - 76,6	
Menopausal situation					
	Premenopausal	12 (35,3)	40 (47,1)	52 (43,7)	0.3063 ††

	Postmenopausal	21 (61,8)	44 (51,7)	65 (54,6)	
	Unknown/Not informed	1 (2,9)	1 (1,2)	2 (1,7)	
Subtype					
	Invasive ductal carcinoma (NPS)	32 (94,1)	83 (97,7)	115 (96,6)	1,0000 ††
	Invasive lobular carcinoma	1 (2,9)	2 (2,3)	3 (2,5)	
	Other	1 (2,9)	0 (0,0)	1 (0,8)	
Histological Grade					
	1	2 (5,9)	3 (3,5)	5 (4,2)	
	2	15 (44,1)	45 (52,9)	60 (50,4)	0.6316 †
	3	17 (50,0)	37 (43,5)	54 (45,4)	
Clinical Stage					
	I	2 (5,9)	4 (4,7)	6 (5,1)	
	II	19 (55,9)	51 (60,0)	70 (58,8)	0.9072 †
	III	13 (38,2)	30 (35,3)	43 (36,1)	
Estrogen receptor					
	Positive	22 (64,7)	58 (68,2)	80 (67,2)	0.8292 ††
	Negative	12 (35,3)	27 (31,8)	39 (32,8)	
TILs score					
	N	34	84	118	
	Median	15	10	10	
	0% - 10%	16 (47,1)	49 (58,3)	65 (55,1)	
	11% - 20%	6 (17,6)	13 (15,5)	19 (16,1)	
	21% - 30%	3 (8,8)	10 (11,9)	13 (11,0)	
	31% - 40%	2 (5,9)	4 (4,8)	6 (5,1)	
	41% - 50%	1 (2,9)	2 (2,4)	3 (2,5)	
	51% - 60%	0 (0,0)	1 (1,2)	1 (0,9)	
	61% - 70%	1 (2,9)	3 (3,6)	4 (3,4)	
	71% - 80%	2 (5,9)	0 (0,0)	2 (1,7)	
	81% - 90%	2 (5,9)	2 (2,4)	4 (3,4)	
	91% - 100%	1 (2,9)	0 (0,0)	1 (0,9)	
	< 20%	18 (52,9)	58 (69,1)	76 (64,4)	0.1367 ††
	>= 20%	16 (47,1)	26 (30,9)	42 (35,6)	
	< 50%	27 (79,4)	76 (90,5)	103 (87,3)	0.1287 ††
	>= 50%	7 (20,6)	8 (9,5)	15 (12,7)	
RCB					
	0	4 (11,8)	8 (9,4)	12 (10,1)	0,1168 †
	I	0 (0,0)	9 (10,6)	9 (7,6)	
	II	21 (61,8)	37 (43,5)	58 (48,7)	
	III	8 (23,5)	27 (31,8)	35 (29,4)	
	Lost	1 (2,9)	4 (4,7)	5 (4,2)	

Note: '†': Pearson Chi-squared test. '††': Fisher's Exact test. '†††': Student t test. '*': when shown, it means that there is a statistically significant result.

Table 2: A - Distribution of TIL in relation to HER2 and Estrogen Receptor (ER); B - Distribution of TIL regarding HER2 status in the negative ER; C- Distribution of TIL regarding to HER2 status in the positive ER

Table 2A. Distribution of TIL in relation to HER2 and Estrogen Receptor (ER) (N = 118).

		TIL < 20% N = 76 (64,4%)	TIL >= 20% N = 42 (35,6%)	Total N = 118 (100%)	p-value
HER2 - RE					
	HER2-low - RE Negative	13 (48,2%)	**14 (51,8%)**	27 (22,9%)	<0,0001†*
	HER2-low - RE Positivo	**45 (78,9%)**	12 (21,1%)	57 (48,3%)	
	HER2-zero - RE Negativo	2 (16,7%)	**10 (83,3%)**	12 (10,2%)	
	HER2-zero - RE Positivo	16 (72,7%)	6 (27,3%)	22 (18,6%)	

Table 2B. Distribution of TIL regarding HER2 status in the negative ER;

		TIL < 20% N = 15 (38,5%)	TIL >= 20% N = 24 (61,5%)	Total N = 39 (100%)	p-value
HER2					
	HER2-zero	2 (16,7%)	10 (83,3%)	12 (30,8%)	0,0832††
	HER2-low	13 (48,1%)	14 (51,9%)	27 (69,2%)	

Table 2C. Distribution of TIL regarding HER2 status in the positive ER

		TIL < 20% N = 61 (77,2%)	TIL >= 20% N = 18 (22,8%)	Total N = 79 (100%)	p-value
HER2					
	HER2-zero	16 (72,7%)	6 (27,3%)	22 (27,8%)	0,5513††
	HER2-low	45 (78,9%)	12 (21,1%)	57 (72,2%)	

'†': Pearson Chi-squared test. '††': Fisher's Exact test. '*':statistically significant result

Table 3: A – Distribution of TILs by tumor grade; B- Distribution of TILs by tumor grade regarding HER2 status

Table 3A. Distribution of TILs by tumor grade (N = 118).

		Grade 1	Grade 2	Grade 3	Total	p-value
		N = 5 (4,2%)	N = 60 (50,8%)	N = 53 (44,9%)	N = 118 (100%)	
TILs score						
	Median	10	5	30	10	NA
	0% - 10%	5 (100,0)	44 (73,3)	16 (30,2)	65 (55,1)	NA
	1. 11% - 20%	0 (0,0)	9 (15,0)	10 (18,9)	19 (16,1)	
	21% - 30%	0 (0,0)	4 (6,7)	9 (17,0)	13 (11,0)	
	31% - 40%	0 (0,0)	2 (3,3)	4 (7,6)	6 (5,1)	
	41% - 50%	0 (0,0)	0 (0,0)	3 (5,7)	3 (2,5)	
	51% - 60%	0 (0,0)	0 (0,0)	1 (1,9)	1 (0,9)	
	61% - 70%	0 (0,0)	0 (0,0)	4 (7,6)	4 (3,4)	
	71% - 80%	0 (0,0)	1 (1,7)	1 (1,9)	2 (1,7)	
	81% - 90%	0 (0,0)	0 (0,0)	4 (7,6)	4 (3,4)	
	91% - 100%	0 (0,0)	0 (0,0)	1 (1,9)	1 (0,9)	
	< 20%	5 (100,0)	**51 (85,0)**	20 (37,7)	76 (64,4)	<0,0001 †*
	>= 20%	0 (0,0)	9 (15,0)	**33 (62,3)**	42 (35,6)	
	< 50%	5 (100,0)	**59 (98,3)**	39 (73,6)	103 (87,3)	0,0003† *
	>= 50%	0 (0,0)	1 (1,7)	**14 (26,4)**	15 (12,7)	

Table 3B. Distribution of TILs by tumor grade regarding HER2 status(N = 118)

		TIL < 20%	TIL >= 20%	Total	p-value
		N = 76 (64,4%)	N = 42 (35,6%)	N = 118 (100%)	
HER2 - Grau					
	HER2-low - Grau 1	3 (100,0%)	0 (0,0%)	3 (2,5%)	<0,0001† *
	HER2-low - Grau 2	**39 (86,7%)**	6 (13,3%)	45 (38,1%)	
	HER2-low - Grau 3	16 (44,4%)	**20 (55,6%)**	36 (30,5%)	
	HER2-zero - Grau 1	2 (100,0%)	0 (0,0%)	2 (1,7%)	
	HER2-zero - Grau 2	12 (80,0%)	3 (20,0%)	15 (12,7%)	
	HER2-zero - Grau 3	4 (23,5%)	**13 (76,5%)**	17 (14,4%)	

'†': Pearson Chi-squared test. '*':statistically significant result.

