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PÓS-GRADUAÇÃO EM ONCOLOGIA

JESSÉ LOPES DA SILVA

CÂNCER DE MAMA TRIPLO-NEGATIVO: AVALIAÇÃO DE FATORES CLÍNICOS E BIOMARCADORES TUMORAIS

Orientadora: Profa. Dr. Andréia Cristina de Melo
Co-orientador: Prof. Dr. Luiz Claudio Santos Thuler

RIO DE JANEIRO-RJ
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Tese apresentada ao Instituto Nacional de Câncer como parte dos requisitos para obtenção do título de Doutor em Oncologia

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CÂNCER DE MAMA TRIPLO-NEGATIVO: AVALIAÇÃO DE FATORES CLÍNICOS E BIOMARCADORES TUMORAIS

JESSÉ LOPES DA SILVA

RESUMO

Introdução: O câncer de mama triplo negativo é amplamente conhecido por se tratar de uma doença com comportamento biológico e clínico heterogêneos. Diferentes subclassificações moleculares já são propostas baseadas em biomarcadores específicos pelo perfil de imunohistoquímica (IHQ) no tumor e no microambiente tumoral. **Objetivo:** Avaliar a influência de fatores sociodemográficos e clinicopatológicos de IHQ (LITs, CD8+, RA, PD-L1, PD-1, PD-L2, FOXP3, CD4, CD3, CD 56, CD68, CK 14, CK 17, CD 117, níveis de Ki67 e p53) na predição de resposta clínica a quimioterapia neoadjuvante assim como nos desfechos de sobrevida. **Material e métodos:** Coorte retrospectiva envolvendo toda população de mulheres com CMTN localmente avançado, matriculadas no INCA entre 2010 e 2014, que iniciaram quimioterapia neoadjuvante e que foram submetidas à abordagem cirúrgica curativa. **Resultados:** 235 mulheres foram avaliadas no artigo 1. O tempo mediano de seguimento foi de 64,3 meses. A maioria das pacientes apresentava estadio clínico avançado (III: 85,1%; cT3/T4: 86,4%; cN1-3: 74,4%) e tumores de alto grau (72,1%). O estadiamento clínico (III versus II, *hazard ratio* [HR] ajustado = 2,95, p = 0,012) influenciou significativamente a taxa de resposta patológica completa (RPC). A ingestão de álcool influenciou negativamente a sobrevida livre de eventos (SLE, HR ajustado = 1,67, p = 0,006) e a sobrevida global (SG, HR ajustado = 1,89, p = 0,005). Mulheres com RPC apresentaram melhor SLE (HR bruto = 0,15, p < 0,001) e SG (HR bruto = 0,12, p < 0,001) em comparação com não-RPC. Nos artigos 2 e 3, foram avaliados 171 casos (para 64 casos, não foi possível recuperar o material patológico). Pacientes com carga residual de câncer (RCB) 0-1 corresponderam a 28,7% dos casos e as com razão linfonodal (LNR) de baixo risco representaram 77,2%. A alta expressão de Ki67 mostrou correlação significativa apenas com tumores de grau 3 (p = 0,0157). A prevalência de marcadores tumorais de imunohistoquímica (IHQ) na core biópsia foi bastante variável e nenhum deles impactou significativamente desfechos de resposta ou sobrevida. Neste ponto, em relação à análise de SLE e SG, estadio clínico (p = 0,014 e p = 0,042, respectivamente), RCB (p < 0,0001 e p < 0,0001, respectivamente) e LNR (p < 0,0001 e p < 0,0001, respectivamente) mostraram associação significativa. Nenhum dos marcadores imunes de IHQ na core biópsia mostrou uma associação significativa com SLE ou SG. Na avaliação de marcadores dos tumores residuais dos 134 pacientes com amostra disponível, os pacientes com alta expressão de CD14 tiveram SLE significativamente mais curta (p = 0,015), enquanto os pacientes com alta expressão dos marcadores CD3 (p = 0,025), CD8 (p = 0,030), FOXP3 (p = 0,005), altas proporções CD4/FOXP3 (p = 0,034) e CD8/FOXP3 (p = 0,008), foram associados com SLE. Apenas a alta expressão de CD4 foi associada com o desfecho de SG (p = 0,038). **Conclusão:** O estágio clínico III foi associado a menor taxa de resposta e pior sobrevida. A ingestão de álcool, pCR e RCB mostraram influência estatisticamente significativa com os desfechos de sobrevida. Nenhum dos marcadores tumorais ou de microambiente tumoral avaliados por IHQ na core biópsia mostrou associação significativa com desfechos de resposta ou sobrevida. O painel de marcadores imunes em tumores residuais na peça cirúrgica pode ser um fator determinante no prognóstico de pacientes com CMTN.

Palavras-chave: 1. Câncer de mama triplo negativo 2. Quimioterapia neoadjuvante 3. Biomarcadores tumorais 4. Infiltrado linfocitário tumoral 5. Microambiente tumoral.

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TRIPLE NEGATIVE BREAST CANCER: EVALUATION OF CLINICAL FACTORS AND TUMOR BIOMARKERS

JESSÉ LOPES DA SILVA

ABSTRACT

Introduction: Triple negative breast cancer (TNBC) is widely known to be a disease with aggressive but heterogeneous biological and clinical behavior. Different molecular subclassifications are already proposed based on specific biomarkers by the immunohistochemical (IHC) profile in the tumor and tumor microenvironment. **Objective:** To evaluate the influence of sociodemographic and clinical pathological factors of IHC (LITs, CD8 +, RA, PD-L1, PD-1, PD-L2, FOXP3, CD4, CD3, CD 56, CD68, CK 14, CK 17, CD 117, Ki67 and p53 levels) in predicting clinical response to neoadjuvant chemotherapy as well as survival outcomes. **Materials and methods:** Retrospective cohort involving the entire population of locally advanced women with TNBC enrolled at INCA between 2009 and 2014 submitted to neoadjuvant chemotherapy (NACT) and underwent curative surgical approach. **Results:** 235 women were evaluated in article 1. The median follow-up was 64.3 months. Most patients had advanced clinical stage (III: 85.1%; cT3 /T4: 86.4%; cN1-3: 74.4%) and high-grade tumors (72.1%). Clinical staging (III versus II, adjusted hazard ratio [HR] = 2.95, p = 0.012) significantly influenced the pathological complete response (pCR) rate. Alcohol intake negatively influenced event-free survival (EFS, adjusted HR = 1.67, p = 0.006) and overall survival (OS, adjusted HR = 1.89, P = .005). Women with pCR showed better EFS (crude HR = 0.15, P < 0.001) and OS (crude HR = 0.12, p < 0.001) compared with non-pCR. In articles 2 and 3, 171 cases of were evaluated (for 64 cases, the pathological sample was unavailable). The residual cancer burden (RCB) 0–1 corresponded to 28.7% of cases and low-risk lymph node ratio (LNR) represented 77.2%. High Ki67 expression only showed a significant correlation with grade 3 tumors (p = 0.0157). The prevalence of immunohistochemistry (IHC) markers in core biopsy was quite variable and none of them significantly impacted response or survival outcomes. At this point, regarding the analysis of EFS and OS, clinical stage (p = 0.014 and p = 0.042, respectively), RCB (p < 0.0001 and p < 0.0001, respectively) and LNR (p < 0.0001 and p < 0.0001, respectively) showed significant association. None of the pre-NACT immune IHC markers showed a significant association with EFS or OS. As for post-NACT markers, patients with high expression of CD14 had significantly shorter EFS (p = 0.015), while patients with high expression of the markers CD3 (p = 0.025), CD8 (p = 0.030), FOXP3 (p = 0.005), high ratios CD4/FOXP3 (p = 0.034) and CD8/FOXP3 (p = 0.008), showed longer EFS. As for the 134 patients with available sample, only high expression of post-NACT CD4 showed significant association with OS (p = 0.038). **Conclusion:** Clinical stage III were associated with lower response rate and worse survival. Alcohol intake, pCR, and RCB have shown considerable influence on survival outcomes. The tumor markers and tumor microenvironment evaluated by IHQ in the core biopsy did not show a significant association with a response or survival outcomes. Post-NACT TILs subtype can be a determining factor in the prognosis of patients with TNBC.

Keywords: 1. Triple-negative breast cancer 2. Neoadjuvant chemotherapy 3. Tumor biomarkers 4. Tumor lymphocyte infiltrate 5. Tumor microenvironment.

LISTA DE ABREVIATURAS

AJCC	Do inglês, *American Joint Cancer Committee*
ARPC	Área Representativa de Pesquisa Clínica
CDI	Carcinoma *ductal invasor*
CDIS	Carcinoma *ductal in situ*
CIM	Carcinoma inflamatório de mama
CK	Citoqueratina
CLIS	Carcinoma lobular *in situ*
CMLA	Câncer de mama localmente avançado
CMTN	Câncer de mama triplo negativo
CTIs	Clusters de células tumorais isolados
DIPAT	Departamento de patologia
eCRF	Ficha clínica eletrônica
EGFR	Do inglês, epidermal growth factor receptor
FDA	Do inglês, Food and drug administration
GEP	Do inglês, gene expression. profile
GIST	Do inglês, gastrointestinal stromal tumor
HER2	Human epidermal growth factor receptor 2
HR	Hazard Ratio
IBGE	Instituto Brasileiro de Geografia e Estatística
IC	Intervalo de confiança
IHQ	Imunohistoquímica
INCA	Instituo Nacional de Câncer
LITs	Linfócito infiltrante tumoral
LOH	Do inglês, *Loss of heterogeneity*
LRA	*Luminal receptor* de androgênio
NA	Do inglês, *not available*
OMS	Organização Mundial de Saúde
OR	Odds Ratio
PARP	Poly (ADP-ribose) polymerase
PD-1	Do inglês, *Programmed cell death protein* 1
PD-L1	Do inglês, *Programmed death-ligand* 1
RA	Receptor de androgênio
RE	Receptor de estrogênio
RP	Receptor de progesterona
RPC	Resposta patológica completa
RR	Risco relativo
RT-PCR	do inglês, *reverse transcriptase polymerase chain reaction*
SG	Sobrevida Global
SLD	Sobrevida livre de doença
SLP	Sobrevida livre de progressão
SLE	Sobrevida livre de evento
TAI	Do inglês, *Telomeric allelic imbalance*
TCGA	Do inglês, The Cancer Genome Atlas
TRG	Taxa de resposta global
TRG	Taxa de resposta global
VEGFR	do inglês, Vascular *Endothelial Growth Factor Receptor*

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SUMÁRIO

1 INTRODUÇÃO

Atualmente, no contexto de saúde pública nacional, o câncer de mama é considerado um grande desafio nos planejamentos estratégicos de gestão para a saúde da mulher. A experiência pessoal na intensa rotina de preceptoria no ambulatório de oncologia clínica do Hospital do Câncer III do Instituto Nacional de Câncer faz com que este terreno se torne muito fértil para reflexões profundas acerca do comportamento biológico do câncer de mama. Dentre os diversos cenários clínicos, o subtipo câncer de mama triplo negativo (CMTN), em estágio localmente avançado, chama a atenção por apresentar padrões de resposta distintos ao tratamento com quimioterapia neoadjuvante, resultando em desfechos diferentes de recidiva e sobrevida. Algumas das pacientes apresentavam expressiva redução do tamanho tumoral com sobrevida satisfatória. Outras deterioravam rápido e agressivamente com progressão clínico-radiológica logo nos primeiros ciclos de tratamento, evoluindo com um tempo de vida muito curto. Estudar profundamente os fatores que possivelmente determinam esses diferentes padrões no contexto de CMTN é entender que diante de um subtipo neoplásico que, pela sua vasta heterogeneidade celular e influenciado pelo microambiente tumoral, merece uma subclassificação patológica mais apropriada, podendo futuramente possibilitar tratamentos personalizados e mais eficientes.

2 REVISÃO DA LITERATURA

2.1 Epidemiologia

Globalmente, o câncer de mama é o segundo tumor maligno mais comumente diagnosticado, logo após o câncer de pulmão. De acordo com as estatísticas do câncer pelo GLOBOCAN 2020, a estimativa mundial de incidência de câncer de mama em 2020 foi de mais de 2,2 milhões de casos novos diagnosticados (distribuição proporcional estimada de 47,8 por 100 mil mulheres ajustada pela idade) ao redor do mundo, alcançando quase 1 em cada 4 cânceres entre mulheres (Figura 2.1 e Figura 2.2), com prevalência estimada de mais de 6 milhões de casos num período de 5 anos (Figura 2.3). Portanto, trata-se da mais frequente neoplasia em mulheres, na grande maioria dos países do mundo acessíveis, para dados epidemiológicos (154 de 185), assim como da principal causa de morte por câncer em 159 países. Na América Latina, a incidência estimada em 2020 foi de mais de 200 mil novos casos (distribuição proporcional estimada de 51,9 por 100 mil mulheres ajustado pela idade), com prevalência estimada em 5 anos de mais de 710 mil mulheres com diagnóstico de câncer de mama (YIP *et al.*, 2008; BRAY *et al.*, 2018; CANCER TODAY, [*s. d.*]).

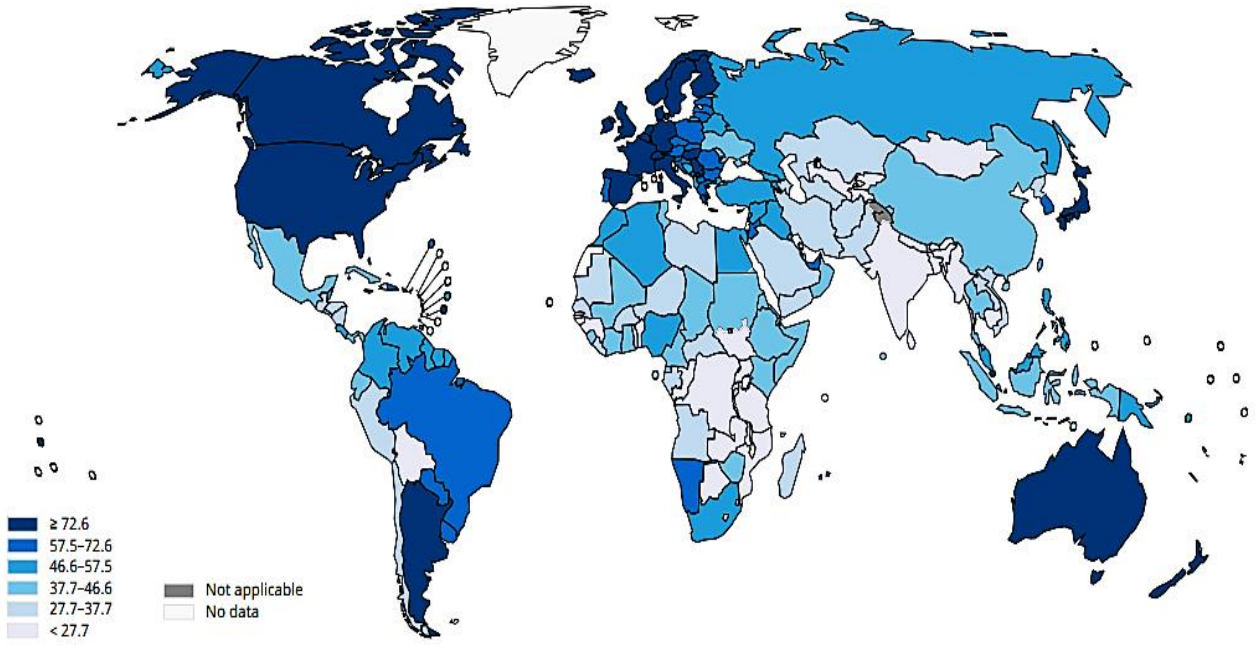

Figura 2.1 - Mapa com distribuição proporcional de incidência de câncer de mama padronizado por idade (mundo), todas as idades.
Fonte: GLOBOCAN 2020. Representação geográfica da incidência de câncer de mama no mundo para homens e mulheres. As cores mais escuras representam regiões de alta incidência, enquanto as cores mais claras representam regiões de baixa incidência. Valores ajustados por idade, por 100.000 habitantes.