Table 4: A- Distribution of TIL and RCB; B- Distribution of TIL regarding HER2 and RCB 0 / III.

Table 4A. Distribuition of TIL and RCB (N = 113).

		RCB 0	RCB 1	RCB 2	RCB 3	Total	p-value
		N = 12 (10.6%)	**N = 9 (8.0%)**	**N = 57 (50.4%)**	**N = 35 (31.0%)**	**N = 113 (100%)**	
TIL							
	Mediana	37,5	15	10	5	10	NA
	0% - 10%	1 (8,3)	4 (44,4)	30 (52,6)	27 (77,1)	62 (54,9)	
	11% - 20%	3 (25,0)	1 (11,1)	11 (19,3)	2 (5,7)	17 (15,0)	
	21% - 30%	1 (8,3)	2 (22,2)	7 (12,3)	3 (8,6)	13 (11,5)	
	31% - 40%	3 (25,0)	0 (0,0)	2 (3,5)	1 (2,9)	6 (5,3)	
	41% - 50%	0 (0,0)	0 (0,0)	3 (5,3)	0 (0,0)	3 (2,7)	NA
	51% - 60%	1 (8,3)	0 (0,0)	0 (0,0)	0 (0,0)	1 (0,9)	
	61% - 70%	0 (0,0)	2 (22,2)	1 (1,8)	1 (2,9)	4 (3,5)	
	71% - 80%	0 (0,0)	0 (0,0)	1 (1,8)	1 (2,9)	2 (1,8)	
	81% - 90%	2 (16,7)	0 (0,0)	2 (3,5)	0 (0,0)	4 (3,5)	
	91% - 100%	1 (8,3)	0 (0,0)	0 (0,0)	0 (0,0)	1 (0,9)	
	< 20%	3 (25,0)	5 (55,6)	35 (61,4)	**29 (82,9)**	72 (63,7)	0,0033† *
	>= 20%	**9 (75,0)**	4 (44,4)	22 (38,6)	6 (17,1)	41 (36,3)	
	< 50%	8 (66,7)	7 (77,8)	50 (87,7)	33 (94,3)	98 (86,7)	0,3213†
	>= 50%	4 (33,3)	2 (22,2)	7 (12,3)	2 (5,7)	15 (13,3)	

Tabela 4B. Distribution of TIL regarding HER2 and RCB (0 / III) (N = 47).

		TIL < 20%	TIL >= 20%	Total	p-value
		N = 32 (68,1%)	**N = 15 (31,9%)**	**N = 47 (100%)**	
HER2 – RCB					
	HER2-low – RCB 0	2 (25,0%)	**6 (75,0%)**	8 (17,0%)	
	HER2-low – RCB III	**24 (88,9%)**	3 (11,1%)	27 (57,5%)	0,0013† *
	HER2-zero – RCB 0	1 (25,0%)	3 (75,0%)	4 (8,5%)	
	HER2-zero – RCB III	5 (62,5%)	3 (37,5%)	8 (17,0%)	

'†': Pearson Chi-squared test. '*':statistically significant result

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Table 5: A – Kappa coefficient (cutoff point 20%); B- Kappa coefficient (cutoff point 50%)

Table 5A. Results of the Kappa Agreement Coefficient – with cutoff point 20% (N = 32).

		Revisor 2			
		TIL < 20%	**TIL >= 20%**	**Total**	**p-value**
		N = 17 (53,3%)	**N = 15 (46,9%)**	**N = 32 (100%)**	
Revisor 1					
	TIL < 20%	16 (94,1%)	4 (26,7%)	20 (62,5%)	< 0,0001†
	TIL >= 20%	1 (5,9%)	11 (73,3%)	8 (37,5%)	

Table 5B. Results of the Kappa Agreement Coefficient – with cutoff point 50% (N = 32).

		Revisor 2			
		TIL < 50%	**TIL >= 50%**	**Total**	**p-value**
		N = 30 (93,8%)	**N = 2 (6,3%)**	**N = 32 (100%)**	
Revisor 1					
	TIL < 50%	29 (96,7%)	0 (0,0%)	29 (90,6%)	< 0,0001†
	TIL >= 50%	1 (3,3%)	2 (100,0%)	3 (9,4%)	

'†': p-value Kappa.

7.2 ARTIGO 2 - ARTIGO DE REVISÃO PUBLICADO

Foi realizado um artigo de revisão com foco em infiltrado inflamatório tumoral como biomarcador preditivo e prognóstico em câncer de mama inicial triplo negativo. O Artigo intulado “Implications of tumor-infiltrating lymphocytes in early-stage triple-negative breast cancer: clinical oncologist perspectives” foi publicado online (https://tbcr.amegroups.org/article/view/79763) na revista *Translational Breast Cancer Research* – DOI: 10.21037/tbcr-23-43

Review Article

Implications of tumor-infiltrating lymphocytes in early-stage triple-negative breast cancer: clinical oncologist perspectives

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Contributions: (I) Conception and design: All authors; (II) Administrative support: All authors; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Breast cancer (BC) is the most common neoplasm in women worldwide and one of the leading causes of female death. The triple-negative subtype, characterized by the absence of hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2), tends to occur in younger patients, be more aggressive and less differentiated. Furthermore, this subtype is considered the most immunogenic and associated with higher levels of tumor cell infiltration, mainly lymphocytes. Tumor-infiltrating lymphocytes (TILs) play a crucial role in the interaction of the host's immune system and cancer cells. The microenvironment is critical in tumor development and progression. Assessment of infiltrating lymphocytes can provide valuable information about the immune response and, given the lack of biomarkers to guide treatment decisions and predict outcomes in triple-negative tumors and can be considered as a potential biomarker. Some evidence suggests that higher levels of these lymphocytes are associated with better responses to systemic treatment, longer progression-free survival and overall survival (OS). However, treatment escalation or de-escalation strategies for triple-negative BC (TNBC) currently do not consider the presence or density of TILs for therapeutic decisions. TILs appear to be useful predictive and prognostic indicators. Further clinical studies are needed to confirm these relationships and integrate TILs as a biomarker consistently into clinical practice. This article summarizes key concepts relating to the role of the immune infiltrate in BC, along with the current status and future prospects regarding TILs as a predictive and prognostic biomarker.

Keywords: Breast neoplasm; triple-negative breast cancer (TNBC); tumor-infiltrating lymphocyte (TIL); biomarker

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Introduction

Breast cancer (BC) is the most common malignant tumor and the leading cause of cancer death in women worldwide (1). This heterogeneous disease is caused by several genetic changes in breast epithelial cells, with different clinical manifestations and outcomes (2,3). Gene expression profiling studies have identified at least four categories of BC (4). These molecular categories correlate with immunohistochemical markers of hormone receptor (HR), estrogen receptor (ER), progesterone receptor (PgR), and

human epidermal growth factor receptor 2 (HER2), with triple-negative subtype defined as the absence of these receptors (5).

The triple-negative BC (TNBC) subtype accounts for 11% to 20% of all BC cases and affects, more commonly, premenopausal patients, women with African ancestry, and carriers of a hereditary mutation in *BRCA1/2* genes (6). Characteristically, TNBCs have a biologically aggressive behavior, tend to be larger, more undifferentiated, and with more frequent lymph node involvement at diagnosis (6). This profile is associated to higher recurrence and mortality rates (7).

The immune system plays an essential role in BC initiation and progression (7). The intensity of the tumor's immune response has been shown to influence the effectiveness of cancer therapy and prognosis (8). In this context, immunotherapy with immune checkpoint inhibitors (ICIs) has emerged as a promising treatment strategy for TNBC. It is currently standard clinical practice in the neoadjuvant setting in combination with chemotherapy, as well as first-line treatment in select patients with metastatic disease (9,10). Despite these recent advances, we still lack biomarkers to help personalize the treatment of BC in general and TNBC in particular. Compared to other subtypes, TNBC is considered more immunogenic and is often associated with higher levels of immune cell infiltration, particularly tumor-infiltrating lymphocytes (TILs) (11). The presence of TILs in TNBC is an independent prognostic biomarker, and it can be potentially used as a predictive biomarker of response to systemic therapies, such as chemotherapy and ICIs (8).

Other several biomarkers are being studied to guide treatment decisions and predict patient outcomes in TNBC, including expression of programmed cell death ligand 1 (PD-L1) or androgen receptors (ARs) and the presence of *BRCA* mutations (12). Available evidence suggests they may play different roles in early *vs.* late disease settings. Although TILs have been shown to be a reliable prognostic biomarker, their predictive role for escalation or de-escalation strategies still needs to be better established. Concentrating on early-stage TNBC (eTNBC), this manuscript summarizes essential concepts about the role of the immune infiltrate in BC and the current status and future perspectives of TILs as a prognostic and predictive biomarker.

Current status of systemic therapy in TNBC

Compared to other BC subtypes, eTNBC has high recurrence rates and an unfavorable prognosis (13). This has been attributed not only to its biologically aggressive behavior but also to limited therapeutic options (14). In recent years, outcomes have been improved with the approval of new agents and the use of the neoadjuvant approach as a strategy for individualizing treatment (15).

The treatment of eTNBC is multimodal, including surgery, radiotherapy, and systemic therapy (16). The main goal has been to combine and sequence these different modalities according to the clinical scenario (17). In clinically stage Ia and Ib disease, upfront surgery may be considered appropriate, usually followed by adjuvant chemotherapy, particularly in tumors larger than 5 mm (18). Neoadjuvant therapy is currently the recommended approach in tumors greater than 1 cm and stages II and III (19). Radiotherapy of the breast and regional nodes follows surgery and systemic therapy according to the stage, nodal involvement, and the selected surgical procedure. The response to the neoadjuvant systemic therapy [pathological complete response (pCR) *vs.* non-pCR] is used to tailor further systemic and locoregional treatment. The objective is to escalate treatment in non-responders or incomplete responders and de-escalate therapy in those with complete response (20). The high chemosensitivity of TNBC confers pCR rates of approximately 40% with the combination of anthracyclines and taxanes (21-23). With the high frequency of homologous recombination defects (HRDs) in these tumors, the addition of carboplatin to neoadjuvant regimens was investigated in phase II and III studies with favorable results consistently increasing pCR rates (24).

Despite a rough start with several phase III trials failing to meet key survival endpoints (25-27) and withdrawn of initially approved agents (atezolizumab), ICIs have been incorporated in the treatment of TNBC. Although initially evaluated in the metastatic setting, early-stage disease represents a promising scenario for the adoption of these agents, since tumor burden is limited and the tumor microenvironment (TME) is less impacted by previous systemic treatments (28).

KEYNOTE-522 is a practice-changing phase III trial that randomized 784 patients with stage II and III eTNBC to receive neoadjuvant chemotherapy (NACT) with concomitant pembrolizumab or placebo (29). The chemotherapy backbone consisted of weekly paclitaxel plus carboplatin followed by anthracycline plus cyclophosphamide every 3 weeks. After surgery, patients continued on adjuvant pembrolizumab or placebo for up to 9 cycles. The study showed a significant increase in the

pCR rate (63% *vs.* 55.6%, P=0.0005) and a prolongation of the event-free survival (EFS) at 3 years [84.5% *vs.* 76.8%; hazard ratio, 0.63; P=0.0003], the two co-primary endpoints, favoring the group treated with pembrolizumab (29). These results, have established the KEYNOTE-522 regimen as the standard of care for patients with stage II and III eTNBC (19).

However, some caveats and difficulties remain regarding the potential toxicity and the selection of patients who benefit from the addition of programmed cell death 1 (PD-1)-blockade (30). The unique side-effect profile of immunotherapeutic agents is particularly relevant for patients with curable disease. In KEYNOTE-522, almost 13% of patients in the pembrolizumab arm experienced grade 3–5 immune-related adverse events (irAEs), *vs.* only 1% in the placebo arm (29). Recommendations for a standardized approach to evaluate and treat irAEs have been published and patients should be monitored closely for these events (31).

Importantly, the prognosis of patients who achieve a pCR is highly favorable whether or not they receive immunotherapy (3-year EFS: 92.5% in the control arm *vs.* 94.4% in the pembrolizumab arm). Although this analysis was exploratory and not powered to make a definitive conclusion, it questions whether adjuvant pembrolizumab adds additional benefits post-pCR (32). The toxicity of adjuvant pembrolizumab was not negligible, with a 6.3% of high-grade irAEs. The OptimICE-PCR study (NCT05812807), is an ongoing clinical trial, that will address the continuation of adjuvant pembrolizumab in patients with pCR. Until the results of this trial are available, a shared decision process should be used to determine whether to continue adjuvant pembrolizumab post-pCR in an individual patient (33).

Notably, a significant proportion of the population treated with the KEYNOTE-522 regimen has residual disease after surgery. This subgroup carries an unfavorable prognosis with 5-year recurrence rates ranging from 30% to 70% depending on the residual cancer burden (RCB) (34). Patients with residual disease had 3-year EFS rates of 56.8% and 67.4% in the control and experimental arms, respectively. In this setting, there is no room for treatment de-escalation and adjuvant pembrolizumab should be prescribed if no contraindication exists. Furthermore, other adjuvant therapies must be considered to improve the outcomes in these patients (35).

The CREATEx trial evaluated the role of capecitabine in patients with residual disease after surgery (36). This study included 910 patients with HER2-negative BC (both HR-positive and TNBC) with non-pCR after NACT. Most of them (80%) received anthracycline and taxane-based regimens (36). Patients were randomized to receive adjuvant endocrine therapy (ET) with or without capecitabine for 6 months. The trial showed positive results in the overall population, with significantly improved disease-free survival (DFS) (74.1% *vs.* 67.6% at 5 years) and overall survival (OS) (89.2% *vs.* 83.6%) greater in the capecitabine group compared to the control group, respectively (36). Subgroup analysis demonstrated that most of the OS benefit occurred in the population with TNBC (hazard ratio, 0.58; 95% CI: 0.39–0.87) *vs.* the HR-positive tumors (hazard ratio, 0.84; 95% CI: 0.57–1.23). This study was the first to confirm that the response to neoadjuvant therapy is discriminating to escalate adjuvant therapy potentially improving survival outcomes (36).

Despite significant advances in our molecular understanding of BC and particularly the heterogeneity of TNBC, there have been very few validated advances in biomarker development to optimize therapy in this context. We mostly continue to treat our patients with a 'one-size fits all' approach. Basically, all therapeutic decisions in stage I, II, and III TNBC are based on traditional clinicopathological criteria considering tumor size and nodal status. Importantly, we remain unable to identify who are the patients that will not reach a pCR with neoadjuvant treatment and can only escalate therapy after surgery once the pathological response is known. Therefore, all patients are routinely treated with aggressive and intensive multidrug regimens. In this setting, a predictive biomarker could potentially allow us to de-escalate the neoadjuvant regimen in those patients destined to achieve a pCR and escalate therapy in the others before surgery.