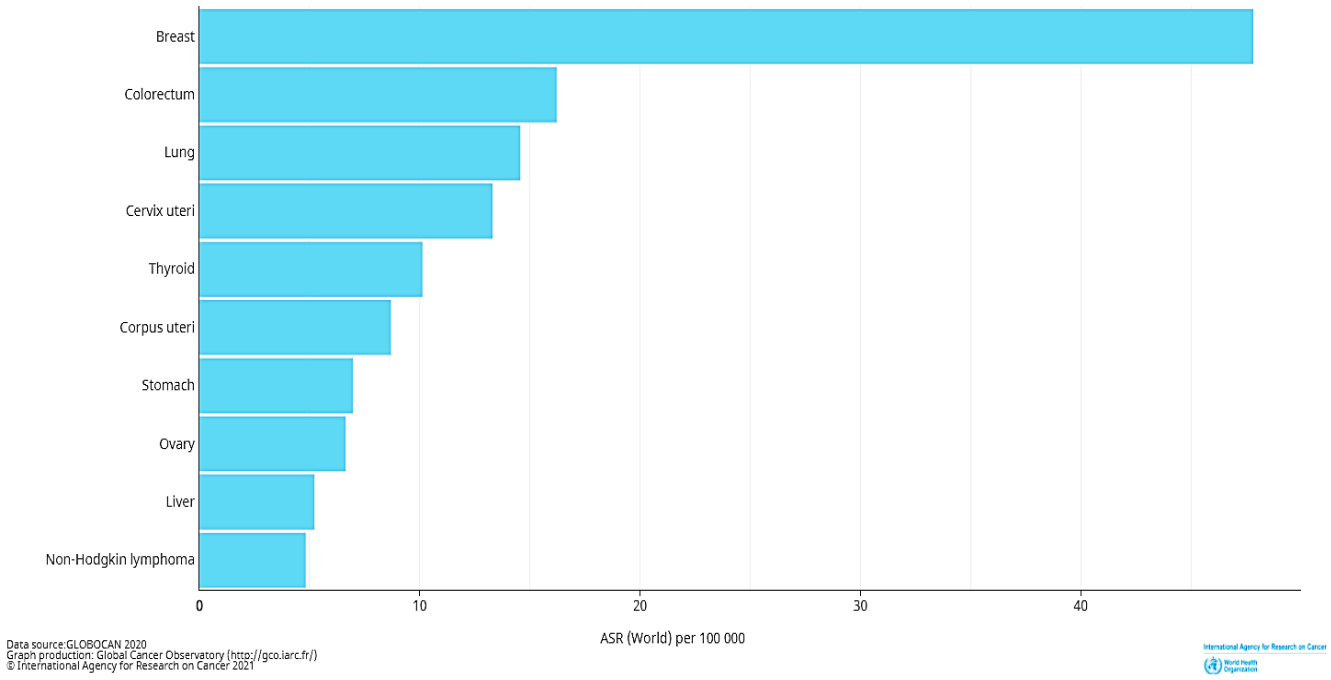

Figura 2.2 - Gráfico com distribuição proporcional de incidência de câncer de mama para mulheres no mundo.
Fonte: GLOBOCAN, 2020.

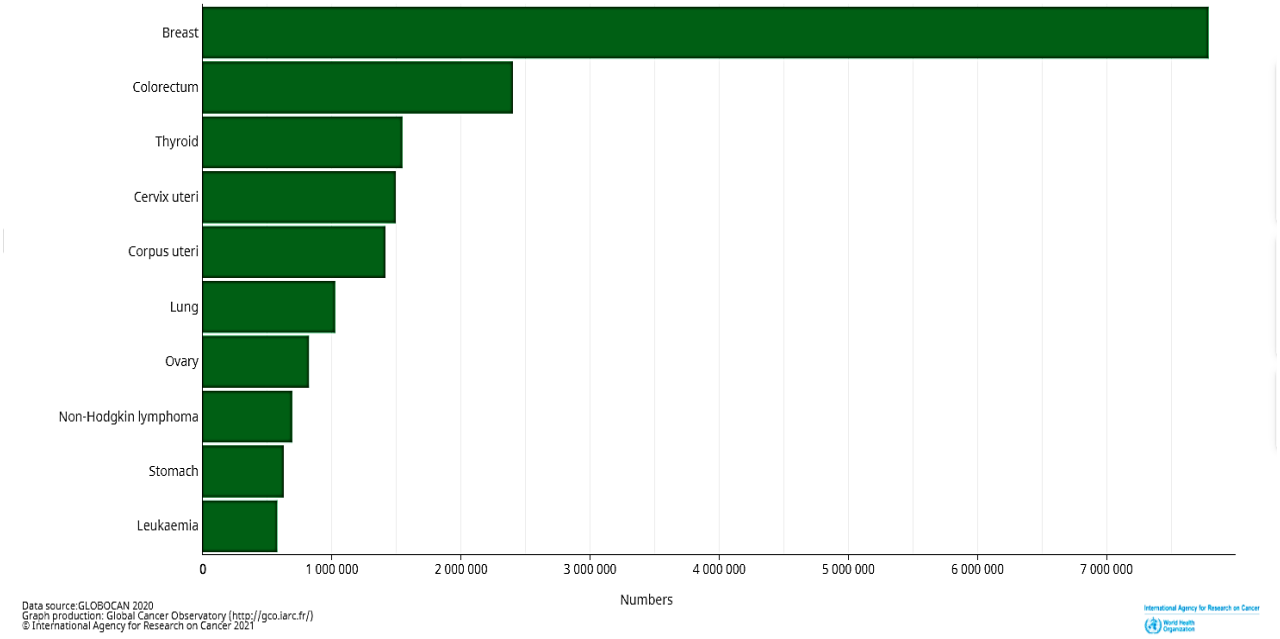

Figura 2.3 - Gráfico de prevalência em 5 anos para mulheres (Mundo).
Fonte: GLOBOCAN, 2020.

Segundo o Instituto Nacional de Câncer (INCA), foram estimados 66.280 novos casos de câncer de mama no Brasil para cada ano do triênio 2020/2022 (distribuição proporcional estimada de 61,61 por 100 mil mulheres ajustado pela idade) (Figura 2.4). Desconsiderando os tumores de pele não melanoma, esse tipo de câncer também é o mais frequente nas mulheres das regiões Sul (71,16 por 100 mil), Sudeste (81,06 por 100 mil), Centro-Oeste (45,24 por 100 mil) e Nordeste (44,29 por 100 mil). Na Região Norte, é o segundo tumor mais incidente (21,34 por 100 mil) (Figura 2.5) (INSTITUTO NACIONAL DE CÂNCER, [s.d.]a).

Apesar das taxas de mortalidade por câncer de mama terem se reduzido desde os anos 70, ainda assim esta comorbidade foi estimada como a quinta maior causa de morte por câncer em 2018 no mundo (KOHLER *et al.*, 2015; CARIOLI *et al.*, 2018). A curva de redução pode provavelmente ser atribuída à melhora nos sistemas públicos de rastreamento e incorporação de novas drogas mais eficazes no tratamento adjuvante (MUNOZ *et al*., 2014; GELDER *et al*., 2015). Foram estimadas 684.996 mortes por câncer de mama em todo mundo em 2020 (distribuição proporcional estimada de 13,6 mortes por 100 mil mulheres) (SUNG *et al.*, [*s. d.*]).

Na população brasileira, a taxa de mortalidade por câncer de mama ajustada pela população mundial revela uma curva ascendente, tornando-se a primeira causa de morte, 13,68 óbitos por 100 mil mulheres em 2015. As regiões Sul e Sudeste são as que apresentam as maiores distribuições proporcionais, com 15,26 e 14,56 óbitos por 100 mil mulheres em 2015, respectivamente. Quanto a mortalidade proporcional por câncer em mulheres, com 16,4% do total de óbitos em 2019, o câncer de mama ocupou o primeiro lugar no país no período 2011-2019. Percebe-se um padrão regional de evolução temporal muito similar, exceto na região Norte, onde os óbitos por câncer de mama ocupam o segundo lugar, com 12,5%. Os maiores índices de mortalidade proporcional por câncer de mama foram os do Sudeste (16,5%) e Centro-Oeste (16,1%), seguidos pelos Sul (15,2%) e Nordeste (14,8%) (ESTIMATIVA 2020, 2019).

Localização Primária	Casos	%
Próstata	65.840	29,2%
Cólon e reto	20.520	9,1%
Traqueia, brônquio e pulmão	17.760	7,9%
Estômago	13.360	5,9%
Cavidade oral	11.180	5,0%
Esôfago	8.690	3,9%
Bexiga	7.590	3,4%
Linfoma não Hodgkin	6.580	2,9%
Laringe	6.470	2,9%
Leucemias	5.920	2,6%

Homens

Mulheres

Localização Primária	Casos	%
Mama feminina	66.280	29,7%
Cólon e reto	20.470	9,2%
Colo do útero	16.590	7,4%
Traqueia,brônquio e pulmão	12.440	5,6%
Glândula tireoide	11.950	5,4%
Estômago	7.870	3,5%
Ovário	6.650	3,0%
Corpo do útero	6.540	2,9%
Linfoma não Hodgkin	5.450	2,4%
Sistema nervoso central	5.220	2,3%

*Números arredondados para múltiplos de 10.

Figura 2.4 - Incidência do câncer de mama no Brasil para ambos os sexos comparativamente com outros sítios.
Fonte: MS/INCA/Estimativa de Câncer no Brasil, 2020.

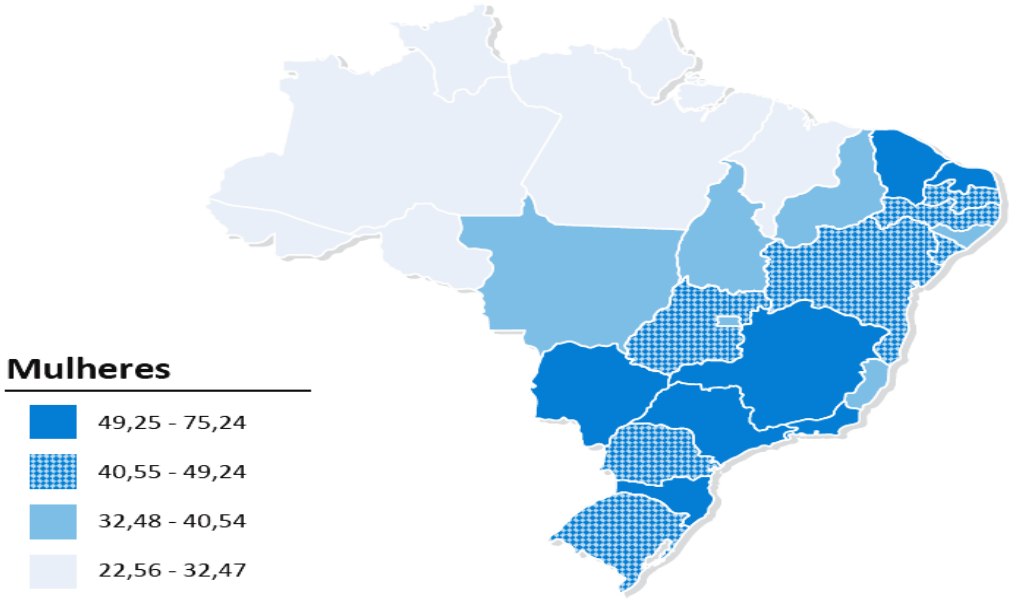

Figura 2.5 - Mapa da taxa de incidência de mama no Brasil.
Fonte: MS/INCA/Estimativa de Câncer no Brasil, 2020. Representação geográfica da incidência de câncer de mama no Brasil para mulheres. As cores mais escuras representam regiões de alta incidência, enquanto as cores mais claras representam regiões de baixa incidência. Valores ajustados por idade, por 100.000 habitantes.

Tabela 2.1 – Distribuição proporcional dos óbitos de acordo com a localização primária do tumor em mulheres estimada para o Brasil no ano de 2016.

- Em mulheres, Brasil, 2019

Localização Primária	Óbitos	%
Mama	18.068	16,4
Traqueia, Brônquios e Pulmões	12.621	11,4
Cólon e Reto	10.385	9,4
Colo do útero	6.596	6,0
Pâncreas	5.893	5,3
Estômago	5.475	5,0
Sistema Nervoso Central	4.663	4,2
Fígado e Vias biliares intrahepáticas	4.584	4,2
Ovário	4.123	3,7
Leucemias	3.356	3,0
Todas neoplasias	**110.344**	**100,0**

Fonte: MS/SVS/DASIS/CGIAE/Sistema de Informação sobre Mortalidade, 2021. MS/INCA/Coordenação de Prevenção e Vigilância/Divisão de Vigilância e Análise de Situação, 2021.

2.2. Fatores de risco

Fatores demográficos estão diretamente ligados ao câncer de mama. Trata-se de uma doença muito mais frequente em mulheres e mais rara em homens, que representam menos de 1% de todos os casos (BUZDAR; GIORDANO; HORTOBAGYI, 2002). Após o gênero, a idade é o fator de risco mais importante para o câncer de mama. A curva de incidência de câncer de mama aumenta consideravelmente com a idade, atingindo um pico logo após a menopausa, podendo evoluir com oscilações a partir de então (GOODMAN; KIM; YOO, 2015). Os fatores reprodutivos também estão relacionados ao câncer de mama. Essa correlação pode ser explicada pela hiperexposição aos hormônios ovarianos nos casos de menarca precoce e menopausa tardia (FIORETTI *et al.*, 1999).

Quanto maior o número de gestações, menor o risco de câncer de mama entre mulheres, sendo a gestação a termo um fator protetor contra o câncer de mama (MA *et al.*, 2010). O papel das pílulas contraceptivas hormonais na incidência do câncer de mama é bastante debatido com dados distintos (FIORETTI *et al.*, 1999). Do mesmo modo, a associação da terapia hormonal na pós-menopausa com câncer de mama ainda permanece como um tema controverso (BERAL, 1997; BERAL, 2003).

Apesar de muitas síndromes genéticas aumentarem o risco de câncer de mama, a grande maioria dos casos de câncer de mama hereditário ocorrem devido a mutação dos genes *BRCA 1* e *BRCA 2* (COBAIN; MERAJVER; MILLIRON, 2016). Alguns estudos sugerem risco vitalício de desenvolver câncer de mama em 55% - 65% dos portadores da mutação *BRCA 1* e em 45% dos portadores da mutação *BRCA 2,* até os 70 anos de idade (GODET; GILKES, 2017). Numa coorte com análise prospectiva de dados, o risco cumulativo de câncer de mama aos 80 anos de idade chegou a 72% nos portadores de mutação *BRCA1* (IC 95%, 65% - 79%) e 69% nos portadores de *BRCA2* mutação (IC 95%, 61% - 77%) (KUCHENBAECKER *et al.*, 2017). Já alguns dados sugerem que mulheres com história familiar de câncer de mama (dois ou mais casos em mulheres com menos de 50 anos ou três ou mais casos em qualquer idade), mesmo sendo negativas em termos de mutações no *BRCA*, têm aproximadamente 11 vezes mais chances de desenvolver câncer de mama. Nesses casos, deve-se estar atento para possíveis medidas de intensificação de triagem com imagens mais frequentes (METCALFE *et al.*, 2009).

Obesidade e sobrepeso têm sido correlacionados com câncer de mama em diversos estudos, muito atribuído às maiores taxas de conversão periférica de precursores androgênicos em estrogênio, através da aromatização periférica de tecido adiposo (LAHMANN *et al*., 2004; GOODMAN; KIM; YOO, 2015; BRAVI; DECARLI; RUSSO, 2018; MILLER *et al*., 2018). Outros dados sugerem que o índice de massa corporal (IMC) também pode influenciar no prognóstico das mulheres com câncer de mama, sendo um preditor independente de sobrevida (BERCLAZ *et al.*, 2004).

O consumo de álcool também tem sido apontado como fator de risco para câncer de mama (MILLER, E. R. *et al.*, 2018). Similarmente, tabagismo ativo, especialmente em mulheres pós-menopausa e jovens nulíparas, está associado ao risco aumentado de desenvolver câncer de mama (TONG *et al.*, 2014). O hábito de fazer atividades físicas tem sido sugerido como fator protetor contra o câncer de mama em mulheres (MCTIERNAN *et al.*, 2003). Além disso, alguns autores sugerem que a atividade física após o diagnóstico pode reduzir o risco de óbito por câncer específico (HOLMES *et al*., 2005; HOLICK *et al*., 2008). Por fim, consumo e nível séricos adequados de 25-hidroxi vitamina D estão inversamente ligados ao risco de câncer de mama em alguns estudos (PARK *et al*., 2015; KHODAIE; SHEKARRIZ-FOUMANI, 2016).

2.3 Diagnóstico diferencial de câncer de mama

O diagnóstico diferencial histopatológico do carcinoma ductal invasor (CDI) de mama é muito vasto, podendo incluir neoplasias que se desenvolvem a partir de origem epitelial, mesotelial, adenomioepitelial, progenitor luminal e células tronco basais (BLANPAIN, 2013). Através da core biópsia de lesões suspeitas vistas nos exames de imagem com alta probabilidade pré-analítica, além do CDI, outras neoplasias podem ser diagnosticadas.

O carcinoma ductal *in situ* (CDIS) é representado por grupos heterogêneos de lesões pré-cancerosas restritas aos ductos e lóbulos mamários, sendo um precursor potencial para CDI. O carcinoma microinvasor é definido por tumores com maior foco de invasão, não mais do que 1mm, estando fortemente acompanhados de CDIS de alto grau do tipo comedocarcinoma. Sarcoma de mama, doença de *Paget*, tumor filóide e linfoma são outras histologias possíveis nesse sítio (LIAO *et al.*, 2018).