A significant development in this area is the recognition that 10–15% of patients with TNBC carry germline mutations in *BRCA1/2* (gBRCA), critical genes for homologous DNA recombination (37). Addressing this particular population of patients, the phase III OlympiA randomized clinical trial evaluated the role of adjuvant treatment with the PARP inhibitor olaparib in high-risk HR-positive, HER2-negative, and TNBC patients with gBRCA (38). In this fantastic collaborative effort, patients were included if they had high-risk characteristics or residual disease after NACT and if they had high-risk characteristics in the adjuvant setting. Patients were randomized to receive olaparib or placebo for 1 year. The study was positive for invasive DFS (hazard ratio, 0.63; 95%

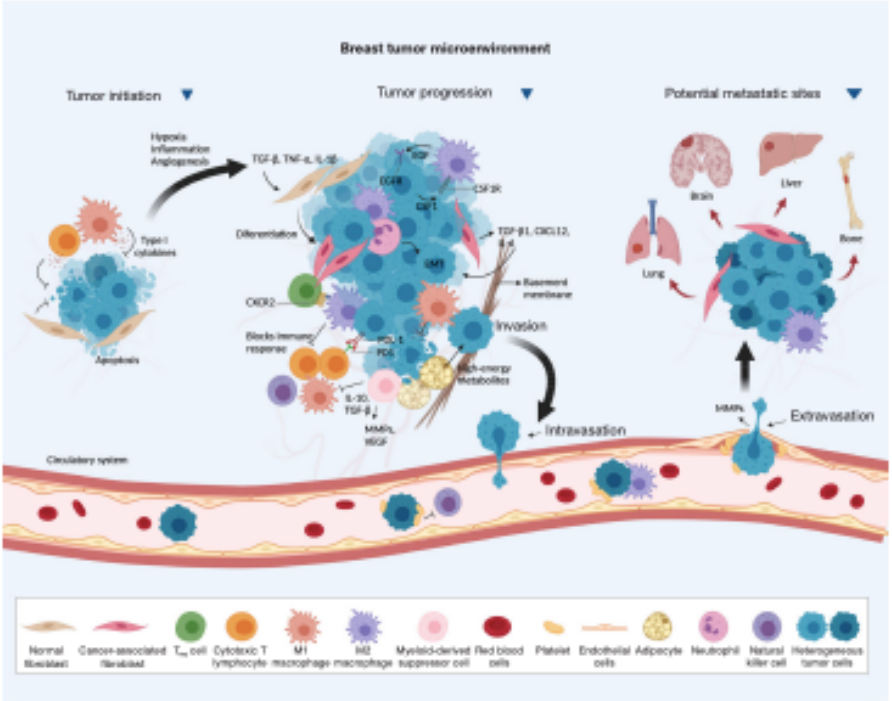

Figure 1 Microenvironment and process of tumor initiation and progression. Reproduced from Terceiro *et al.* [2021] (40) with permission.

CI: 0.50–0.78) and OS (hazard ratio, 0.68; 95% CI: 0.47–0.97; P=0.009) (38). These results establish gBRCA status as a useful biomarker in this setting.

A clear unmet need, there is great interest in developing predictive biomarkers that can help personalize therapeutic decisions in clinical practice. Besides tumor-related biomarkers, the TME role has been increasingly recognized as a critical player influencing tumor evolution and progression, with essential roles in treatment response and resistance development (39). Within the complexity of the TME, immune infiltrates, particularly TILs, have been extensively studied and may be considered as a promising biomarker.

Role of the immune system and the TME in BC

The TME is critically important in tumor development and progression. Cancer cells actively interact with non-malignant cells such as immune system cells, lymphatic vasculature, fibroblasts, and pericytes (*Figure 1*) (40,41). Regulation of the immune response results from interaction with different classes of T cells such as CD8 T lymphocytes, CD4 T lymphocytes, and regulatory T cells (Tregs) (42).

The immune system can recognize and eliminate cancer cells. Still, tumors can evade the immune system and create an immunosuppressive environment, favoring the development and progression of the disease (43). During cancer immunoediting, the host's immune system shapes tumor fate in three phases through the activation of innate and adaptive immune mechanisms (44). In the first phase, elimination, cancerous cells are destroyed by a competent immune system. Sporadic tumor cells that manage to survive immune destruction may then enter an equilibrium phase where editing occurs. The escape phase represents the third and final phase of the process, where immunologically sculpted tumor cells can grow progressively, become clinically evident, in an established an immunosuppressive TME (44).

In TNBC, the TME significantly influences the malignant behavior and growth of both tumor and surrounding cells. The microenvironment has the ability to reprogram neighboring cells, can counteract the progression

of cancer cells, and defining the signaling of cellular pathways, impact the results of therapies. Therefore, the characteristics of the TME define the interaction with the host's immune system and can affect the response to therapies (45). While specific responses and innate reactions can be harnessed to control TNBC development impeding tumor cells' initiation, progression, and metastasis, immunosuppressive cells can facilitate immune evasion. The TME in question is closely associated with the characteristic features of TNBC itself, resulting in immune system suppression, evasion of immune detection, and drug resistance (46). The inflammatory infiltration is constituted by all cells with a lymphocytic nature that infiltrate tumor tissues (14). Three TME categories have been defined in different types of tumors: immune desert, comprising tumors devoid of lymphocytes; excluded immune, in which lymphocytes are present only in the peritumoral stroma; and inflamed ("hot"), with high infiltration of T cells (45).

Definition of TILs and standard evaluation

By definition, TILs are mononuclear immune cells that leave the blood and enter the TME, comprising a mixture of cytotoxic and helper T (Th) cells, B cells, macrophages, natural killer cells, and dendritic cells (47). T lymphocytes account for about 75% of TILs (48), and $CD8^+$ cells are abundant in the TNBC microenvironment. Several studies have established a correlation between TILs and TNBC prognosis, indicating that increased expression of $CD8^+$ T lymphocytes is associated with better clinical outcomes (49,50).

The presence of $CD3^+$ T cells is frequently observed in the TME representing the mature T cell population with co-differentiated antigens on their surface, serving as markers for total T lymphocytes within the tissue. Further characterization of infiltrating cell population reveals distinct subsets, including $CD8^+$ T lymphocytes, $CD4^+$ Th lymphocytes, and $CD4^+$ Tregs.

The $CD4^+$ Th lymphocytes can be categorized into Th1 and Th2 subtypes based on their secretion of cytokines and participate in cellular and humoral immunity, respectively. $CD4^+$ Th lymphocytes assist in $CD8^+$ T lymphocyte-mediated cell killing, actively contributing to the tumor immune response. On the other hand, Tregs, which constitute 10% of all $CD4^+$ T lymphocytes in the peripheral blood of healthy individuals, can increase to 30–50% within tumor lesions, inhibit the activation of $CD8^+$ T and $CD4^+$ T lymphocytes, playing a crucial role in immune suppression and angiogenesis and potentially hampering the body's anti-tumor immune response. This significant accumulation of immunosuppressive Treg subsets, can include high infiltration of forkhead box P3 ($FOXP3^+$) cells (46). These $FOXP3^+$ Tregs can suppress immune responses against self-antigens, hinder anti-tumor immunity, and are a prognostic indicator of poor outcome (51).

Within the TNBC TME, TILs should be assessed by analyzing immune cells' presence, density, and distribution within the tumor. This can be done through various techniques, including histological examination, immunohistochemistry, flow cytometry, and gene expression profiling (52). The TILs Working Group is a cooperative group of researchers who has developed guidelines to standardize and allow greater reproducibility of TILs assessment in BC (53). In summary, the general rules for evaluating TILs range from pre-analytical guidelines, such as slide preparation and fixation (the ideal thickness of the slide should be 4 to 5 µm of tissue fixed in formalin and embedded in paraffin) to analytical issues (53). TILs should be evaluated within the limits of the invasive component of a tumor. However, only the stromal component should be considered and importantly, areas occupied by carcinoma cells should not be included in the total surface area evaluated. TILs in areas with crushing artifacts, regressive hyalinization, and necrosis and those at a previous biopsy site should be excluded from consideration. The percentage of TILs present in a given tumor should be calculated by dividing the area occupied by mononuclear inflammatory cells by the total area of the tumoral stroma. The value should be given as a percentage and is a continuous variable. If the percentage of TILs is uncertain, the case should be discussed with a second pathologist (53). Standardization of the evaluation allows for greater reproducibility and agreement and, therefore, facilitates the development of studies evaluating TILs as a potential biomarker of impact in clinical practice.

Prevalence of TILs in eTNBC

The molecular subtype of BC impacts the interaction with the immune system. TNBC is more often infiltrated by TILs than luminal tumors (45). Although we should value the recommendations from the International TILs Working Group, there is no consensus on the ideal cutoff point for determining high and low TILs (53). The German Breast Cancer Group conducted a study evaluating the predictive and prognostic value of TILs and defined three groups:

low (0–10%), intermediate (11–59%), and high (≥60%) TILs (45). Determining a specific cut-off point is an important and complex issue, as the impact of TILs expression, both on the prognosis and on the predictive importance of this biomarker, appears to be linear (45).

Some studies use the term lymphocytic predominantly BC (LPBC) to define tumors that have a TIL density greater than or equal to 50–60% (54-59), considered as having "high TILs". Other studies have used different cutoff points to assess the impact of TILs. It is important to emphasize that although TNBC is considered the most immunogenic among BC subtypes, most early TNBC have a low or intermediate density of TILs (60). *Table 1* summarizes some recent trials assessing the prevalence of stromal TILs (sTILs) in eTNBC. Not all studies cited follow the evaluation rules defined by the TILs working group, which hampers comparative analyses. Despite this, the studies agree regarding the prevalence of sTILs in the studied populations.

TILs as a prognostic biomarker in eTNBC

Evidence of the impact of TILs as a prognostic biomarker highlights the importance of the immune response and the role of the TME in tumor development and control. A meta-analysis of approximately 13,100 patients demonstrated an association between a high density of TILs and better prognosis in BC, including better DFS and OS (73). The systemic immune response, defined by the TILs score and the systemic inflammation index (determined by platelet × neutrophil/lymphocyte) has also been correlated with survival outcomes (74). It is important to highlight the dynamic characteristic of TILs density during the evolution of the disease. The cellular population in the TME is impacted by systemic treatment. An increase in TILs during neoadjuvant treatment appears to be associated with better outcomes in TNBC. The survival benefit of higher levels of infiltration was demonstrated in a meta-analysis that analyzed studies that performed paired analyses of TILs density before and after NACT (75). The NeoTRIP study also demonstrated an increase in TILs after 1 cycle of neoadjuvant systemic therapy (76). The prognostic value was assessed in both initial and residual diseases after neoadjuvant therapy, where a greater lymphocytic infiltrate also demonstrated an association with favorable outcomes (77). Importantly, better definition of the international standards for assessment of TILs in residual disease and in surgical specimens with pCR is needed to validate the potential role of dynamic changes in TILs after neoadjuvant therapy.

As described in *Table 2*, several studies have evaluated the prognostic role of the presence and density of TILs on survival outcomes of patients with eTNBC. Although some studies were carried out prior to the standardization of TILs assessment, the positive correlation between TILs and better prognosis has been consistent. The findings consistently show a linear behavior, where for each 10% increase in the density of sTILs, there is a reduction in the risk of the event (recurrence or death) in the order of 5% to 20%. Two studies (57,64) describe excellent survival outcomes, with 10-year OS rates of around 95% in patients with eTNBC and high TILs. Loi *et al.* conducted a pooled analysis of 2,148 patients undergoing adjuvant treatment for TNBC (83). The study showed that stage II high TILs patients had better prognosis and improved OS compared to clinical stage I with lower infiltration of TILs (79). This important and surprising result underscores the concept that biological characteristics should be better understood and further evaluated for risk classification of BC patients. They suggest that TILs can be superior to the anatomical staging system used dogmatically in breast oncology for decades.

The prognostic correlation was also evident in a cohort of patients who did not undergo chemotherapy treatment. Park *et al.*, evaluated approximately 480 patients with early disease, exclusively submitted to local treatment and not exposed to any systemic therapy, and showed an impressive long-term OS in those patients with a high percentage of sTIL (≥30%) (80). In the same context, a Dutch cohort evaluated more than 400 patients aged <40 years and with node-negative disease, not submitted to any systemic treatment. Patients with high TILs (more than 75%) had better prognosis and a reduced risk of distant recurrence and death than those with low TILs (78). The 15-year incidence of metastases or death of was only 2.1%, and compared favorably to the 38% observed in the low TIL cohort (those with less than 30%) (64). These data raise the question of whether TILs could be used in clinical practice to guide de-escalation strategies for patients with early disease and "high TILs". This provocative concept is summarized in *Figure 2* (84), although it is important to emphasize that it requires further validation.

In addition to the quantitative analysis of TILs, evaluating the subtype of T cells in the TME is extremely relevant and has been addressed in several studies. As mentioned, different T cell subtypes can play an activating or suppressing role in carcinogenesis, progression and treatment response, depending on the cell subpopulation

Table 1 Prevalence of TILs in eTNBC

Study	Number of TNBC patients	Cutoff to define "high TILs"	Prevalence
Kimura *et al.*, 2023, (61)	54	50%	High: 27.80%
			Low: 62.20%
Agarwal *et al.*, 2023, (62)	108	60%	High (≥60%): 15.7%
			Intermediate (11–59%): 29.9%
			Low (≤10%): 34.4%
Candelaria *et al.*, 2022, (63)	284	20%	High: 32%
			Low: 68%
de Jong *et al.*, 2022, (64)	441	75%	High (≥75%): 21%
			Intermediate (31–74%): 27%
			Low (≤30%): 52%
Stecklein *et al.*, 2023, (65)	110	20%	High: 51%
			Low: 49%
Sharma *et al.*, 2022, (66)	117	30%	High: 47%
			Low: 53%
Gluz *et al.*, 2022, (67)	336	60%	High: 13%
			>10%: 33.8%
			≤10%: 66.2%
Loibl *et al.*, 2019, (68)	174	60%	High (≥60%): 14%
			Intermediate (11–59%): 48%
			Low (≤10%): 38%
Denkert *et al.*, 2018, (55)	906	60%	LPBC: 30%
Galvez *et al.*, 2018, (69)	86	50%	High: 50%
O'Loughlin *et al.*, 2018, (70)	75	50%	LPBC: 12%
Adams *et al.*, 2014, (56)	481	50%	LPBC (≥50%): 5%
			Intermediate (11–49%): 75%
			Low (≤10%): 20%
Pruneri *et al.*, 2016, (71)	897	50%	LPBC: 21.9%
Pruneri *et al.*, 2016, (57)	647	50%	LPBC: 18%
Tian *et al.*, 2016, (58)	425	50%	LPBC: 3.5%
Denkert *et al.*, 2015, (72)	314	60%	LPBC: 28.3%
Denkert *et al.*, 2010, (54)	1,058	60%	High: 61%

TILs, tumor-infiltrating lymphocytes; eTNBC, early-stage TNBC; TNBC, triple-negative breast cancer; LPBC, lymphocytic predominantly breast cancer.