2.4 Subclassificações do câncer de mama triplo-negativo

O câncer de mama é uma doença altamente heterogênea, o que faz com que tumores em estágio clínico similar expressem perfis patológicos com desfechos clínicos diversos. Os tipos de câncer de mama podem ser identificados a partir de testes de perfil de expressão gênica que levam a diferentes implicações clínicas, biológicas e terapêuticas. Na prática oncológica atual, como tais testes ainda não são acessíveis na rotina ambulatorial para todos os pacientes, os subtipos moleculares ainda são projetados pela expressão de imunohistoquímica (IHQ) tumoral (PEROU *et al.*, 2000).

O CMTN é caracterizado por uma falta de expressão de receptores de estrogênio (RE), receptores de progesterona (RP) e do *Human Epidermal Growth Factor 2* (HER2). A depender da série de referência, estima-se que represente 15% a 20% de todos os cânceres de mama (BAUER *et al.*, 2007; CAREY *et al.*, 2007; TISCHKOWITZ *et al.*, 2007). Geralmente, são tumores de alto grau histológico, manifestando-se em idade precoce, estando associados a pior prognóstico em geral, com risco aumentado de recidiva precoce a distância, além de óbito precoce pela doença. As metástases tendem a ser viscerais, sendo mais comuns em sítio pulmonar, hepático e cerebral. Tais características clínicas configuram um grande desafio médico no manejo dessas pacientes. Conceitualmente, não há uma resposta eficaz com terapia hormonal e nem com drogas anti-HER2 (HINES *et al.*, 2008; LIN *et al.*, 2008; NAM *et al.*, 2008; SCHNEIDER *et al.*, 2008).

Sabe-se atualmente que o CMTN se define como uma doença heterogênea. Neste aspecto, diferentes subclassificações são propostas através de biomarcadores moleculares baseados num painel mais amplo de IHQ, que se mostra muito importante para a seleção de potenciais terapias-alvo molecular ou individualização do tratamento quimioterápico sistêmico. Alguns desses marcadores podem se mostrar fortes preditores de resposta ou prognóstico.

Levando-se em consideração os estudos clínicos que atribuem classificações moleculares correlacionando com resultados de IHQ (*claudin-low*) (PRAT *et al.*, 2010), subtipagem PAM-50 (SORLIE *et al.*, 2001; LEHMANN *et al*., 2011; BURSTEIN *et al.*, 2015), sugere-se agrupar os CMTN em 5 subtipos: o grupo *basal-like*, caracterizado

predominantemente por deficiência de reparo de DNA e também por expressão de fatores de crescimento, expressando citoqueratinas basais, incluindo CK5/6, CK14 e CK17, além de EGFR; o grupo imunomodulatório, conhecido como CMTN immune-associado; o grupo *mesenquimal-like*, com transição epitelial para mesenquimal com características de células tronco; o grupo apócrino/luminal receptor de androgênio (LRA), com hiperexpressão de RA; e por fim o grupo HER2 - enriquecido.

2.5 Biomarcadores em câncer de mama triplo-negativo

Segundo a Organização Mundial de Saúde (OMS), por definição, biomarcadores (ou “marcadores biológicos”) referem-se a uma ampla subcategoria de fatores (substâncias, estruturas ou processos) que podem ser acuradamente medidos de forma reprodutível no corpo, ou mesmo produtos do seu metabólito, podendo influenciar ou predizer um agravo ou desfecho específico (INCHEM, [s.d.]).

Outra definição, ainda mais ampla, considera não apenas a incidência e os desfechos dos agravos, mas também os efeitos de tratamentos, intervenções e até mesmo exposição ambiental não intencional, como há produtos químicos ou nutrientes. Em seu relatório sobre a validade dos biomarcadores na avaliação de risco ambiental, a OMS afirmou que uma verdadeira definição de biomarcadores inclui “quase todas as medições que refletem uma interação entre um sistema biológico e um perigo potencial, que pode ser químico, físico ou biológico. A resposta medida pode ser funcional, fisiológica e bioquímica no nível celular ou uma interação molecular” (INCHEM, [s.d.]).

Biomarcadores de câncer são biomoléculas produzidas pelas células tumorais ou por outras células do corpo em resposta ao tumor. Cada tipo de célula tem sua assinatura molecular única com características identificáveis, tais como níveis ou atividades de uma miríade de genes, proteínas ou outras características moleculares. Portanto, os biomarcadores podem facilitar a definição molecular do câncer. O desafio e a perspectiva dos biomarcadores, ao facilitar a combinação de terapêuticas com diagnósticos, prometem desempenhar um papel importante no desenvolvimento da medicina personalizada. Embora tanto os biomarcadores prognósticos quanto os preditivos de câncer previnem o resultado clínico, o termo "biomarcador preditivo" é reservado para a

associação de uma terapia específica com um desfecho clínico específico (BELKOWSKI; ILYIN; PLATA-SALAMÁN, 2004; CHO, 2007; GOOSSENS *et al*., 2015).

Uma revisão de literatura detalhada sobre o papel dos biomarcadores no CMTN, performada por Silva et al, foi publicada recentemente (anexo A).

2.5.1 Gene *TP53*, proteína p53 e Ki-67

O gene 53 da proteína do tumor (*TP53*) é localizado no cromossoma 17 (17p13.1) e codifica a proteína p53, um fator da transcrição que suprime o crescimento do tumor e é essencial no processo de resposta celular aos danos do DNA. Quando esse dano ocorre, há um aumento na transcrição do p53, causando a interrupção do processo mitótico e o reparo do DNA, ou mesmo, morte celular. Isso é efetivamente estabelecido pela interação com alvos como p21, cinase dependente de ciclina (CDK), proteínas de reparo (PARP, BRCA) e PTEN (LEHMANN et al., 2011; BIOMARKERS IN RISK ASSESSMENT: VALIDITY AND VALIDATION, [sd], 2019; BIOMARKERS IN RISK ASSESSMENT: CONCEPTS AND PRINCIPLES, [sd], 2019).

Os dados da literatura sugerem que a mutação de *TP53* é a mais frequentemente encontrada em tumores malignos, ocorrendo em aproximadamente 30% dos casos de câncer de mama e em 75% - 80% dos casos de CMTN, de acordo com o projeto *The Cancer Genome Atlas (TCGA)* (CANCER GENOME ATLAS NETWORK, 2012). A proteína mutante p53 pode exercer funções aberrantes, interagindo de forma diferente com alvos distintos, aumentando a regulação do CDK-1 e da via de transdução de sinais PI3K/AKT/mTOR, reduzindo a ação de proteínas supressoras tumorais, como p63 e p73, estimulando a proliferação celular e evasão da apoptose (TURNER *et al.*, 2013).

A expressão da proteína p53 em tecidos tumorais de CMTN pode variar de acordo com o tipo de mutação que ocorre no gene *TP53*: os pacientes com algumas mutações *missense* tendem a mostrar maior expressão de p53, já que produzem normalmente uma proteína mais estável no comprimento total, em contraste com pacientes que apresentam mutações de deleção e não expressam a proteína (YEMELYANOVA *et al.*, 2011). Diversos estudos tentaram determinar o impacto das mutações deletérias do *TP53* no prognóstico das pacientes com CMTN. Em um estudo com 174 mulheres com CMTN

portadoras da mutação em *TP53*, a baixa expressão do mRNA foi associado a um prognóstico desfavorável, com pior sobrevida livre de recorrência a distância em 5 anos (5 anos-SLRD: baixa expressão versus alta expressão, 50,0% *versus* 87,8%; p = 0,009) (KIM, J.-Y. *et al.*, 2016). Analisando a expressão da proteína p53, os dados de um estudo retrospectivo de uma coorte queniana revelaram que as mulheres com CMTN linfonodo-negativas e com expressão p53 por IHQ apresentaram sobrevida global (SG) significativamente maior do que pacientes sem expressão p53 (p53 + versus p53-; HR, 2,8; IC 95%, 1,1-7,1; p = 0,022) (BAE, 2016).

No campo de terapias direcionadas, embora a proteína p53 tenha sido considerada como *undruggable*, estudos recentes apontam novos compostos que podem segmentar seletivamente a proteína p53 mutante e restaurar suas propriedades de tipo selvagem em células de câncer de mama, como PRIMA-1 e PRIMA-1^{Met} (reativação p53 e indução de apoptose maciça) (SYNNOTT *et al.*, 2017). Como os compostos PRIMA-1 e PRIMA-1^{Met} mostraram atividade anticâncer em linhas celulares CMTN, estudos de modelos animais foram realizados, e posteriormente estudos de fase I não demonstraram evidências de toxicidade limitante (LEHMANN *et al.*, 2012), sendo então considerados uma terapia muito atrativa para serem avaliadas nos futuros estudos clínicos dos pacientes com CMTN.

Ao longo dos anos, a pesquisa desenvolveu várias técnicas moleculares para medir as taxas de proliferação celular, uma das quais é a quantificação por IHQ de antígenos nucleares relacionados à proliferação. Ki-67, uma proteína codificada pelo gene *MKI67* (marcador de gene da proliferação Ki-67), é o marcador celular mais comumente utilizado para determinar a proliferação celular, sendo, portanto, considerado um biomarcador prognóstico e preditivo no câncer de mama, mas exibindo um valor incerto no contexto de pacientes com CMTN (BLOWS *et al*., 2010; VIALE *et al*., 2008). Estes tumores tendem a ter uma expressão aumentada de Ki-67, com estudos que mostram prevalências de 44,7% a 53,4% dos tumores com expressão de Ki-67 acima de 20% (NAKAGAWA *et al.*, 2011).

Recentemente, os resultados de uma coorte retrospectiva que avaliaram 363 mulheres com CMTNs iniciais operáveis através da análise multivariada sugeriram que o escore Ki-67, mais elevado, é um fator de risco independente para a sobrevida livre de doença (SLD; RR, 2,83; IC 95%, 1,58-5,06; p < 0,001) e sobrevida global (SG; RR, 3,18; IC 95%, 1,48-6,79; p = 0,003) (WANG, W. *et al.*, 2016). Em outra coorte, o Ki-67 foi

significativamente correlacionado com o fenótipo CMTN. O valor médio foi de 44,7% e 22,2% nos pacientes com CMTN e não-CMTN, respectivamente, e também foi associado ao estágio patológico mais avançado (p = 0,004) e ao acometimento nodal mais extenso (p = 0,033) (ILIE *et al.*, 2018).

2.5.2 Receptor do Fator de Crescimento Epidérmico, c-KIT e citoqueratinas (CK 5/6, CK 14, CK 17

O receptor do fator de crescimento epidérmico (EGFR) faz parte de uma família de glicoproteínas transmembranas com um domínio tirosina cinase que ativa as vias de transdução de sinal, desempenhando um papel importante na proliferação celular e na inibição da apoptose (LEHMANN *et al.*, 2011). A prevalência de superexpressão de EGFR nos casos de CMTN é bastante variável entre os estudos, variando de 13 - 78% (GLUZ *et al.*, 2009; GUMUSKAYA *et al.*, 2010), devido à falta de padronização na mensuração dos resultados do IHQ e a uma ampla variação demográfica.

Embora alguns resultados sugiram uma associação forte do número de cópia mais elevado do gene do EGFR, muito comum no CMTN, com sobrevida desfavorável, maior grau tumoral e comprometimento nodal axilar no câncer de mama em geral, os dados da superexpressão da proteína de EGFR no CMTN são controversos (LIU *et al.*, 2012; NAKAJIMA *et al.*, 2014; PARK *et al.*, 2014). Os estudos de fase II que avaliaram a eficácia dos inibidores da EGFR, tais como TKIs (inibidores da tirosina cinase - gefitinibe, afatinibe e erlotinibe) e anticorpos monoclonais (cetuximabe e panitumumabe), não mostraram resultados efetivos (BASELGA *et al*., 2005; DICKLER *et al*., 2008; CAREY *et al*., 2012).

A sinalização por c-KIT (CD117) parece desempenhar um papel importante na transformação celular e diferenciação. A expressão da proteína c-KIT é detectada em aproximadamente metade dos tecidos tumorais da CMTN (JANSSON *et al.*, 2014). A ativação aberrante do gene *c-KIT* é parte do processo de carcinogênese e de mecanismos de formação de metástases de várias malignidades humanas. Estudar a hiperativação e as alterações da via c-KIT torna-se muito interessante porque é alvo direto do tratamento com o TKI imatinibe, já tradicionalmente utilizado no tratamento de algumas doenças onco-hematológicas, como a leucemia mieloide crônica, assim, no tratamento de tumores

sólidos tais como tumor estromal gastrintestinai (GIST) e dermatofribrossarcoma protuberans, agindo sobre a proteína de fusão BRC-ABL (SHAMS; SHAMS, [*s. d.*]).

Em um estudo retrospectivo de 58 pacientes com CMTN, o status positivo de c-KIT por IHQ foi associado com mutações *missense* do gene *TP53* (p = 0,031), invasão vascular, recidiva precoce e maior índice de proliferação de Ki-67. A presença concomitante da expressão da proteína do c-KIT e de mutações *missense TP53* nas amostras de tumor primário foi um fator prognóstico independente para maior risco de óbito (LUO *et al.*, 2018a). Em outra coorte com 653 mulheres com CMTN, o status positivo para c-KIT também foi acompanhado por sobrevida mais curta (p = 0,036) (THIKE *et al.*, 2010).

Câncer de mama *basal-like*, considerado um subtipo agressivo de CMTN, geralmente expressa citoqueratinas basais como CK 5/6, CK 14 e CK 17 (CHEANG *et al.*, 2008). A expressão CK 5/6 varia de 24% a 72% dos casos de CMTN (ZHANG *et al.*, 2014). Alguns resultados sugeriram que o CMTN positivo para CK 5/6 tenha um prognóstico mais pobre, independente das características clínico-patológicas conhecidas (VAN DE RIJN *et al.*, 2002a). Outros resultados sugerem que o status positivo de CK 5/6 tem maior correlação com a metástase nodal e o tamanho do tumor (INANC *et al.*, 2014).

Por outro lado, uma coorte com 150 pacientes não conseguiu determinar uma correlação da expressão de CK 5/6 com características clinicopatológicas ominosas, provavelmente devido à baixa expressão de CK 5/6 nessa amostra (HASHMI *et al.*, 2018). De forma geral, a expressão de CK 14 e CK 17 ocorre em sobreposição com a de CK 5/6. A positividade para CK 17 isoladamente não foi identificada como um biomarcador de prognóstico confiável (VAN DE RIJN *et al.*, 2002b). Num estudo transversal de 150 pacientes com CMTN, a expressão de CK 14 foi positiva em 50,8% dos casos (LESAR *et al.*, 2016), enquanto num estudo com mulheres asiáticas foi em torno de 39,6% (LIU *et al.*, 2016).

2.5.3 Fator de Crescimento Endotelial Vascular (VEGF)

A sinalização da angiogênese mediada pelo fator de crescimento endotelial vascular (VEGF), que desencadeia a neovascularização, é crucial no processo de crescimento e disseminação tumoral. O VEGF compreende uma família de 6 proteínas: VEGF A, B, C, D e o fator de crescimento placentário. O *splicing* alternativo de seu mRNA cria 4 isoformas, sendo a mais comum VEGF165. Os mediadores da expressão gênica são: hipóxia, fator de crescimento, óxido nítrico, oncogenes, HER2 e genes supressores do tumor (CLAESSON-WELSH; GERWINS; SKÖLDENBERG, 2000; HOLMES; ZACHARY, 2005). Alta expressão de VEGF é frequente em CMTN, em torno de 30 a 60% dos casos (LINDERHOLM *et al.*, 2009). Adicionalmente, a mensuração de micro-densidade vascular por IHQ tem sido usada como um biomarcador prognóstico em CMTN. Conceitualmente, uma alta densidade vascular média no câncer de mama pode estar associada a um pior prognóstico com sobrevida mais curta (ALI; SHETA MOHSEN, 2011).

Na coorte retrospectiva que avaliou mulheres suecas com câncer de mama operável, o nível sérico médio de VEGF de 87 pacientes com CMTN foi estatisticamente maior do que o de pacientes não-CMTN, 62% dos pacientes com CMTN tinham uma expressão de VEGF maior que o valor mediano quando comparado com 47% dos casos não-TNBC (p = 0,036) (LINDERHOLM *et al.*, 2009). Da mesma forma, num pequeno ensaio clínico de 60 pacientes, o efeito antiangiogênico da terapia metronômica de baixa dose, com capecitabina e ciclofosfamida, no câncer de mama metastático foram proporcionais à redução dos níveis séricos basais de VEGF após 6 meses de tratamento (p = 0,001) (EL-ARAB; EL MAHDY; SWELLAM, 2012). Os resultados do estudo que recrutou 41 mulheres egípcias com CMTN metastático, o VEGF se apresentou em níveis mais elevado nos pacientes com tumores maiores comparados àqueles com tumores menores (p = 0,053), assim como nos pacientes com doença mais progressiva comparados aqueles que tiveram doença com benefício clínico a terapia instituída (p = 0,016) (TAHA *et al.*, 2009).

O VEGF tem sido amplamente estudado como um alvo para o tratamento da CMTN em diversos estudos que testaram a eficácia do bevacizumabe, anticorpo monoclonal humanizado de VEGF A. Dos 663 doentes com CMTN incluídos em um ensaio clínico, a

adição de bevacizumabe à quimioterapia neoadjuvante aumentou significativamente a taxa de resposta patológica completa (RPC) de 27,9% para 39,3% (p = 0,003) (BUCHERINI *et al.*, 2012). Da mesma forma, 46 pacientes com CMTN metastático, incluídos num estudo de fase II, apresentaram taxa de resposta global (TRG) de 65,2% (IC 95%, 52,9%-80,4%) (SALOUSTROS *et al.*, 2018). Apesar de mostrar aumento da SLP, três estudos de fase III[55–57] não mostraram uma melhoria estatisticamente significativa na sobrevida global. Em relação ao tratamento adjuvante, o bevacizumabe não demonstrou benefício nos desfechos de sobrevida(CAMERON *et al.*, 2013). Outras terapias com anticorpos monoclonais ou TKIs anti-VEGFR não demonstraram eficácia (RIBATTI *et al.*, 2016).

2.5.4 Receptor de Androgênio

O receptor de andrógeno (RA) faz parte de um complexo de receptores hormonais esteroidais que modulam os fatores de transcrição, controlando a expressão gênica em diferentes processos celulares, às vezes dualísticos. Nesse sentido, o RA pode estimular a proliferação e a desdiferenciação, assim como induzir a apoptose e a morte celular, a depender das vias de sinalização ativada. Embora os estudos iniciais sugerissem um efeito prognóstico negativo do RA nos CMTN, os dados mais recentes sugerem que os pacientes com CMTN RA-positivo têm um prognóstico mais favorável. De acordo com a coorte, a frequência de expressão IHQ do RA no CMTN pode variar de 10 até 90% (NIEMEIER *et al.*, 2010; HE *et al.*, 2012; JAM *et al.*, 2019).

Uma revisão sistemática com meta-análise avaliou dados agrupados de 13 ensaios clínicos que recrutaram no total 2826 pacientes com CMTN de 2007 a 2015. Nessa análise, 24,4% dos casos foram RA-positivos, estando associados a baixo grau tumoral (40,8% dos pacientes RA-positivos) positivos, status pós-menopausa (26,9% dos pacientes RA-positivos) e menor risco de acometimento nodal (28,8% dos pacientes RA-positivos) (HE *et al.*, 2012). Do mesmo modo, outros dados sugeriram que pacientes com CMTN mais agressivos eram negativos para RA, enquanto os casos com maior expressão de RA estavam associados à doença de estadiamento clínico mais precoce, baixa

expressão de Ki-67 e baixo grau nuclear (MCNAMARA *et al*., 2013; GASPARINI *et al*., 2014; MAEDA *et al*., 2016).

Quanto à análise agrupada de 12 estudos que recrutaram um total de 5270 mulheres com CMTN, o grupo RA-positivo teve 48% de redução do risco de progressão ou morte em comparação com pacientes RA-negativos (SLD; HR, 0,52; IC 95%, 0,43-0,64) (QU *et al.*, 2013). Em outra meta-análise com 521 pacientes com CMTN, o OR para SLD foi de 0,44 (p = 0,002) (KIM; JAE; YOON, 2015). No entanto, não houve correlação do status de RA com o desfecho de SG em nenhum desses estudos.

Em relação ao valor preditivo do status de RA em CMTN, alguns dados sugerem que os pacientes RA-positivos são mais propensos a serem resistentes a quimioterapia do que os pacientes RA-negativos. A análise de 637 amostras de biópsia de core biópsia, de tumores primários de pacientes incluídos no estudo Gepartrio, mostrou que no câncer de mama RA-positivo, a taxa de RPC foi de 12,8% em comparação com 25,4% nos tumores RA-negativos (p < 0,0001) (HILBORN *et al.*, 2016). Resultados semelhantes foram observados numa coorte retrospectiva japonesa, na qual os CMTNs RA-positivos apresentaram menor taxa de RPC que o RA-negativo numa análise univariada (HR, 5,26; IC 95%, 1,39-19,86; p = 0,014) (ASANO *et al.*, 2016).

Alguns ensaios clínicos avaliaram a eficácia e segurança de antiandrogênicos em pacientes com CMTN avançado ou metastático. Um estudo de braço-único com bicalutamida como monoterapia em pacientes pesadamente tratados mostrou uma taxa de benefício clínico (TBC) de 19% com uma mediana de SLP de 12 semanas (variando entre 6,25 e 57,5 meses) (GUCALP; TRAINA, 2016). Da mesma forma, um ensaio de fase II com pacientes CMTN RA-positivos tratados com agente único enzalutamida mostrou uma TBC com 16 e 24 semanas de tratamento de 35% e 29%, respectivamente (TRAINA *et al.*, 2018). E, finalmente, num estudo de fase II com abiraterona em monoterapia, a TBC foi de 20% e o PFS mediano foi de 2,8 meses com eventos adversos manejáveis (BONNEFOI *et al.*, 2016).

2.5.5 Deficiência de Recombinação Homóloga (HRD) e mutações de *BRCA 1/2*

Todas as células do corpo humano sofrem agressões externas constantes no DNA, entretanto, contam com uma eficiente maquinaria de reparo de dano (DDR). As quebras de fita-dupla são formas sérias de dano e que podem ser reparadas por duas vias principais: recombinação homóloga livre de erro e extremidade-junção não-homóloga (NHEJ) (ABKEVICH *et al.*, 2012; TELLI *et al.*, 2016). Inicialmente descrita em pacientes com mutações genéticas *BRCA1* e *BRCA2*, a deficiência de recombinação homóloga (HRD) pode ocorrer em cânceres esporádicos por inativação genética e epigenética de outros componentes (PALB2, BARD1, BRIP1, RAD51B, RAD51C, RAD51D, ATM, FAAP20, CHEK2, FAN1, FANCE, FANCM, e POLQ), uma condição definida como *BRCAness* (LORD; ASHWORTH, 2016). Os tumores deficientes para recombinação homóloga (HRd) são mais sensíveis à quimioterapia a base de platina, assim como os novos inibidores da enzima do reparo do DNA poli-ADP ribose polymerase 1 (PARP1) (SASTRE-GARAU *et al.*, 2012).

As mutações germinativas *BRCA* 1/2 estão presentes em aproximadamente 14 a 20% dos casos de CMTN, mas uma proporção maior de pacientes parece ser portadora de HRD (GONZALEZ-ANGULO *et al.*, 2011; TELLI *et al.*, 2015; LOIBL *et al.*, 2018). Num estudo retrospectivo de 45 mulheres com CMTN, entre as pacientes selvagens para *BRCA1/2*, as portadoras de HRD tinham maiores taxas de RPC (OR, 16; IC 95%, 1,65-160,41; p = 0,0041) comparadas com as pacientes não-HRD (TELLI *et al.*, 2018). Da mesma forma, em uma coorte de 77 pacientes com CMTN, 19,5% eram mutadas para *BRCA* (*BRCA*m), e as estimativas de sobrevida livre de recorrência (SLR) em 5 anos foram de 51,7% para *BRCA* selvagem (*BRCA*wt) versus 86,2% para BRCAm (p = 0,031) e estimativas de SG em 5 anos foram de 52,8% e 73,3% (p = 0,225) para *BRCA*wt e BRCAm, respectivamente (GONZALEZ-ANGULO *et al.*, 2012).

Um estudo de fase II avaliou o papel da adição do inibidor de PARP veliparibe com carboplatina à quimioterapia neoadjuvante padrão nos pacientes com CMTN localmente avançado, identificando que a taxa de RPC foi maior no grupo velipribe/carboplatina do que no grupo controle com paclitaxel isoladamente (168 [53%] de 316 pacientes versus 49 [31%] de 158, p < 0,0001) (LOIBL *et al.*, 2018). Recentemente, outro ensaio clínico de

fase III comparou a monoterapia com olaparibe com quimioterapia padrão em pacientes que apresentavam mutação germinativa de *BRCA* diagnosticadas com câncer de mama metastático HER2-negativo previamente tratadas com dois ou mais regimes quimioterápicos. No subgrupo de 150 pacientes com CMTN, o HR para SLP foi de 0,43 (IC 95%, 0,29-0,63) a favor da monoterapia com olaparibe (ROBSON *et al.*, 2017).

Além das mutações deletérias de *BRCA* e HRD, a instabilidade genômica também foi avaliada por técnicas como perda de heterozigosidade (LOH), desequilíbrio alélico telomérico (TAI) e transições de estado em grande escala (LST) (ABKEVICH *et al.*, 2012; BIRKBAK *et al.*, 2012; SASTRE-GARAU *et al.*, 2012). Um estudo de fase II avaliou a eficácia da gemcitabina, carboplatina e iniparibe como terapêutica neoadjuvante para doentes *BRCA*m com CMTN. A taxa global de RPC na população por intenção de tratar (n= 80) foi de 36% (IC 95%, 27-46%) e os escores médios de HRD-LOH foram maiores nos respondedores comparados com os não respondedores (p = 0,02) (TELLI *et al.*, 2015).

2.5.6 Linfócitos Infiltrantes Tumorais e expressão de PD-L1/PD-1

Estudos mais recentes têm se concentrado no microambiente tumoral como determinante de resposta a terapia sistêmica e de sobrevida em CMTN. O tecido normal da mama geralmente não contém células imunes, mas o tecido do tumor e o estroma circunvizinho podem conter níveis elevados de infiltrado celular imune (DEGNIM *et al.*, 2014). Há uma evidência crescente do papel do infiltrado imune linfocítico na tumorigênese do CMTN. O conceito de imunoensaio supõe que a imunidade do hospedeiro, dependendo da composição peritumoral e intratumoral, pode estimular o crescimento do tumor ou erradicar a doença, alicerçando a definição da evasão imune e da imunogenicidade, respectivamente. Nesse contexto, as células tumorais são inicialmente rejeitadas pelo sistema imunológico, permanecendo num estado de dormência. Após regulação positiva das vias de transdução de sinais celulares pró-sobrevida, passam a expressar moléculas que promovem a supressão imunológica e angiogênese. Define-se aqui então os três estágios de imunoedição: as fases de eliminação, equilíbrio e escape (KIM, 2007).

Durante o doutorado, foi publicada uma minuciosa revisão de literatura sobre imunoterapia, desde mecanismos imunobiológicos para modificação do microambiente tumoral, até biomarcadores em validação para melhor seleção de contextos específicos para o uso das novas drogas, tendo sido muito enfatizado o tratamento de CMTN (Anexo B).

Linfócitos T-*Helper* CD4+ e CD8+ são parte do complexo de resposta do tipo Th1, necessário para a identificação e destruição de células cancerígenas. Tanto o sistema imunológico inato (neutrófilos, monócitos, macrófagos, células apresentadoras de antígeno) quanto células adaptativas do sistema (linfócitos B e T) são fundamentais para alertar o reconhecimento e a resposta a patógenos, bem como células *non-self* ou receptores tumorais. Muitos marcadores presentes na membrana celular do câncer de mama podem ativar e estimular as células T, induzindo a resposta imune regulatória. No entanto, a habilidade de supressão imune é crítica para a sobrevivência de células normais do corpo (SOCIETY, 2015).

Os linfócitos infiltrantes tumorais (LITs) são altamente prevalentes em aproximadamente 20% de casos de CMTN. Alguns estudos sugerem que os LITs no câncer de mama podem ser um biomarcador substituto para resposta imune adaptativa, principalmente para o subtipo CMTN, considerado um dos mais imunogênicos. Há um consenso de que o efeito citotóxico da quimioterapia é parcialmente influenciado pela reação imune contra as células tumorais. Da mesma forma, a quimioterapia pode proporcionar uma melhor resposta imune modificando o microambiente, assim como o aumento da imunogenicidade, levando a resposta tumoral (COUSSENS; DENARDO, 2007; SCHMIDT *et al.*, 2008).

Em dois estudos com quimioterapia baseada em antraciclina e taxane, as taxas de RPC no câncer de mama com padrão predominantemente linfocítico foram maiores que nos tumores sem LITs, 42% e 3%, respectivamente (DENKERT *et al.*, 2010). Em uma meta-análise com estudos de quimioterapia neoadjuvante, o CMTN com níveis mais elevados de linfócitos TCD8+ e inferior FoxP3+ apresentou melhores taxas de RPC (OR, 2,49; IC 95%, 1,16-3,83) (MAO *et al.*, 2014).

Em um estudo retrospectivo multicêntrico, 278 casos de CMTN com doença residual foram analisados após quimioterapia neoadjuvante. A taxa SG em 5 anos foi de

91% (IC 95%: 68%-97%) para pacientes com LITs alto (n= 27) versus 55% (IC 95%, 48%-61%) para pacientes com LITs baixo (HR, 0,19; IC 95%, 0,06-0,61; p = 0,0017) (DIECI *et al.*, 2014b). Similarmente, numa análise agrupada dos casos de CMTN de dois ensaios de câncer de mama adjuvante randomizados de fase III (ECOG 2197 e ECOG 1199), para cada aumento de 10% nos LITs, foi observada uma redução de 14% do risco de recorrência ou morte (p = 0,02), uma redução de 18% do risco de recorrência distante (p = 0,04) e 19% de redução do risco de óbito (p = 0,01) (ADAMS *et al.*, 2014).

A atividade antitumoral dos inibidores de *checkpoint* imune tem sido extensivamente estudada no CMTN. A proteína de morte celular programada 1 (PD-1) é um receptor de *checkpoint* imune que limita a ação das células T efetoras dentro dos tecidos, exercendo um papel crucial no processo da evasão imune do tumor. Os dois ligantes de PD-1, com perfis distintos da expressão em tipos do tumor são PD-L1 e PD-L2 (ISHIDA *et al.*, 1992a). A regulação do PD-L1 pode ocorrer por vários processos: resposta à ação do IFN-Gamma, sinalização oncogênica, deleção ou silenciamento do PTEN com consequente superexpressão da via PI3K (CASTANEDA *et al.*, 2016).

A imunoterapia com inibidores de PD-1 e PD-L1 resulta na ativação de células T, restaurando a atividade imune antitumoral do hospedeiro, demonstrando atividade de longa duração e aumento da sobrevida em tumores selecionados. Devido às diferenças nos métodos de detecção, amostragem e tamanho do tumor, a taxa de expressão de PD1/PD-L1 é variável em vários estudos (GHEBEH *et al.*, 2006). Através da análise por IHQ nos pacientes com CMTN, o PD-L1 foi relatado entre 15,8% e 30% (GHEBEH *et al.*, 2006; BECKERS *et al.*, 2016a; CASTANEDA *et al.*, 2016). Já pela técnica de hibridização *in situ* do mRNA, foi detectado mRNA do PD-L1 em 55% a 60% dos TMAs do tecido tumoral (SCHALPER *et al.*, 2014).

Numa grande coorte retrospectiva envolvendo pacientes com câncer de mama, a regulação aumentada do PD-L1, detectada através da análise do mRNA, foi associada com propriedades prognósticas ominosas (tamanho grande do tumor, RE negativo, RP negativo, status HER2-positivo, índices de proliferação elevados, subtipos de CMTN *basal-like* e HER2-enriquecidos), com maiores taxas de RPC (50% contra 21%) e uma taxa de sobrevida livre de metástase em 5 anos de 61% (IC 95%, 0,58-0,64) (SABATIER *et al.*, 2015). Da mesma forma, outro estudo retrospectivo mostrou uma associação

positiva significante entre o estado de PD-L1 ≥ 25% e a maiores taxas de RPC (p = 0,024) (CERBELLI *et al.*, 2017).