Table 2 TILs as a prognostic biomarker in eTNBC

Study	N	Outcomes	Results
Agarwal *et al.*, 2023, (62)	108	DFS and OS	High sTIL associated with better DFS and OS
de Jong *et al.*, 2022, (64)	441	OS and DRFS	Each 10% sTIL decrease risk of death in 19% (hazard ratio: 0.81)
De Jong *et al.*, 2020, (78)	481	OS and DRFS	Each 10% sTIL decrease risk of event in 17% (hazard ratio: 0.83)
Loi *et al.*, 2022, (79)	2,148	IDFS, DDFS, and OS	Each 10% sTIL decrease risk of event in 13–17% (IDFS, hazard ratio: 0.87; DDFS, hazard ratio: 0.83; OS, hazard ratio: 0.84)
Gluz *et al.*, 2022, (67)	336	IDFS, DDFS, and OS	High sTIL associated with better IDFS, DDFS, and OS
Gao *et al.*, 2020, (59)	18,170	DFS and OS	High sTIL associated with increased DFS (hazard ratio: 0.907) and OS (hazard ratio: 0.869)
Park *et al.*, 2019, (80)	476	IDFS, DDFS, and OS	High sTIL stage I: 5-year IDFS 91%; 5-year DDFS 97%; 5-year OS 98%
Denkert *et al.*, 2018, (55)	906	DFS and OS	Each 10% sTIL decrease risk of event in 7–8% (DFS, hazard ratio: 0.93; OS, hazard ratio: 0.92)
Leon-Ferre *et al.*, 2018, (81)	605	IDFS and OS	Lower TIL associated with worse IDFS and OS
Pruneri *et al.*, 2016, (71)	897	DFS, DDFS, and OS	Each 10% TIL better DFS, DDFS, and OS
Pruneri *et al.*, 2016, (57)	647	BCFI, DFS, DRFI, and OS	Each 10% TILs decrease risk of event 11–17% (BCFI, hazard ratio: 0.87; DFS, hazard ratio: 0.89; DRFI, hazard ratio: 0.84; OS, hazard ratio: 0.83)
Tian *et al.*, 2016, (58)	425	DFS, DDFS, and OS	Each 10% sTILs decrease risk of event 5% (recurrence or death)
Dieci *et al.*, 2015, (82)	199	OS	OS 10-year: HT 89%, LT 68%
Adams *et al.*, 2014, (56)	481	DFS, OS, and DRFI	Each 10% sTIL decrease risk of event in 14%
Dieci *et al.*, 2014, (77)	278	MFS and OS	Each 10% sTIL decrease risk of event in 21% (mets or death)
Loi *et al.*, 2013, (83)	256	DFS and OS	Each 10% sTIL decrease risk of event 15–17%

TILs, tumor-infiltrating lymphocytes; eTNBC, early-stage TNBC; TNBC, triple-negative breast cancer; DFS, disease-free survival; OS, overall survival; sTIL, stromal TIL; DRFS, distant relapse-free survival; IDFS, invasive DFS; DDFS, distant DFS; BCFI, breast cancer-free interval; DRFI, distant recurrence-free interval; HT, high TILs; LT, low TILs; MFS, metastasis-free survival.

identified (85). A TME with predominantly $CD8^+$ and $CD4^+$ T cells infiltration has been correlated with good prognosis in various cancer types (48,50,86). In contrast, a greater infiltration with Tregs (associated with immunosuppression) determines a worse prognosis and associates with more aggressive tumors, unfavorable clinicopathological characteristics (high tumor grade, high-risk disease), late relapses, and worse survival (87-90). The correlation between infiltrating Treg and $CD8^+$ T lymphocytes has been shown to have prognostic and predictive significance, depending on the location and density of each subpopulation (88). Although the prognostic impact is evident and consistent in several studies, so far there is no support in the literature for using the TILs as a biomarker to omit or escalate systemic treatment (49).

TILs as a predictive biomarker in eTNBC

The neoadjuvant approach is considered an optimal scenario to evaluate the interaction between anticancer drugs and tumor response (88). Studies have shown that higher levels of TILs within the TME are associated with a greater likelihood of achieving a pCR following NACT in eTNBC. A pCR is correlated with better long-term outcomes and survival rates. Studies have elicited a linear relationship between the level of TILs and clinical and pathological response rates in TNBC patients submitted to NACT, as summarized in *Table 3*. The addition of platinum use in the neoadjuvant regime was evaluated in the GeparSixto study, which included 314 patients with eTNBC. The study demonstrated a positive association for pCR in patients with

LPBC. In this study, the TNBC patients with LPBC treated with a platinum salt had a pCR rate of 75% and the chance of a pCR was 3.71-fold compared to that of non-LPBC

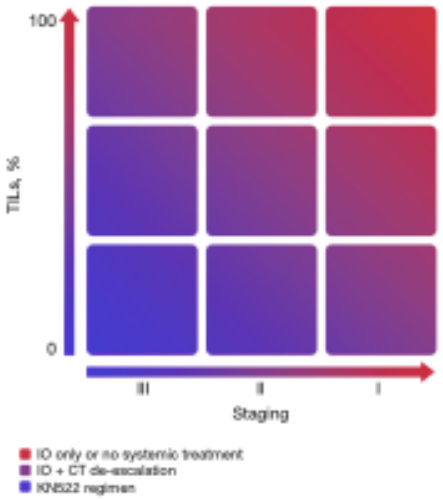

Figure 2 TILs density as a neoadjuvant de-escalation. Adapted from Bonadio *et al*. [2022] (84) with permission. TILs, tumor-infiltrating lymphocytes; IO, immunotherapy; CT, chemotherapy; KN522, KEYNOTE-522 trial.

population (72). Another study demonstrated numerically higher pCR, but no statistical difference, between platinum-based and non-platinum-based regimens in the high TIL subgroup (91). These data suggest that the subpopulations of T cells involved in the inflammatory infiltrate in the TME may have an impact on the response to treatment. This is in line with the rationale for an immunomodulatory function of the immune infiltrate and with the prognostic impact we previously discussed. Furthermore, available data also suggests that higher $CD8^+/FOXP3^+$ ratios and high levels of $CD4^+$ and $CD8^+$ TILs are associated with a greater probability of obtaining pCR in eTNBC (88,96). In addition, the presence of TILs both at presentation and in the residual disease after exposure to systemic treatment is associated with a favorable outcome and seems to be inversely related to the activation of the cell proliferation RAS/MAPK pathway (88).

The impact of TILs has also been evaluated in tumors treated with immunotherapy in combination with NACT. A correlation between *s*TILs and pCR has been demonstrated, suggesting a predictive value for TILs in response to ICI. These data must be cautiously considered, as they often result from exploratory analyses (97). Studies evaluating the use of pembrolizumab in the context of neoadjuvant therapy in the KEYNOTE-173 (92), I-SPY (98), and NeoPACT (66)

Table 3 TILs as a predictive biomarker in eTNBC

Study	Number of TNBC	High TILs cutoff	pCR high TILs	pCR low TILs
Abdullaeva *et al*., 2023, (91)	132	40%	63.3%	46.1%
Agarwal *et al*., 2023, (62)	108	60%	52.90%	21.10%
Sharma *et al*., 2022, (66)	117	30%	78%	45%
Gluz *et al*., 2022, (67)	336	60%	59.30%	29%
Bianchini *et al*., 2020, (76)	260	40%	71%	–
Schmid *et al*., 2020, (92)	60	40%	74–78%	–
Denkert *et al*., 2018, (55)	906	60%	50%	31%
Loibl *et al*., 2019, (68)	174	60%	OR 3.09 HT × IT	
Herrero-Vicent *et al*., 2017, (93)	164	40%	88%	9%
Tomioka *et al*., 2018, (94)	32	30%	30%	21%
Hida *et al*., 2016, (95)	48	50%	63%	17%
Denkert *et al*., 2015, (72)	314	60%	75%	–
Denkert *et al*., 2010, (54)	1,058	60%	40%	7%

eTNBC, early-stage TNBC; TNBC, triple-negative breast cancer; TILs, tumor-infiltrating lymphocytes; pCR, pathological complete response; OR, odds ratio; HT, high TILs; IT, intermediate TILs.

studies, explored the association of high TILs and pCR. An analysis of the NeoPACT study hypothesizes that lymphocyte-dependent response mechanisms dominate the therapeutic response in sTIL-high tumors. In contrast, in sTIL-low tumors, the response may be more related to the proliferation index, identified by proliferation gene expression signatures, with no impact from the TILs population (65). Studies evaluating other checkpoint inhibitors, such as Atezolizumab and Durvalumab, also demonstrate a positive association between TILs and pCR rates. The NeoTRIP study found a pCR rate of 74.1% in the population with TILs >40% before cycle 2 (76). The NeoMONO study evaluated biomarkers that can predict early response or resistance to immunotherapy in a cohort of 101 patients. Preliminary data showed that high TILs at the onset or increased TILs after 2 weeks of atezolizumab monotherapy are associated with an increased likelihood of pCR, with rates around 85% (99). The GeparNuevo study evaluated the combination of Durvalumab or placebo with chemotherapy and stratified patients by TIL density. The population with high TILs (>60%) had higher pCR rates in both groups (100).

In summary, TILs are emerging as a valuable prognostic and potentially predictive biomarker in eTNBC, providing important information about the tumor's immune landscape and response to therapy. Some international consensuses, such as European Society for Medical Oncology (ESMO) and St. Gallen (101,102), endorse the prognostic relevance of TILs and advocate the routine inclusion of TILs count in the pathological report. However, we recognize that the current data are mostly based on retrospective exploratory analyses and do not meet the criteria for clinical utility, defined by Hayes *et al.*, which consider the analytical validity of the test, the significance of related results, and the magnitude of impact, in addition to the level of evidence that determines the applicability of the test (103). Further validation with prospective data are lacking to support the use of this biomarker to optimize and individualize our current therapeutic strategies for these patients.

Future perspectives

Biomarker development remains a very challenging endeavor. We clearly recognize different populations in clinical practice but lack effective tools to set apart groups of patients that may have different biology and require different therapeutic strategies. Translating knowledge of TILs into clinical practice and their use as an effective and reliable biomarker faces several difficulties related to tumor heterogeneity, dynamic variability of TILs, data interpretation, different assessment techniques and the complexity of the TME. In addition to the proposal of the TILs working group, new technologies, such as automated methods of immunofluorescence image analysis, next-generation sequencing (NGS) and the use of transcriptomic data, can also contribute to a greater understanding and precision in the quantification of TILs and potentially improve clinical applicability. Among other references, a transcriptomic signature was correlated with TILs assessed by histology in a cohort of patients with early BC. The declared signature was found to be a good biomarker associated with DFS and OS in an analysis adjusted for molecular and clinical variables, with better survival in basal and HER2 tumor types (104). The use of multiplexed immunofluorescent imaging and NGS that can determine the spatial distribution of specific immunophenotypes has also showed an impact on TNBC. A study evaluated the development and validation of a gene classifier for spatial immunophenotype and found positive results with response to checkpoint inhibitor (anti-PD1) treatment independently of currently used clinical markers (105). In the future, digital pathology, machine learning techniques, and better identification of TILs subpopulations will help to standardize pathology evaluation and expand our ability to validate tumor immune infiltration as a clinically meaningful biomarker (106).

Despite the growing amount of information on the subject, more clinical research and prospective translational studies are needed to unravel the potential role of TILs in guiding therapy choices. Several ongoing clinical trials are exploring the use of TILs and their potential impact on the treatment and outcomes of eTNBC. High TILs are being considered as an inclusion criterion for therapeutic personalization strategies. For example, the NeoTRACT study (NCT05645380) uses an early assessment combining radiologic response and TILs status to de-escalate chemotherapy (anthracycline-free protocol). This is a non-randomized phase II study that evaluates a treatment de-escalation strategy according to the expression of initial TILs (<5%, 5–29%, and ≥30%) whose primary objective is pCR rate in a high-sTIL cohort with radiographic complete response. The Dutch BELLINI (107) study demonstrates that a significant proportion of patients with TNBC and high TILs had marked immune activation after 4 weeks of neoadjuvant therapy with ICI, highlighting the potential for developing randomized clinical trials with immunotherapy

without chemotherapy for selected patients in this setting.

On a different perspective, the outstanding achievements of chimeric antigen receptor (CAR)-T cell therapy in hematological malignancies and the promising effects of adaptive cell therapies (ACTs) in solid tumors have prompted the search for more suitable targets or combination programs to expand this therapeutic approach to solid tumors. ACT with expansion of TILs may more accurately identify targets in tumor cells (108). Strategies in development have been focused on metastatic disease, where case reports with favorable outcomes have already been described. However, it is possible to envisage applying strategies involving ACT in treating earlier stages of BC. Other forms of immunotherapy, such as vaccines and antibody-drug conjugates, are also being extensively studied in eTNBC. TILs may also be useful as biomarkers in these scenarios (109).

Conclusions

Even though we have advanced in our understanding of the disease, the increasingly and evolving complex biology of cancer remains a challenge and compromises our ability to improve outcomes. Both progression of the disease and resistance development represent formidable hurdles. As the current tumor genetic sequencing associated excitement seems to be wearing off, progressively, we are focusing on other important aspects of the disease and discovering host-related tumor enabling properties that are potential treatment targets. The study of TILs falls in this scenario. The expression of TILs in early TNBC has shown clinical relevance in several studies that are consistent in showing the association of higher density of TILs and favorable outcomes in terms of both survival and response to neoadjuvant treatment. This impact supports the development of studies that evaluate the expression of TILs as stratification factors or the conduct of studies that can evaluate the usefulness and clinical validation of the biomarker. While the mere presence of TILs seems rather consistently to have a prognostic impact, more granular characterization of the type of lymphocytes seems to be required to better understand the biological significance of the infiltrate and adds value to prognostication.

Although the therapeutic impact of the immune system has been clearly established in many different tumors, better characterization of the specific immune response in a given tumor will certainly better inform and qualify our therapeutic strategy. Imbedded in the very challenging endeavor of biomarker development, we need more well-conducted clinical studies to address both the prognostic and predictive value of TILs. The evidence we present, supports that the lymphocyte infiltration in early BC seems to have a prognostic impact that can be clinically useful to characterize patient populations with very different biology and that can be amenable to escalation or de-escalation strategies. Ongoing and future studies should shed light on the clinical practical application of the immune infiltration in early BC.

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Footnote

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8 CONSIDERAÇÕES FINAIS

O desenvolvimento de biomarcadores continua sendo ponto de fundamental relevância para definição de terapêutica personalizada. Apesar da crescente quantidade de informações sobre o impacto dos TILs na evolução da doença, e da padronização de avaliação pelo TILs working group (Salgado *et al.*, 2015), mais pesquisas clínicas e estudos translacionais prospectivos são necessários para compreender o papel potencial dos TILs como biomarcador. O "subtipo" HER2-low, ganhou destaque recentemente tendo em vista a alta sensibilidade a terapia alvo antiHER2 com o desenvolvimento dos novos ADCs (Modi *et al.*, 2022).

O presente estudo faz parte de uma linha de pesquisa que visa avaliar o comportamento da doença HER2low e possíveis individualidades que pudessem classificá-la como um novo subtipo tumoral. A análise avaliou o impacto de TILs na população HER2 negativo com foco no papel do infiltrado celular no microambiente tumoral do câncer de mama HER2-low. De acordo com nosso resultado os pacientes com doença HER2-low compartilham características com casos HER2 zero. A mediana de distribuição de TILs foi numericamente inferior na população HER2-low versus HER2 zero (10 e 15%, respectivamente). Avaliando dados apresentados na literatura, análise de uma coorte de 529 pacientes, enriquecida por população luminal (57,6%), encontrou diferença significativa com menor densidade de TILs na população HER2-low quando comparado a HER2-zero. Interessantemente, ainda neste estudo a população HER2-low também apresentou expressão gênica relacionada a pior prognóstico e depleção da imunidade, sugerindo que tumores HER2-low possam ter menor resposta imune quando comparados a HER2-zero (van den Ende *et al.*, 2022). Contrariando estes achados, estudo avaliou 178 pacientes com câncer de mama estágio I a III e demonstrou que a população HER2-low apresenta porcentagem de TIL discretamente superior quando comparado a HER2 zero (Fernandes *et al.*, 2023). Já outro estudo avaliando pacientes com câncer de mama avançado demonstrou semelhança nas característica clínico-patológica, incluindo infiltração de sTILs, sem diferença nas populações HER2 zero e low.