Num estudo clínico de fase I de mulheres com CMTN tratados com atezolizumabe, um agente anti-PD-L1, a taxa de resposta objetiva (TRO) para IC2/3 (definidos como pacientes escore de PD-L1 ≥ 5%, alta expressão considerando como numerador célula imune infiltrante tumoral positiva para o reagente PD-L1 sobre o total de células imunes do infiltrado intratumoral) foram 17% versus 8% para IC0/1 (PD-L1 negativo, escore <1%). O grupo com mais de 10% do escore de LITs, ou com mais de 1,35% de células CD8 + no tumor primário, apresentou maior TRO e mediana de SG mais longa (SCHMID, Peter *et al.*, 2017). Recentemente, a combinação de atezolizumabe e nab-paclitaxel foi avaliada como terapia de primeira linha para a TNBC num estudo de fase III. Numa análise mais específica do subgrupo representado pelos pacientes com status positivo para PD-L1 (41% do total), o risco de progressão ou morte foi reduzido 38% no braço de atezolizumabe com nab-paclitaxel comparado com o braço controle do placebo com nab-paclitaxel (HR, 0,62; IC 95%, 0,49-0,78; p < 0,001) e a mediana de SG foi de 25,5 e 15,5 meses, respectivamente (HR, 0,62; IC 95%, 0,45-0,86) (EMENS *et al.*, 2018).

No cenário de CMTN metastático, os resultados do estudo de fase III KEYNOTE 355 sugeriu que a adição de pembrolizumabe ao esquema de quimioterapia paliativa aumentou discretamente SLP (7,5 versus 5,6 meses; HR 0,82, IC 95% 0,69-0,97). Os resultados também foram estratificados de acordo com CPS que coram para PD-L1). Esses resultados sugerem que o benefício é limitado àqueles com CPS ≥10, nos quais a adição de pembrolizumabe à quimioterapia melhorou a PFS mediana em aproximadamente dois meses (9,7 versus 5,6 meses; HR 0,65, IC 95% 0,49-0,86). Os eventos adversos de grau 3 a 4 foram comparáveis entre os dois grupos (aproximadamente 70 por cento), embora um paciente no braço do pembrolizumabe morreu de toxicidade relacionada ao tratamento(CORTES et al., 2020).

A Figura 2.6 faz uma representação das vias de transdução de sinais referentes a diversos receptores de membrana celular tumoral a serem analisados através de reações de IHQ no presente estudo.

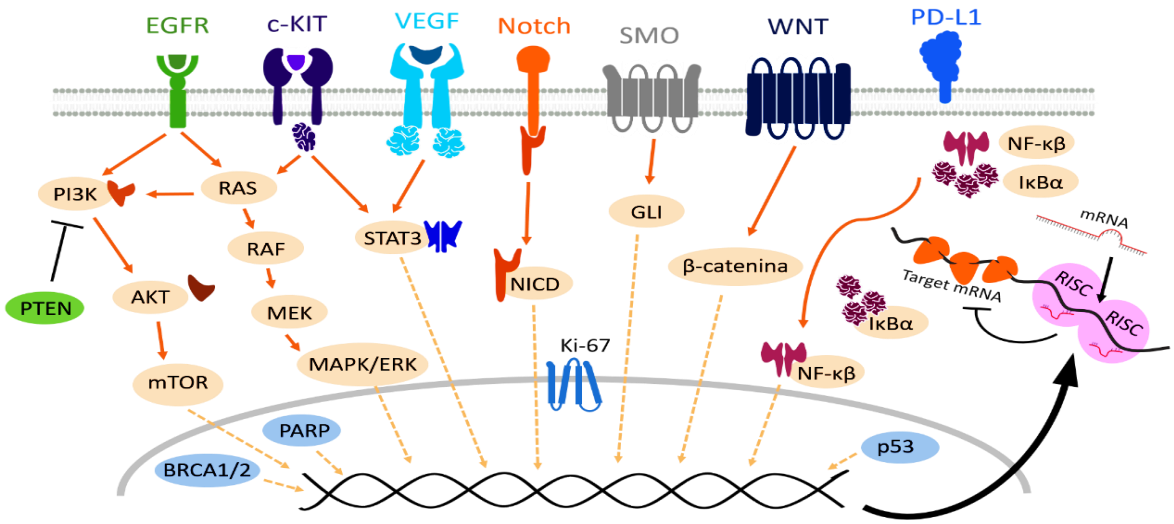

Figura 2.6 - Descrição das vias de transdução de sinal celular na tumorigênese.
Fonte: Triple negative breast cancer: A thorough review of biomarkers (DA SILVA *et al.*, 2020).

2.6 Estadiamento e definições de câncer de mama localmente avançado

Comumente aplicado para os tumores sólidos, o estadiamento é um processo em que se propõe determinar a extensão da doença para orientar o planejamento terapêutico. O sistema mais amplamente usado é o da *American Joint Cancer Committee* (AJCC), tanto para o estadiamento clínico pré-cirúrgico na core biópsia quanto para o estadiamento patológico na peça cirúrgica, conferindo a classificação baseada no TNM. Comparado com edições anteriores, a 8ª edição do AJCC resultou em mudança de mais de 35% do estadiamento das pacientes diagnosticadas com câncer de mama (PLICHTA *et al.*, 2018).

O novo sistema fornece dois grupos principais para o estadiamento do câncer de mama. O grupo anatômico é baseado na extensão do câncer como definido pelo tamanho do tumor (T), *status* linfonodal (N) e metástase à distância (M). O grupo prognóstico inclui o TNM anatômico, o grau tumoral e o *status* do HER2, RE e RP. Este último passou a ser o preferido recentemente para atendimento ao paciente com câncer de mama, já que define melhor questões prognósticas. No geral, espera-se que a transição para nova classificação direcione melhor o atendimento clínico, as recomendações de tratamento e as pesquisas futuras no câncer de mama (Tabela **2.2** a Tabela **2.5**).

A definição de câncer de mama localmente avançado (CMLA) é muito variável entre os estudos e os centros de tratamento, geralmente incluindo tumores primários em estadio avançado com extenso envolvimento nodal e carcinomas inflamatórios de mama. Engloba

o câncer de mama que é inoperável inicialmente ou ressecável apenas por cirurgia radical. Trata-se de um grande desafio clínico, já que a grande maioria dos pacientes com CMLA poderá apresentar recidiva precoce da doença ou eventualmente óbito, mesmo com tratamento multimodal agressivo (COSTA; GRADISHAR; HANSEN, 2018).

O CMLA geralmente engloba a doença estadio III, abrangendo tumores primários T0-T3 com axila clinicamente detectável (emaranhados ou fixos), infraclavicular ipsilateral, linfonodos mamários supraclaviculares ou internos (N2 ou doença N3), ou extensão do tumor na parede torácica ou pele (T4) independentemente do estado nodal (RUSSO *et al.*, 2019). Nos casos de pacientes com subtipos mais agressivos como CMTN, a definição de CMLA é ampliada para incluir pacientes com doença do estadio clínico IIB, como tumor primário ≥5cm e sem envolvimento nodal (T3N0). Nesse sentido, a maioria dos especialistas considera pacientes com estadio IIB-IIIA (T3 N0–1) como câncer de mama "de grande porte", em contraste com casos inoperáveis com inflamação e/ou extenso envolvimento cutâneo, linfonodo axilar fixo ou muito volumoso, doença nodal supraclavicular ou mamária interna (GIULIANO *et al.*, 2011).

O carcinoma inflamatório de mama (CIM) é um tipo de CMLA pouco frequente e agressivo. No diagnóstico clínico, o CIM é definido como eritema e edema dérmico chamado *peau d'orange* ocorrendo numa extensão substancial área da mama com evolução rápida. O diagnóstico clínico é geralmente acompanhado por achados patológicos de embolia tumoral linfática na derme, embora este achado não seja necessário ou suficiente (se faltarem sintomas clínicos) para confirmar o diagnóstico de CIM (YAMAUCHI *et al.*, 2012). Os doentes com CMLA devem ser tratados com terapia que emprega modalidades sistêmicas e locorregionais, demandando um cronograma de tratamento bem coordenado e uma estreita cooperação multiprofissional entre médicos mastologistas, oncologistas clínicos e rádio-oncologistas.

Tabela 2.2 - Definição clínica e patológica do tumor primário (T).

Categoria T	Critério T
Tx	Tumor primário não pode ser avaliado.
T0	Nenhuma evidência de tumor primário.
Tis (CDIS)*	Carcinoma ductal *in situ.*
Tis (Paget)	Doença de paginação do mamilo não associada a CDI ou CDIS no parênquima mamário subjacente. Os carcinomas do parênquima mamário associados à doença de Paget são categorizados com base no tamanho e nas características da doença parenquimatosa, embora a presença de doença de Paget deva ser ainda observada.
T1	Tumor ≤ 20 mm em maior dimensão.
T1 mi	Tumor ≤ 1 mm na maior dimensão.
T1a	Tumor> 1 mm mas ≤ 5 mm em maior dimensão
T1b	(arredondar qualquer medida> 1,0-1,9 mm a 2 mm).
T1c	Tumor> 5 mm mas ≤ 10 mm em maior dimensão.
T2	Tumor> 10 mm mas ≤ 20 mm em maior dimensão.
T3	Tumor> 20 mm mas ≤ 50 mm em maior dimensão.
T4	Tumor> 50 mm em maior dimensão.
T4a	Tumor de qualquer tamanho com extensão direta à parede torácica e / ou à pele (ulceração ou nódulos cutâneos), não incluindo invasão da derme.
T4b	Extensão da parede torácica, não incluindo apenas aderência / invasão do músculo peitoral.
T4c	Ulceração e / ou nódulos satélites ipsilaterais e / ou edema (incluindo peau d'orange) da pele, que não preenchem os critérios para o carcinoma inflamatório.
T4d	T4a e T4b.
	Carcinoma Inflamatório.

Fonte: AJCC Cancer Staging Manual (8th edition). Springer International Publishing: American Joint Commission on Cancer; 2017.
*Nota: Carcinoma lobular in situ é considerado uma doença benigna e foi removido da classificação TNM no 8° AJCC.

Tabela 2.3 - Definição de linfonodo regional clínico (cN).

Categoria cN	Critério cN
cNx	Linfonodos regionais não podem ser avaliados (por exemplo, removidos anteriormente).
cN0	Nenhuma metástase linfonodal regional (em exames de imagem ou exame clínico).
cN1	Metástase para nível móvel ipsilateral I, linfonodo(s)
cN1mic	axilar(es) II.
	Micrometástases (aproximadamente 200 células, maiores
cN2	que 0,2 mm, mas nenhuma maior que 2,0 mm).
	Em metástases ipsilaterais nível I, II linfonodos axilares que estão clinicamente fixos ou emaranhados; ou em nódulos mamários internos ipsilaterais, na ausência de
cN2a	metástases linfonodais axilares clinicamente evidentes.
	Metástases nos linfonodos axilares de nível I e II
cN2b	ipsilaterais fixados um ao outro (emaranhados) ou a outras estruturas.
cN3	Metástases apenas em nódulos mamários internos ipsilaterais e na ausência de metástases linfonodais axilares.
	Metástases em linfonodo (s) infraclavicular (axilar(I) nível ipsilateral), com ou sem envolvimento do nódulo axilar de nível I ou II, ou em linfonodo(s) mamário(s) interno (s)
cN3a	ipsilateral com metástase em linfonodos axilares de nível
cN3b	I e II; ou metástases em linfonodo supraclavicular ipsilateral, com ou sem comprometimento linfonodal axilar
cN3c	ou interno de mama.
	Metástase em linfonodo infraclavicular ipsilateral.
	Metástase em linfonodo (s) mamário interno ipsilateral e linfonodo (s) axilar (es).
	Metástase em linfonodo supraclavicular ipsilateral.

Fonte: AJCC Cancer Staging Manual (8th edition). Springer International Publishing: American Joint Commission on Cancer; 2017

*Nota: Nota: os sufixos (sn) e (f) devem ser adicionados à categoria N para denotar a confirmação de metástase por biópsia de linfonodo sentinela ou biópsia por agulha fina / punção com agulha grossa, respectivamente.

Tabela 2.4 - Definição de linfonodo regional patológico (pN)

Categoria pN	Critério pN
pNx	Linfonodos regionais não podem ser avaliados (por exemplo, previamente removidos ou não removidos para estudo patológico).
pN0	Na metástase linfonodal regional, identificou-se histologicamente, ou clusters de células tumorais isolados (CTIs) apenas. Nota: as ITC são definidas como pequenos aglomerados de células ≤ 0,2 mm, ou células tumorais isoladas, ou um aglomerado de <200 células em uma única seção histológica; Os ITC podem ser detectados por histologia de rotina ou por métodos IHQ; os nós contendo apenas CTIs são excluídos da contagem total de nós positivos para fins de classificação N, mas devem ser incluídos no número total de nós avaliados.
pN0 (i)	Não há metástases linfonodais regionais histologicamente negativas IHQ.
pN0 (i+)	CTIs apenas em linfonodo(s) regional(ais).
pN0 (mol)	Nenhumas metástases linfonodais regionais histologicamente, achados moleculares negativos (reação em cadeia da polimerase via transcriptase reversa [RT-PCR]).
pN0 (mol+)	Achados moleculares positivos por RT-PCR; nenhum ITC detectado.
pN1	Micrometástases; ou metástases em 1-3 gânglios linfáticos axilares e / ou em nódulos mamários internos; e / ou em nódulos mamários internos clinicamente negativos com micrometástases ou macrometástases por biópsia de linfonodo sentinela.
pN1mi	Micrometástases (200 células,> 0,2 mm, mas nenhuma> 2,0 mm).
pN1a	Metástases em 1-3 gânglios linfáticos axilares (pelo menos 1 metástase> 2,0 mm).
pN1b	Metástases em linfonodos mamários internos ipsilaterais, excluindo ITC, detectadas por biópsia de linfonodo sentinela
pN1c	Metástases em 1-3 linfonodos axilares e linfonodos sentinelas mamários internos (isto é, pN1a e pN1b combinados).
pN2	Metástases em 4-9 linfonodos axilares; ou linfonodos mamários internos ipsilaterais positivos por imagem na ausência de metástases linfonodais axilares.
pN2a	Metástases em 4-9 linfonodos axilares (pelo menos 1 depósito de tumor> 2,0 mm).

pN2b	Metástases detectadas clinicamente* em linfonodos mamários internos com ou sem confirmação microscópica; com linfonodos axilares patologicamente negativos.
pN3	Metástases em 10 linfonodos axilares; ou em nódulos linfáticos infraclaviculares (axilares de nível III); ou linfonodos mamários internos ipsilaterais positivos por exames de imagem na presença de um ou mais linfonodos axilares positivos de nível I e II; ou em> 3 linfonodos axilares e micrometástases ou macrometástases por biópsia de linfonodo sentinela em linfonodos mamários internos ipsilaterais clinicamente negativos; ou nos gânglios linfáticos supraclaviculares ipsilaterais.
pN3a	Metástases em 10 linfonodos axilares (pelo menos 1 depósito tumoral> 2,0 mm); ou metástases nos linfonodos infraclaviculares (linfonodos axilares de nível III).
pN3b	pN1a ou pN2a na presença de cN2b (linfonodos mamários internos positivos por imagem) ou pN2a na presença de pN1b.
pN3c	Nas metástases linfáticas supraclaviculares ipsilaterais.

Fonte: AJCC Cancer Staging Manual (8th edition). Springer International Publishing: American Joint Commission on Cancer; 2017.

*Nota: "Detectada clinicamente" é definida como detectada por exames de imagem (excluindo a linfocintilografia) ou por exame clínico e com características altamente suspeitas de malignidade ou presumível macrometástase patológica com base na biópsia da PAAF com exame citológico.

Tabela 2.5 - Definição de metástase a distância

Categoria M	Critério M
M0	Nenhuma evidência clínica ou radiográfica de metástase à distância.
cM0 (i+)	Nenhuma evidência clínica ou radiográfica de metástases à distância na presença de células tumorais ou depósitos não maiores que 0,2 mm detectados microscopicamente ou por técnicas moleculares no sangue circulante, medula óssea ou outro tecido nodal não regional em um paciente sem sintomas ou sinais de metástase.
cM1	Metástases à distância detectadas por meios clínicos e radiográficos.
pM1	Quaisquer metástases histologicamente provocadas em órgãos distantes; ou se nos linfonodos não regionais, metástases> 0,2 mm.

Fonte: AJCC Cancer Staging Manual (8th edition). Springer International Publishing: American Joint Commission on Cancer; 2017.

Tabela 2.6 - Grupos de estadios anatômicos AJCC

Estadio	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1mi	M0
	T1	N1mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	IIIC	IIIC	IIIC
Qualquer T	Qualquer T	Qualquer T	Qualquer T

Fonte: AJCC Cancer Staging Manual (8th edition). Springer International Publishing: American Joint Commission on Cancer; 2017.

Nota: A designação do estadio pode ser alterada se os estudos de imagem pós-cirúrgicos revelarem a presença de metástases à distância, desde que os estudos sejam realizados dentro de 4 meses do diagnóstico na ausência de progressão da doença e desde que o paciente não tenha recebido terapia neoadjuvante. O estadiamento após a terapia neoadjuvante é designado com o prefixo “yc” ou “yp” para a classificação T e N. No grupo de estadio anatômico é atribuído se houver uma resposta patológica completa (RPC) à terapia neoadjuvante, por exemplo, ypT0ypN0cM0.