Quanto ao padrão hormonal, em nosso estudo a população com doença luminal apresentou menor infiltrado linfocitário do que tumores triplo-negativos, tanto na doença HER2-low quanto HER2 zero. Quando avaliada isoladamente a população triplo

negativa, percebemos uma tendência de maior frequência de TILs alto (>=20%) na doença HER2-zero, em relação ao HER2-low, porém esta diferença não teve diferença estatística. Vale ressaltar que a análise foi exploratória e com grande desbalanço entre os grupos. Resultado discrepante foi demonstrado em outra análise retrospectiva, sugerindo que tumores luminais tivessem maior densidade TILs em tumores HER2 zero, enquanto em tumores com RE negativo a densidade de TILs foi inferior no HER2 zero quando comparado a HER2-low (Lu *et al.*, 2023).

Houve correlação positiva entre grau tumoral e maior densidade de TILs. Quando analisado o impacto em resposta patológica, a mediana de TILs foi maior naqueles pacientes que atingiram pCR, e a correlação foi estatisticamente significativa, independente do status HER2 (zero ou low). Apesar do caráter linear do impacto de TILs, tanto como fator preditivo, quanto prognostico, conforme demonstrado em inúmeros estudos, encontramos um ponto de corte de 20% com impacto significativo em desfecho de reposta patológica.

Nosso estudo não avaliou o potencial impacto prognóstico. Lu et al., não encontraram impacto de TILs em sobrevida especificamente na doença RE positivo, mas demonstrou TILs como fator prognóstico independente na população em geral (Lu *et al.*, 2023). Em contraste a este achado, Sun et al (Sun *et al.*, 2023) avaliaram 1763 pacientes e encontraram associação positiva de altos níveis de TILS e melhora de sobrevida, especialmente no subtipo RE positivo. Enquanto Yue et al. não demonstrou impacto prognóstico relacionado ao status HER2-low ou a expressão de TILs (Yue *et al.*, 2023). Percebemos que os achados são discordantes e o número de estudos abordando o tema ainda permanece bastante reduzido.

Ponto fundamental para a determinação de biomarcador é a confiabilidade na avaliação da variável. Em nosso estudo encontramos boa concordância entre patologistas, sendo 84,4% quando o limiar foi 20% e 96,9% quando usamos o limiar de 50%, com coeficiente de Kappa de 0,6825 e 0,7838, respectivamente. Esses dados são semelhantes ao encontrado por Denkert et al. (Denkert *et al.*, 2016). O estudo avaliou a concordância na avaliação de TILS em 120 amostras entre vários patologistas em duas rodadas. Os valores de Kappa ao final da segunda rodada, nos cortes referidos 20% e 50%, foram 0,65 e 0,72, com taxas de concordância 85% e 93%, respectivamente (Denkert *et al.*, 2016).

A principal limitação do presente estudo inclui o pequeno tamanho da amostra, principalmente na análise de subgrupos, além do caráter retrospectivo e o desequilíbrio de pacientes com diferentes status de HER2. Todas as análises são exploratórias, sem

cálculo amostral prévio, portanto, mesmo nos casos onde se encontrou associação positiva na análise estatística, a credibilidade do resultado é baixa. Dentre os pontos fortes do estudo, destaca-se a metodologia que permitiu análise centralizada padronizada de TILs conforme diretrizes internacionais de todas as pacientes, e a originalidade. Apesar de atualmente existirem algumas publicações avaliando TILs na população HER2-low, nenhuma delas foca no impacto preditivo para resposta patológica como desfecho.

Os achados do estudo e da revisão de artigos publicados até o momento permitem concluir que a baixa expressão de HER2 isoladamente não confere um papel preditivo de resposta a QT neoadjuvante e o impacto de TILs nesta população específica permanece incerto. Sugerimos que a doença HER2-low não se trata de um novo subtipo tumoral, mas sim um marcador de resposta terapia alvo.

9 PERSPECTIVAS

O surgimento e aprovação de fármacos como os ADC estimulam o aprofundamento de estudos que permitam maior compreensão de complexa biologia do câncer. Neste contexto a avaliação de tumores HER2-low como alvo terapêutico ganhou espaço recentemente com a aprovação de droga ativa neste contexto. A necessidade de melhorar os resultados, tanto à resposta ao tratamento, quanto a sobrevida, passa pela descoberta de biomarcadores que permitam o ajuste terapêutico direcionado para cada doença e cada paciente. O estudo dos TILs se enquadra nesse cenário. Vários ensaios clínicos estão em andamento explorando a densidade de TILs (como critérios de inclusão ou de estratificação) e seu impacto potencial no tratamento e nos resultados, especialmente na população de pacientes com câncer de mama triplo negativo (independentemente do status HER2, zero ou low). Não foi encontrado estudo em andamento avaliando TILs de forma prospectiva especificamente na população HER2-low.

Ainda são necessários estudos prospectivos para determinar a aplicabilidade clínica deste biomarcador. Enquanto a simples presença de TILs parece ter um impacto prognóstico bastante consistente, uma caracterização do tipo de linfócitos parece ser necessária para melhor entender o significado biológico do infiltrado. No futuro, a patologia digital deve auxiliar na análise quantitativa e na identificação das subpopulações de TILs permitindo a validação do uso da infiltração imune do tumor como um biomarcador clinicamente significativo.

Perspectivas futuras incluem manter o interesse em compreender a biologia tumoral a fim de constantemente proporcionar a personalização do tratamento do câncer. As evidências que apresentamos suportam que a infiltração linfocitária no câncer de mama inicial tem impacto prognóstico e pode ser clinicamente útil para caracterizar populações de pacientes com biologia tumoral distinta passíveis de estratégias de escalonamento ou descalonamento de tratamento. A linha de pesquisa, desenvolvida em parceria entre a UFRGS e UNICAMP e na qual este estudo está contemplado, prevê a análise de dados clínico-patológicos e genômicos de cerca de 600 pacientes. Pretendemos refazer as análises sobre o impacto dos TILs com o número completo de participantes e também abordar o impacto prognóstico, não avaliado nesta análise. Este será o maior estudo nacional de que temos conhecimento abordando a população HER2 negativo com foco em HER2-low, correlacionando achados de características tumorais (fenotípico e genotípicos) com desfechos clínicos, possibilitando a avaliação de biomarcadores para a individualização terapêutica.

10 ANEXO

10.1 ANEXO 1 - STROBE CHECKLIST

Tabela de itens que devem ser descritos em estudos observacionais, segundo a declaração Streightening the reporting of observational studies in epidemiology (versão em português adaptada da publicação por Malta M et al, Rev Saude Publica 2010, 44(3): 559-65)

	Item No	Pág No
Título e Resumo	1	1, 6
Introdução		
Contexto/Justificativa	2	13, 38
Objetivos	3	39
Métodos		
Desenho do estudo	4	55
Contexto	5	55
Participantes	6	55
Variáveis	7	56
Tamanho do estudo	8	56
Métodos estatísticos	9	56
Resultados		
Dados descritivos	10	56
Resultados principais	11	57
Discussão		
Interpretação	12	58, 85
Limitações	13	59, 87