2.7 Tratamento neoadjuvante do câncer de mama triplo-negativo

A quimioterapia neoadjuvante conceitualmente administrada antes do tratamento loco-regional (cirurgia/radioterapia) é o tratamento padrão para o CMLA (COSTA; GRADISHAR; HANSEN, 2018). A resposta à quimioterapia depende do regime utilizado, assim como do subtipo tratado. Antraciclinas e taxanos são o grupo mais ativo de quimioterapia usado para câncer de mama (ANAMPA; MAKOWER; SPARANO JA, 2015). Estes regimes são geralmente administrados com outras drogas quimioterápicas como a ciclofosfamida, fluorouracil. As antraciclinas incluem doxorrubicina, epirrubicina e mitoxantrona. Por outro lado, os compostos taxanos são originalmente identificados a partir da planta do gênero *Taxus*, sendo docetaxel e paclitaxel os mais utilizados em câncer de mama (ESTÉVEZ; GRADISHAR, 2004).

A quimioterapia neoadjuvante aumenta a chance de cirurgia conservadora da mama, mas não há consenso sobre o papel das drogas quimioterápicas no aumento da taxa de cirurgia conservadora da mama (DIÉRAS *et al.*, 2004; SAURA *et al.*, 2013). Além disso, a obtenção da RPC à quimioterapia neoadjuvante prediz resultados melhores de sobrevida a longo prazo, uma vez que os pacientes com câncer de mama que alcançam a RPC têm melhor sobrevida do que os pacientes que não o fazem (RASTOGI *et al.*, 2008).

Quanto a escolha do melhor regime, os resultados relatados de ensaios clínicos randomizados foram contraditórios, pois alguns favoreceram a quimioterapia baseada em taxanos sobre a quimioterapia baseada em antraciclinas (ROMERO *et al.*, 2013; ABRAHAM *et al.*, 2015) em relação ao RPC, enquanto outros sugerem o contrário (HEYS *et al.*, 2002; EVANS *et al.*, 2005). A eficácia dos taxanos sobre as antraciclinas foi avaliada em alguns estudos de tratamento adjuvante e verificou-se que está associada ao aumento da sobrevida global nesses casos (FUJII *et al.*, 2015). Apesar de não ter influenciado a taxa de RPC, os resultados do estudo institucional de fase II NeoSAMBA com antraciclina-taxane sugeriram uma melhora em EFS e OS com a sequência de quimioterapia neoadjuvante taxano primeiro quando comparado com antraciclina primeiro em pacientes HER2-negativos de mama localmente avançada Câncer (BINES et al., 2020).

Duas revisões sistemáticas divagaram sobre a eficácia relativa dos taxanos, mas não puderam sintetizar os resultados para a resposta local numa metanálise por conta da

escassez de estudos confiáveis naquele momento (NOWAK *et al.*, 2004; TRUDEAU *et al.*, 2005). Além disso, essas revisões não puderam analisar o efeito dos taxanos nos resultados de sobrevida de longo prazo. Já uma revisão sistemática com metanálise recentemente publicada concluiu que a adição de taxanos à quimioterapia baseada em antraciclinas melhora significativamente as taxas de RPC, a sobrevida livre de doença e a sobrevida livre de recidiva loco-regional, mas sem impacto significativo nas taxas cirurgia conservadora da mama (PATHAK *et al.*, 2019).

O uso da carboplatina em associação ao regime padrão, considerando a fisiopatologia favorável de resposta do CMTN a platina, tem apresentado resultados atrativos em alguns estudos após o ano de 2014. Nesse sentido, a adição de platina vem sendo incorporada à quimioterapia neoadjuvante em alguns casos específicos no serviço por meio de discussão em tumor *board*. Entretanto, dados de um estudo brasileiro de fase II braço-único, que analisou o uso de CAP (ciclofosfamida, doxorrubicina e cisplatina) neoadjuvante em 41 pacientes com CMTN estadio IIB/III, mostraram um RPC de 19.5%, mas com um perfil de tolerabilidade muito desfavorável (FERREIRA *et al.*, 2018).

Em torno de 80% dos casos de tumores hereditários de mama associado a mutação de *BRCA 1* são do subtipo TNBC e são particularmente sensíveis a platina devido ao defeito na aparelhagem reparo de dano de DNA pela recombinação homóloga (TURNER; TUTT, 2012). Alguns estudos com CMTN metastáticos resultaram em taxa de resposta de 10-40% à exposição a agentes de platina (LIU *et al.*, 2013). Dois grandes ensaios clínicos mostraram aumento de taxa de RPC com a adição de carboplatina a quimioterapia neoadjuvante. No CALGB 40603 (Alliance), a taxa de RPC passou de 46% para 60% (OR, 1.76; p = 0,0018), por outro lado não houve aumento estatisticamente significativo da sobrevida livre de eventos em 3 anos (HR, 0,84; IC 95%, 0,58 – 1,22; p = 0,36) e nem de SG (HR, 1,15; IC 95% 0,74–1,79; p = 0,53) (SIKOV *et al.*, 2015, 2019). Com resultados claramente melhores, no grupo de pacientes com CMTN do estudo GeparSixto, a taxa de RPC passou de 37% para 53% (p = 0.005), traduzindo num aumento de sobrevida livre de eventos em 3 anos em 9,7% (HR, 0,56; IC 95% 0,33–0,96) (VON MINCKWITZ *et al.*, 2014).

Para as pacientes que fazem tratamento completar neoadjuvante por resposta tumoral insatisfatória ao tratamento padrão, os desfechos são claramente piores quando

comparados ao das pacientes que tiveram resposta clínica. O uso complementar de quimioterapia e/ou radioterapia nesse contexto apresenta resultados conflitantes na literatura. Dados retrospectivos de 57 pacientes tratadas com radioterapia neoadjuvante complementar no INCA mostraram efetividade em reduzir o tamanho dos tumores localmente avançado com baixa ou nenhuma resposta à quimioterapia, permitindo assim a ressecção cirúrgica e melhor os defeitos clínicos dos pacientes (COELHO *et al.*, 2017). Numa coorte retrospectiva com 21 mulheres expostas a quimiorradioterapia concomitante baseada em capecitabina para resgate após quimioterapia neoadjuvante, a comparação pareada com controles mostrou uma sobrevida global significativamente pior (LIU *et al.*, 2018). Por outro lado, em dois estudos de fase II com desenhos semelhantes, pouco mais de 85% tornaram-se operáveis após exposição a radioterapia concomitante com capecitabina (GAUI *et al.*, 2007; ABD; EL-N; HUSSEN, [*s. d.*]).

Na tentativa de intensificar o tratamento das pacientes de alto risco com pouca resposta a quimioterapia neoadjuvante, dados mais recentes são controversos em sugerir que pacientes com carga de doença residual na peça cirúrgica, após quimioterapia neoadjuvante, sejam considerados para um tratamento sistêmico adjuvante complementar prolongado, uma vez que elas têm uma chance maior de recaída e recidiva de doença a distância. O estudo CREATE-X mostrou um potencial benefício de maior sobrevida com o uso da capecitabina como esquema quimioterápico adjuvante padrão no CMTN localmente avançado com alta carga tumoral residual (da sigla em inglês RBC) após o tratamento neoadjuvante (MASUDA *et al.*, 2017).

Similarmente, os resultados de uma metanálise também dão suporte para a adição de capecitabina à quimioterapia padrão neoadjuvante ou adjuvante em pacientes com CMTN (NATORI *et al.*, 2017). Por outro lado, o estudo CIBOMA, que recrutou pacientes com TNBC operadas primariamente e submetidas a quimioterapia adjuvante padrão (antraciclina/taxane ou antraciclina isolada), falhou em mostrar um aumento estatisticamente significativo de SLD ao adicionar capecitabina estendida ao tratamento sistêmico pós-operatório (LLUCH *et al.*, 2019).

Os dados do uso de cisplatina como tratamento de resgate para pacientes sem resposta satisfatória a quimioterapia neoadjuvante padrão, em câncer de mama, também são controversos. Um estudo retrospectivo mostrou resultados de boa resposta e

desfechos de sobrevida interessantes com o uso da cisplatina concomitante com radioterapia após exposição ao esquema de quimioterapia neoadjuvante padrão (RAPHAEL *et al.*, 2017). Numa outra coorte com 19 pacientes CMTN resistentes a quimioterapia neoadjuvante padrão com antraciclina-taxane, tratadas com cisplatina concomitante com radioterapia, a taxa de resposta foi de 64% tornando os tumores operáveis e possibilitando a cirurgia (LEE *et al.*, 2017).

3 JUSTIFICATIVA DO ESTUDO

Os fatores que determinam diferentes padrões de resposta do CMTN à quimioterapia neoadjuvante, num contexto de doença localmente avançada, ainda não foram totalmente elucidados. Logo, há uma forte necessidade de se explorar fatores sociodemográficos e clínico-patológicos a fim de se realizar abordagens mais efetivas com terapias mais direcionadas nesse cenário.

Nesse contexto, muitas questões ainda precisam ser respondidas com relação a interpretação do papel preditivo e prognóstico da expressão por IHQ de biomarcadores como RA, EGFR, CK5/6, CK14, CK17, CD117, p53, Ki67, PD-L2, PD-L1 na célula tumoral, assim como, quando presente, da qualidade do infiltrado linfo-mononuclear intratumoral (PD-1 +, PD-L1+, FOXP3 +, CD4 + ou CD8 +, CD3 +, CD56 +, CD68 + ou CD14+).

Até o momento, os dados brasileiros publicados sobre os tipos de câncer de mama são bastante escassos, bem como não há qualquer menção a uma avaliação, mesmo exploratória, de fatores específicos relacionados ao contexto de CMTN.

Trata-se de um estudo original e inédito numa instituição brasileira de pesquisa clínica oncológica de relevância nacional, em que se propõe uma análise do perfil sócio-clínico-patológico e uma avaliação pareada por imunohistoquímica pré e pós-quimioterapia neoadjuvante das pacientes com CMTN localmente avançado, matriculadas no Instituto Nacional de Câncer no período de janeiro de 2010 a dezembro de 2014, cujos resultados poderão servir como base para gerar hipóteses importantes a serem confirmadas através de estudos prospectivos.

4 HIPÓTESES

I. Pacientes com características sociodemográficas e clinicopatológicas historicamente desfavoráveis apresentam desfechos de resposta e sobrevida piores (*objetivo secundário I a ser citado no item 5.2*).

II. Biomarcadores tumorais, analisados aqui por IHQ *(objetivos secundários II e III do item 5.2),* podem influenciar em cada avaliação específica, os desfechos de resposta e sobrevida das pacientes com CMTN tratadas com quimioterapia neoadjuvante.

5 OBJETIVOS

5.1 Objetivo primário

Avaliar a influência de fatores sócio-clínico-patológicos e biomarcadores tumorais de IHQ na predição de resposta clínica a quimioterapia neoadjuvante assim como nos desfechos de sobrevida.

5.2. Objetivos secundários

I. Avaliar de forma exploratória se há associação das variáveis sociodemográficas (raça, idade ao diagnóstico, escolaridade, tabagismo, etilismo, tempo da confirmação diagnóstica pela core biópsia para início da quimioterapia neoadjuvante padrão, tempo do término da quimioterapia neoadjuvante para a abordagem cirúrgica, distância da residência ao centro de tratamento) e variáveis clínico-patológicas (estádio clínico, TNM, grau tumoral) com RPC, sobrevida livre de eventos (SLE) e SG (*artigo 1*).

II. Apresentar descritivamente o escore da IHQ dos biomarcadores RA, EGFR, CK5/6, CK14, CK17, CD117, p53, Ki67, PD-L2, PD-L1 na célula tumoral, além da descrição da qualidade do infiltrado linfo-mononuclear intratumoral quando presente (PD -1 +, FOXP3 +, CD4 + ou CD8 +, CD3 +, CD56 +, CD68 + ou CD14 +) tanto na core biópsia quanto na peça cirúrgica (*artigos 2 e 3*).

III. Avaliar de forma exploratória a influência do status dos biomarcadores RA, EGFR, CK5/6, CK14, CK17, CD117, p53, Ki67, PD-L2, PD-L1 na célula tumoral, além da qualidade do infiltrado linfo-mononuclear intratumoral quando presente (PD -1 +, FOXP3 +, CD4 + ou CD8 +, CD3 +, CD56 +, CD68 + ou CD14 +) na core biópsia para os desfechos de taxa de RPC, SLE e SG, assim como na peça cirúrgica de tumores residuais para desfechos de SLE e SG (*artigos 2 e 3*).

6 METODOLOGIA

A metodologia detalhada do estudo está descrita no Apêndice A.

Os documentos de aprovação da isenção de TCLE e do projeto no CEP estão apresentados nos anexos C e D.

7 RESULTADOS

7.1 Esta tese será apresentada em formato de artigos

7.1.1 Artigo 1 (Publicado) - Sociodemographic, clinical, and pathological factors influencing outcomes in locally advanced triple negative breast cancer: a Brazilian cohort.

7.1.2 Artigo 2 (Publicado) - Triple-negative breast cancer: assessing the role of immunohistochemical biomarkers on neoadjuvant treatment.

7.1.3 Artigo 3 (Submetido - em revisão) - Prognostic influence of residual tumor-infiltrating lymphocyte subtype after neoadjuvant chemotherapy in triple-negative breast cancer.

7.1.1 Artigo 1

Sociodemographic, Clinical, and Pathological Factors Influencing Outcomes in Locally Advanced Triple Negative Breast Cancer: A Brazilian Cohort

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ABSTRACT

OBJECTIVE: To evaluate the association of sociodemographic, clinical, and pathological factors with response and survival in triple negative breast cancer (TNBC) undergoing neoadjuvant chemotherapy (NACT).

METHODS: Clinical-pathological and sociodemographic data were obtained from medical records of 235 eligible women with TNBC diagnosed between 2010 and 2014 undergoing NACT and surgery at the Brazilian National Cancer Institute. They have been assessed for pathological complete response (pCR), event-free survival (EFS), and overall survival (OS). Both univariate and multivariate Cox regression analyses were performed.

RESULTS: The median follow-up was 64.3 months. Most patients had advanced clinical stage (III: 85.1%; cT3/T4: 86.4%; cN1-3: 74.4%) and high-grade tumors (72.1%). Clinical staging (III vs II, adjusted hazard ratio [HR] = 2.95, $P = .012$) significantly influenced the pCR rate. Alcohol intake negatively influenced EFS (adjusted HR = 1.67, $P = .006$) and OS (adjusted HR = 1.89, $P = .005$). Women with pCR showed better EFS (crude HR = 0.15, $P < .001$) and OS (crude HR = 0.12, $P < .001$) compared with non-pCR. The ypT (<0.001) and ypN (<0.001) gradually influenced survival outcomes.

CONCLUSION: Clinical stage III were associated with lower response rate and worse survival. Alcohol intake, pCR, and burden of post-NACT residual disease have shown considerable influence on survival outcomes.

KEYWORDS: Triple negative breast cancer, neoadjuvant chemotherapy, prognostic factors, predictive factors, complete pathological response

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Introduction

According to GLOBOCAN 2018 cancer statistics, breast cancer is the most common cancer in women in the vast majority of countries around the world, as well as the leading cause of cancer death in more than 100 countries.[1] Triple negative breast cancer (TNBC) accounts for approximately 12% to 17% of primary breast tumors.[2] Histologically, it is defined as tumors lacking estrogen receptor (ER), progesterone receptor (PR) expression, and human epidermal growth factor receptor 2 (HER2).[3] Generally, TNBCs are mostly high-grade tumors with higher incidence in younger women, associated with poorer overall prognosis, increased risk of early distant relapse, and higher risk of premature death. Metastases tend to be visceral, mostly occurring at the pulmonary, pleural, hepatic, and central nervous system sites. These clinical features represent a major medical concern in the management of these patients.[4,5]

Unlike luminal or HER2-positive types, which are known to be sensitive to hormone therapy and anti-HER2 agents, respectively, the treatment of TNBC is based on cytotoxic chemotherapy. In this context, neoadjuvant chemotherapy (NACT) has become the standard of care for most locally advanced TNBCs. This is due to the possibility of greater chances of breast-conserving surgery and consistent evidence of pathologic complete response (pCR) as a strong predictor, or even surrogate, of long-term survival outcomes.[6] Anthracycline and taxane-based chemotherapy regimens are the current standard therapy in most cases, showing pCR rates of around 17% to 40% in some studies.[7] However, recent clinical trials have proposed a refinement of these therapeutic schemes with new drugs such as platinum-based agents, immunotherapy and Poly [ADP-ribose] polymerase 1 (PARP) inhibitors, or even dose-dense regimens.[8,9]

Some sociodemographic, clinical, and pathological factors may influence the outcomes of NACT in patients with locally advanced TNBC. Nevertheless, there is scarce data in the literature about the role some of these variables have in this specific setting. This study aims to evaluate the association of these factors with tumor response and survival outcomes, as well as present the institutional profile of women with TNBC undergoing NACT at the Brazilian National Cancer Institute (INCA).

Box 1. Neoadjuvant chemotherapy regimens.

STANDARD NACT REGIMENS	DOSE/SCHEDULE
FAC	Fluorouracil 500 mg/m², doxorubicin 50 mg/m², and cyclophosphamide 500 mg/m², administered intravenously every 21 days for 6 cycles.
FAC-T	Fluorouracil 500 mg/m², doxorubicin 50 mg/m², and cyclophosphamide 500 mg/m², administered intravenously every 21 days for 3 cycles, followed by docetaxel 100 mg/m² every 21 days for 3 cycles.
AC-T	Doxorubicin 60 mg/m² and cyclophosphamide 600 mg/m², given intravenously every 21 days for 4 cycles, followed by docetaxel 100 mg/m² given intravenously every 21 days for 4 cycles, or followed by weekly paclitaxel 80 mg/m² given intravenously for 12 consecutive weeks without interval, defined here as a total of four 3-week cycles.
CT[a]	Cyclophosphamide 600 mg/m² and docetaxel 75 mg/m² administered every 21 days intravenously for 4 cycles
Complementary chemotherapy[b]	Dose/schedule
Cisplatin	75 mg/m² administered every 21 days intravenously during radiotherapy.
Capecitabine	850 mg/m² orally twice daily for 14 days every 3 weeks concomitant with radiotherapy.

Abbreviation: NACT, neoadjuvant chemotherapy; FAC, fluorouracil, doxorubicin and cyclophosphamide; FAC-T, fluorouracil, doxorubicin and cyclophosphamide followed by docetaxel; AC-T, doxorubicin and cyclophosphamide followed by docetaxel or followed by weekly paclitaxel; CT, cyclophosphamide and docetaxel.
[a]Non-anthracycline option defined by the institutional tumor board for selected cases.
[b]Following the routine of the oncology team, patients with tumors considered unresectable soon after NACT were exposed to complementary chemotherapy and/or salvage radiotherapy to achieve clinical response to enable the surgical approach.

Materials and Methods

Study design and ethical considerations

This retrospective cohort was designed to assess the influence of sociodemographic, clinical, and pathological factors on the prediction of clinical response to NACT and on survival outcomes. The study was approved by the Ethics in Human Research Committee of INCA, Rio de Janeiro, Brazil, under number CAAE 61675516.9.0000.5274, and conducted in accordance with Good Clinical Practice guidelines.

Patient selection

Patients newly diagnosed with breast cancer at INCA between January 2010 and December 2014 were included if all the following criteria were met: (a) women more than 18 years old; (b) diagnosis of TNBC (tumors with ER and PR score $< 1\%$, as well as HER-2 score 0/1+ or 2+ with negative FISH) by the INCA Pathology Department (DIPAT/INCA) following the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines[10,11]; (c) stage IIb-IIIc by the 7th AJCC (T3-4N*any*M0; T*any*N1-3M0); and (d) undergoing NACT and curative surgery at INCA. In turn, patients with synchronic or anachronistic tumors, previously exposed to antineoplastic agents were excluded, as well as patients who remained with unresectable tumors, even after standard NACT and complementary treatment with chemotherapy and/or radiotherapy.

Variables and outcomes

Patients were identified through internal database. Data were collected from electronic hospital records and medical charts. The following sociodemographic and treatment variables were evaluated: age at diagnosis, ethnicity (Caucasian or others according to national institutional statistical classifications, IBGE[12]), schooling (<8 or $\geqslant 8$ years), smoking and alcohol consumption (previous or current habit), body mass index (BMI), distance from home to hospital (set by Google Maps), type of standard NACT (detailed in Box 1: FAC, FAC-T, AC-T, or CT), time from diagnosis to NACT onset, time from the end of standard NACT to surgery, compliance to standard NACT (median cycles; complete vs incomplete treatment), and site of progression. The clinical and pathological variables evaluated were clinical stage (II-III), clinical T stage (cT), clinical nodal stage (cN), pathological T stage (ypT), pathological nodal stage (ypN), lymphovascular invasion (LVI), perineural infiltration (PI), Elston histological grade (1-2: low grade; 3: high grade), and type of surgery (radical or conservative, axillary approach type).

The pCR was defined as no viable tumor in the breast or axilla (ypT0N0).[6] Event-free survival (EFS) was calculated from the date of diagnosis to the earliest date of disease progression, death from any cause, or discontinuation of treatment for initiation of complementary treatment due to poor response to standard NACT. Overall survival (OS) was calculated from the date of diagnosis to the date of death or censored if the patient was known to be alive on the last day of data collection.

Statistical analysis

Statistical analyses were conducted using R environment.[13] All continuous variables were evaluated by the Shapiro-Wilk test of normality. For the pCR outcome, logistic regression was used for each variable assessed to calculate the odds ratio (OR). Survival rates were calculated by Kaplan-Meier curves for each factor and were compared by log-rank test. The crude hazard ratio (HR) for each factor was calculated by the Cox

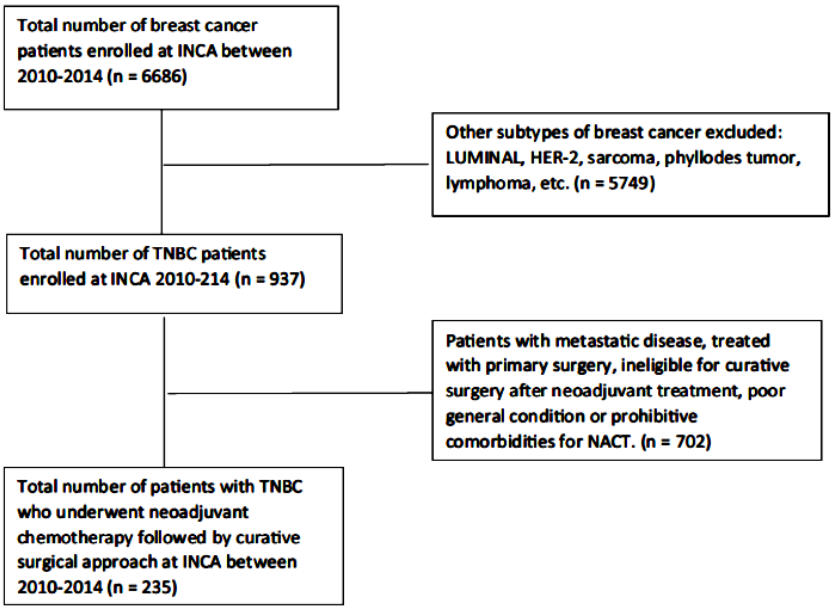

Figure 1. Study profile. HER-2 indicates human epidermal growth factor receptor 2; INCA, Brazilian National Cancer Institute; NACT, neoadjuvant chemotherapy; TNBC, triple negative breast cancer.

proportional hazards model. All variables associated with survival outcomes at $P < .20$ on univariate analysis were included in multivariate models. The Akaike criteria was used to pick the most suitable model for multiple Cox analysis. A P-value of .05 or less was considered to indicate statistical significance. The missing data were excluded from the analysis.

Results

Patients' characteristics

A total of 235 cases of TNBC were eligible for the study (Figure 1). The main characteristics of the patients are summarized in Table 1. The mean age was 50.1 years (range, 23.5-75.8), most women were Caucasian (47.6%) and had completed 8 or more years of education (55.2%). The median BMI was 28.1 kg/m^2 (interquartile range [IQR], 24.4; 32.5). Smoking and alcohol consumption were reported by 24.2% and 22.9% of the patients, respectively. The median home distance to INCA was 28 km (IQR, 17; 40). At diagnosis, most patients had advanced clinical stage tumors (≥IIIa: 85.1%; cT3/T4: 86.4%; cN1-3: 74.4%) and the predominant histological subtype was high-grade invasive ductal carcinoma (72.1%). Metaplastic carcinoma accounted for only 4.7% of cases, with the other patients (95.3%) having non-special-type invasive carcinoma. At surgery, LVI and PI were present in 23.4% and 11% of cases, respectively, and the pathological nodal stage was predominantly ypN0-1 (76.6%).

Treatment data

Mastectomy was the treatment of choice in 97.4% of cases and axillary dissection was performed in 86.8% of the patients, as shown in Table 2. Regarding systemic treatment, 94.1% underwent chemotherapy with anthracycline and taxane-based regimens and 83.4% completed all the cycles of NACT as scheduled. Complementary chemotherapy was performed in only 6.8% of cases and neoadjuvant radiotherapy in 4.7%. The median time from diagnosis to initiation of treatment was 90.0 days (IQR, 58; 126.5) and slightly more than a quarter of patients (27.2%) started treatment in less than 60 days.

Pathological response and survival outcomes

The overall pCR rate was 21.2%. By univariate analysis, patients with clinical stage II (crude OR = 0.99, $P = .005$) and not exposed to alcohol intake (crude OR = 0.38, $P = .036$) had better pCR rate, as shown in Table 3. In the final model selected for multivariate analysis, only clinical stage II (adjusted OR = 2.95, $P = .012$) was associated with higher pCR rate.

The median follow-up was 64.3 months (95% confidence interval [CI]: 60.3-68.2). Locoregional recurrence occurred in 51 patients (21.7%) and distant recurrence was observed in 95 (40.4%). The most common distant sites were pleuropulmonary (23%), nodal (14%), hepatic (10.6%), bone (10.6%), and central nervous system (6.4%) (data not shown). For the general population of the study, with 114 events, the probability of 3-year EFS and 5-year EFS was, respectively, 59.4% (95% CI: 53.4-66.2) and 53.3% (95% CI: 47.0-60.5). The median EFS was 76.5 months (95% CI: 44.76-not reached [NR]). For patients with pCR vs non-pCR, the probability of 3-year EFS and 5-year EFS was, respectively, 98.0% (95% CI: 94.2-100) vs 48.9% (95% CI: 42.2-56.9) and 93.3% (95% CI: 86.3-100) vs 42.5 (95% CI: 35.6-50.7) (data not shown). As shown in Table 4, patients with pCR had an 85% reduction in risk of

Table 1. Baseline sociodemographic characteristics of eligible patients.

VARIABLES	N = 235 (100%)
Mean age, y (SD)	50.1 (± 11.5)
Race/ethnicity White	111 (47.6)
Schooling ⩾ 8 y	127 (54.0)
Smoking	57 (24.2)
Alcohol consumption	54 (22.9)
Median BMI, kg/m² (IQR)	28.1 (24.4; 32.5)
Median home distance, km (IQR)	28 (17; 40)
Clinical staging	
II	35 (14.9)
III	200 (85.1)
Clinical T stage	
cTx	1 (0.4)
cT2	31 (13.2)
cT3	99 (42.1)
cT4	104 (44.3)
Clinical N stage	
N0	60 (25.6)
N1-N3	175 (74.4)
Histological subtype	
Metaplastic	11 (4.7)
Non-special-type invasive carcinoma	234 (95.3)
Histologic grade	
Grade 1	3 (1.4)
Grade 2	61 (25.9)
Grade 3	171 (72.7)
LVI positive status	55 (23.4)
PI positive status	26 (11.1)

Abbreviations: BMI, body mass index; IQR, interquartile range; LVI, lymphovascular invasion; PI, perivascular infiltration; SD, standard deviation. Missing values: race/ethnicity (2; 0.8%), schooling (5; 2.1%), smoking (1; 4.7%), alcohol consumption (13; 5.5%), home distance (5; 2.1%), LVI (35; 14.9%), and PI (64; 27.2%).

events as compared with non-pCR (crude HR = 0.15, 95% CI: 0.06-0.34, $P < .001$). Herein, the gradient of post-NACT residual disease burden, represented by ypT0-4 ($P < .001$) and ypN0-3 ($P < .001$), also showed a gradual effect on EFS. Still in the univariate analysis, alcohol intake increased by 74% ($P = .02$) the risk of presenting an event.

Table 2. Data from neoadjuvant chemotherapy and surgical treatment.

TREATMENT	N = 235 (100%)
Standard NACT	
AC-T	131 (55.8)
FAC-T	90 (38.3)
FAC	6 (2.6)
AC	1 (0.4)
CT	7 (2.9)
Complete standard NACT	196 (83.4)
Median time (days) from diagnosis to NACT (IQR)	90.0 (58; 126.5)
⩽60 days	64 (27.2)
>60 days	171 (72.8)
Median time (days) from the end of NACT to surgery (IQR)	48.0 (36.5; 71.5)
Post-NACT complementary treatment	
Cisplatin	10 (4.2)
Capecitabine	6 (2.6)
Radiotherapy	11 (4.7)
Type of surgery	
Breast-conserving surgery	6 (2.6)
Mastectomy	229 (97.5)
Axillary approach	
Sentinel lymph node biopsy	13 (5.5)
Axillary lymph node dissection	204 (86.8)

Abbreviations: IQR, interquartile range; NACT, neoadjuvant chemotherapy.

Regarding OS, with 101 deaths, the estimated probability of patients being alive at 3 and 5 years was, respectively, 68.2% (95% CI: 62.3-74.6) and 59.6% (95% CI: 53.0-66.5). The median OS was 83.36 months (95% CI: 65.66-NR). For patients with pCR vs non-pCR, the probability of 3-year EFS and 5-year EFS was, respectively, 98% (95% CI: 94.2-100) vs 60.2% (95% CI: 53.3-67.9) and 98% (95% CI: 94.2-100) vs 49.9% (95% CI: 42.8-58.2) (data not shown). As shown in Table 5, patients with pCR had an 89% reduction in risk of death as compared with non-pCR (HR = 0.11, 95% CI: 0.04-0.31, $P < .001$). The residual disease burden gradient, composed of ypT0-4 ($P < .001$) and ypN0-3 ($P < .01$), showed a gradual association with OS. In the univariate analysis, alcohol intake increased by 97% ($P = .002$) the risk of death. Kaplan-Meier survival curves for EFS and OS are, respectively, shown in Figures 2 and 3.

As shown in Table 4, following the Akaike criteria, a model with 3 variables were selected for the EFS multivariate analysis.

Table 3. Univariate and multivariate analysis according to pathological complete response.

	CRUDE OR FOR PCR (95% CI, *P*-VALUE)	ADJUSTED OR FOR PCR (95% CI%, *P*-VALUE)
Age	0.99 (0.96-1.01, *P* = .526)	
Clinical stage		
II	**3.04 (1.39-6.51, *P* = .005)**	**2.95 (1.25-6.86, *P* = .012)**
III[a]	–	
Clinical T stage		–
cT0-cT2[a]	–	
cT3-cT4	0.45 (0.20-1.05, *P* = .056)	
Clinical N stage		–
cN0-cN1[a]	–	
cN2-cN3	0.54 (0.25-1.098, *P* = .102)	
Smoking		–
Not exposed[a]	–	
Exposed	0.72 (0.32-0.15, *P* = .409)	
Alcohol consumption		
Not exposed[a]	–	
Exposed	**0.38 (0.14-0.88, *P* = .036)**	**0.42 (0.15-1.01, *P* = .053)**
Schooling		
⩾8 y	0.66 (0.34-0.25, *P* = .203)	
<8 y[a]	–	
Race		
Caucasian	**1.65 (0.88-3.17, *P* = .126)**	**1.63 (0.82-3.29, *P* = .167)**
Non-Caucasian[a]	–	
BMI		
⩽30 kg/m^2[a]	–	
>30 kg/m^2	**1.58 (0.83-3.02, *P* = .162)**	**1.05 (0.99-1.11, *P* = .082)**
Home distance	0.99 (0.98-1.0, *P* = .411)	–
Time from diagnosis to NACT onset	1.00 (0.99-1.00, *P* = .963)	–
Compliance to NACT		–
Incomplete treatment[a]		
Complete treatment	1.28 (0.56-3.35, *P* = .579)	
Duration of treatment	1.00 (0.99-1.01, *P* = .680)	–
Regimen of NACT		–
FAC-T[a]	–	
AC-T	1.28 (0.66-2.53, *P* = .475)	

Abbreviations: BMI, body mass index; CI, confidence interval; NACT, neoadjuvant chemotherapy; FAC-T, fluorouracil, doxorubicin and cyclophosphamide followed by docetaxel; AC-T, doxorubicin and cyclophosphamide followed by docetaxel or followed by weekly paclitaxel; OR, odds ratio; pCR, pathological complete response. The variables of the final model selected for analysis by the Cox multiple model were highlighted in bold.
Regarding the chemotherapy regimen, only the AC-T vs FAC-T regimens were compared.
[a]Reference.

Table 4. Univariate and multivariate analysis according to event-free survival.

	CRUDE HR FOR EFS (95% CI%, *P*-VALUE)	ADJUSTED HR FOR EFS (95% CI%, *P*-VALUE)
Clinical stage		
II[a]		
III	**2.72 (1.32-5.59, *P* = .007)**	**2.57 (1.19-5.56, *P* = .016)**
Clinical T stage		
cT0-cT2[a]		
cT3-cT4	3.57 (1.57-8.15, *P* = .002)	
Clinical N stage		
cN0-cN1[a]		
cN2-cN3	2.33 (1.60-3.40, *P* < .001)	
Alcohol consumption		
Not exposed[a]		
Exposed	**1.74 (1.17-2.59, *P* = .006)**	**1.67 (1.12-2.48, *P* = .012)**
Smoking		
Not exposed[a]		
Exposed	1.20 (0.79-1.81, *P* = .393)	
BMI		
⩽30 kg/m^2[a]		
>30 kg/m^2	1.09 (0.72-1.66, *P* = .683)	
Schooling		
⩾8 y[a]		
<8 y	1.22 (0.84-1.78, *P* = .299)	
Home distance (median)	1.00 (0.99-1.00, *P* = .626)	
Compliance to NACT		
Incomplete treatment[a]		
Complete treatment	**0.48 (0.30-0.76, *P* = .002)**	**0.54 (0.34-0.85, *P* = .08)**
Pathological T stage		
ypT0[a]		
ypT1	3.56 (1.69-7.53, *P* = .001)	
ypT2	4.28 (2.05-8.96, *P* < .001)	
ypT3	9.62 (4.55-20.34, *P* < .001)	
ypT4	9.94 (4.02-24.57, *P* < .001)	
Pathological N stage		
ypN0[a]		
ypN1	2.44 (1.45-4.09, *P* = .001)	

(Continued)

Table 4. (Continued)

	CRUDE HR FOR EFS (95% CI%, *P*-VALUE)	ADJUSTED HR FOR EFS (95% CI%, *P*-VALUE)
ypN2	4.19 (2.62-6.72, $P < .001$)	
ypN3	7.75 (4.28-14.02, $P < .001$)	
pCR status		
Non-pCR[a]		
pCR	0.15 (0.06-0.34, $P < .001$)	

Abbreviations: BMI, body mass index; CI, confidence interval; EFS, event-free survival; HR, hazard ratio; NACT, neoadjuvant chemotherapy; pCR, pathological complete response; FAC-T, fluorouracil, doxorubicin and cyclophosphamide followed by docetaxel; AC-T, doxorubicin and cyclophosphamide followed by docetaxel or followed by weekly paclitaxel.
The variables of the final model selected for analysis by the Cox multiple model were highlighted in bold.
Regarding the chemotherapy regimen, only the AC-T vs FAC-T regimens were compared.
[a]Reference.

Table 5. Univariate and multivariate analysis according to overall survival.

	CRUDE HR FOR OS (95% CI, *P*-VALUE)	ADJUSTED HR FOR OS (95% CI%, *P*-VALUE)
Clinical stage		
II[a]		
III	**2.28 (1.10-4.70, $P = .026$)**	**2.21 (1.02- 4.81, $P = .046$)**
Clinical T stage		
cT0-cT2[a]		
cT3-cT4	3.70 (1.50-9.10, $P = .004$)	
Clinical N stage		
cN0-cN1[a]		
cN2-cN3	2.56 (1.72-3.82, $P < .001$)	
Alcohol consumption		
Not exposed[a]		
Exposed	**1.97 (1.29-2.98, $P = .002$)**	**1.89 (1.21-2.96, $P = .005$)**
Smoking		
Not exposed[a]		
Exposed	**1.40 (0.91-2.15, $P = .127$)**	**1.03 (0.65-1.64, $P = .903$)**
BMI		
$\leq 30\,kg/m^2$[a]		
$>30\,kg/m^2$	1.12 (0.72-1.74, $P = .620$)	
Schooling		
$\geq 8\,y$[a]		
$<8\,y$	1.10 (0.74-1.64, $P = .639$)	
Home distance (median)	1.00 (0.99-1.00, $P = .357$)	
Compliance to NACT		
Incomplete treatment[a]		
Complete treatment	**0.68 (0.41-1.13, $P = .140$)**	**0.74 (0.44-1.23, $P = .248$)**

(Continued)

Table 5. (Continued)

	CRUDE HR FOR OS (95% CI, *P*-VALUE)	ADJUSTED HR FOR OS (95% CI%, *P*-VALUE)
Pathological T stage		
ypT0[a]		
ypT1	4.08 (1.77-9.40, $P = .001$)	
ypT2	4.61 (2.01-10.57, $P < .001$)	
ypT3	8.85 (3.84-20.40, $P < .001$)	
ypT4	13.41 (5.06-35.55, $P < .001$)	
Pathological N stage		
ypN0[a]		
ypN1	2.86 (1.65-4.96, $P < .001$)	
ypN2	4.85 (2.92-8.03, $P < .001$)	
ypN3	9.31 (5.02-17.24, $P < .001$)	
pCR status		
Non-pCR[a]		
pCR	0.11 (0.04-0.31, $P < .001$)	

Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio; NACT, neoadjuvant chemotherapy; OS, overall survival; pCR, pathological complete response; FAC-T, fluorouracil, doxorubicin and cyclophosphamide followed by docetaxel; AC-T, doxorubicin and cyclophosphamide followed by docetaxel or followed by weekly paclitaxel.
The variables of the final model selected for analysis by the Cox multiple model were highlighted in bold.
Regarding the chemotherapy regimen, only the AC-T vs FAC-T regimens were compared.
[a]Reference.

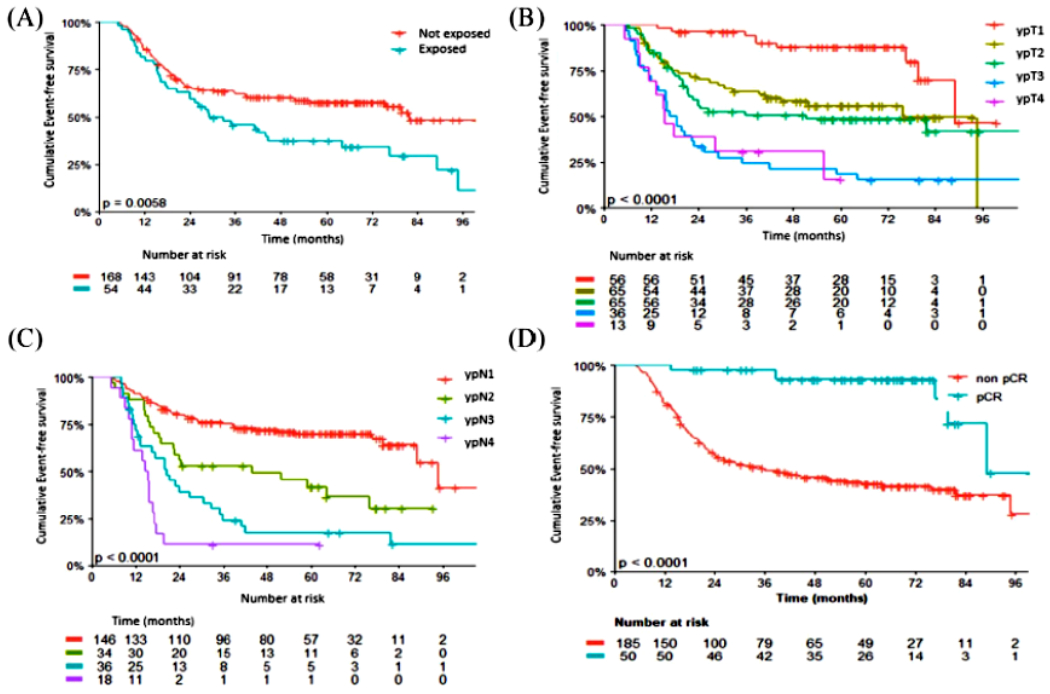

Figure 2. Kaplan-Meier event-free survival estimates according to (A) alcohol consumption, (B) pathological tumor stage, (C) pathological nodal stage, and (D) pathological complete response. pCR indicates pathological complete response.

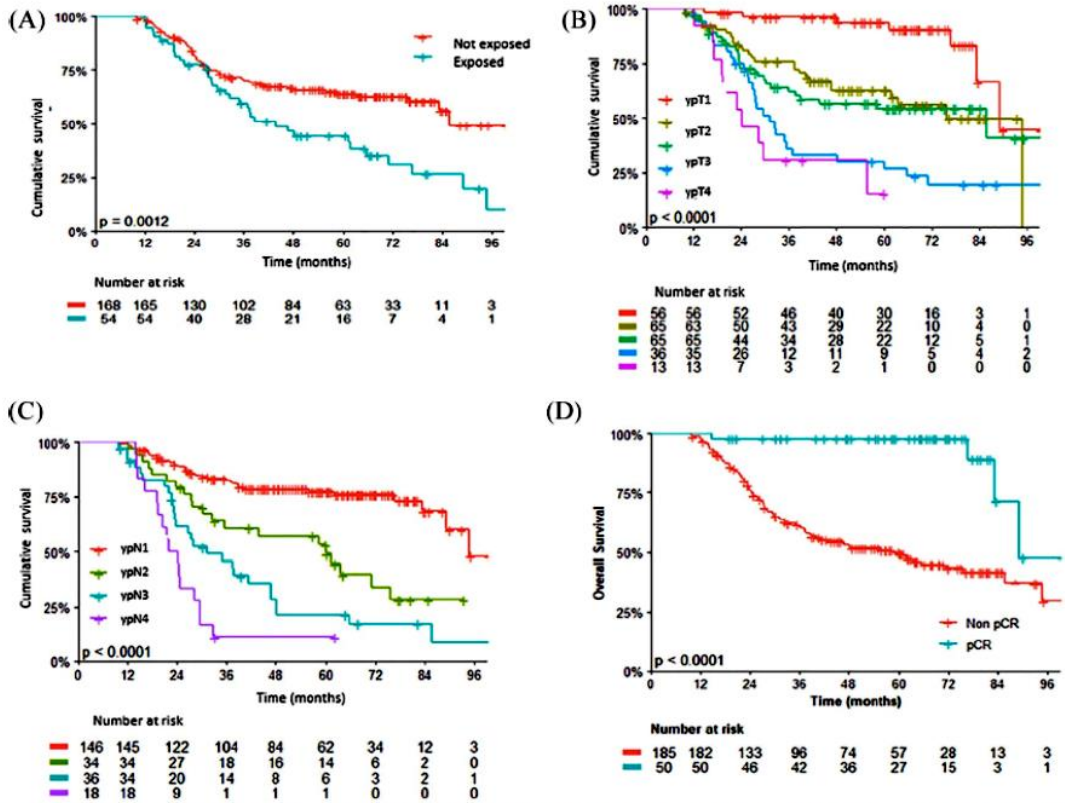

Figure 3. Kaplan-Meier overall survival estimates according to (A) alcohol consumption, (B) pathological tumor stage, (C) pathological nodal stage, and (D) pathological complete response. pCR indicates pathological complete response.

Clinical stage III increased the risk of event vs stage II by 2.57-fold ($P = .016$), alcohol intake increased the risk of recurrence or death by 67% ($P = .010$), and treatment compliance showed no association with EFS ($P = .08$).

The final model for OS consisted of 4 variables (Table 5). Patients with clinical stage III had a risk of death 2.21 times higher than stage II ($P = .046$) and alcohol intake increased the risk of death by 89% ($P = .005$). Smoking ($P = .903$) and compliance to NACT ($P = .248$) did not influence OS.

Discussion

This study evaluated the association of sociodemographic, clinical, and pathological variables with response and survival outcomes in women with TNBC undergoing NACT at INCA. To the best of our knowledge, with 235 women included, these were one of the largest cohorts in this subset. Overall, the patients showed a trend toward early recurrence, mostly as distant metastases. The results showed that clinical stage III and alcohol consumption were associated with lower pCR rate and shorter survival. However, patients who achieved pCR had considerably longer survival.

Early age at diagnosis and overweight were mostly found in the patients of this cohort, corresponding to the data presented in previous series.[14,15] Likewise, other ominous features of TNBC also prevailed such as high-grade tumors and locally advanced disease at diagnosis with axillary nodal involvement, which in some extent explains the fact that almost all patients underwent radical surgery.[16]

The alcohol consumption and smoking rate were quite similar to other series that included women with TNBC.[17-20] Further information on the dose, duration, and type of exposure to alcohol and tobacco unfortunately were not available in the records of the patients in this study. A meta-analysis published in 2013 pointed out that only a more regular and heavy alcohol intake, greater than 20 g/day, would be consistently associated with increased breast cancer mortality and earlier recurrence.[21] Apparently, there may be some interference of alcohol on pharmacokinetics of chemotherapy as well as social implications that lead to lower adherence to treatment. As for smoking, it could not be confirmed as a predictive factor for NACT or as a prognostic factor. The data in the literature is quite controversial, with some negative results contrasting with others where smoking had a negative impact on survival. Interestingly, smoking cessation after the diagnosis of breast cancer is likely to reduce the risk of breast mortality.[20,22,23]

Schooling data were similar to those of another Brazilian cohort[23] and did not show association with response or survival outcomes. Similarly, a Norwegian cohort[24] also showed no influence of schooling on survival or response, whereas other results suggested that higher level of education may be associated with better survival and quality of life.[25,26] In the same way, there is not much data in the literature about distance

from home to the treatment center. The great variability of this social factor among the cases of this cohort may explain the negative results.

The median time from diagnosis to treatment onset of 90 days was quite long. Although not shown to be associated with survival outcomes in this cohort, it is highly suspected that delays in NACT onset can negatively influence treatment outcomes. To avoid long delays in the initiation of cancer treatment, the Brazilian Federal Government decreed the "Law of 60 days" in 2012 (Federal Law number 12.732/12). This law was nationally established in 2013 and defines the maximum range that a patient with cancer has to wait to initiate the specific treatment. However, due to public health system infrastructure issues, this goal is still far from being achieved.[27]

A recent comprehensive patient-level meta-analysis[28] has pointed pCR as a strong surrogate of long-term survival outcomes. In the current cohort, the pCR rate was quite similar to other studies using anthracycline and taxane-based NACT. However, this rate was considerably modest when compared to recent clinical studies, in which pCR reached rates over 50%. Some possible reasons for this may be the narrow definition of pCR (ypT0ypN0) and the proportional greater number of women with larger tumors in this study, as well as the use of dense-dose regimens, and the addition of new drugs to NACT in the other studies, such as PARP inhibitors, immunotherapy, and antiangiogenic agents.[29]

The use of carboplatin and the PARP inhibitor (veliparib) in the Brightness trial has prompted a substantial increase in pCR rate by more than 20%.[9] Preliminary results from KEYNOTE-173[30] and I-SPY 2 trial[31] have shown a pCR rate of over 50% with the association of pembrolizumab. Other agents such as bevacizumab, nab-paclitaxel, capecitabine, and eribulin showed less significant results.[32] A recent meta-analysis suggested that dose-dense chemotherapy with anthracycline and taxane-based regimen may reduce the risk of death for patients with hormone receptor-negative breast cancer by up to 20%.[33]

The ypT and ypN staging were pointed out as reliable prognostic factors. These findings are consistent with the results of a Brazilian cohort that evaluated patients undergoing axillary lymph node dissection, which suggested that the greater the number of positive axillary lymph nodes, the lower the median disease-free survival and OS.[23] Other systems that measure the degree of response to chemotherapy were reported since 2013 and validated in some studies.[34-36] The Residual Cancer Burden score may be more reliable than the TNM system for post-NACT staging and evaluation for prognosis, providing an index with good reproducibility in terms of predicting long-term survival.[37] In this cohort, pCR and residual burden disease (ypT and ypN) showed a considerable association with survival outcomes in all models tested for Cox multiple analysis. However, following Akaike criteria, perhaps because they may strongly influence other variables, the final model selected did not include these variables.

Some strengths of this study must be mentioned. The patient inclusion criteria allowed a more uniform sample for evaluation of a broad panel of factors, some of them with interesting and unpublished data. Besides that, using real-world data, the current cohort has drawn a detailed portrait of the harsh sociodemographic reality of women with TNBC treated at a Brazilian public health institution.

On the contrary, some important limitations must be highlighted. As a single-institutional retrospective cohort with lack of standardization of medical records, there was considerable missing data for some variables and some patients were censored for short-term follow-up. Furthermore, the lack of a specific questionnaire to measure the exposure gradient (dose, duration, and type) to alcohol, considering parameters determined by the case-control study conducted by White et al,[38] and tobacco, considering the cutoff of more than 20 pack-years of smoking suggested by Saquib et al,[39] hindered a more detailed analysis of the influence of these factors both in the outcomes. New treatment regimens such as dose-dense schedules, addition of platinum agents to NACT, and maintenance adjuvant capecitabine for patients with residual disease after NACT are not yet available at the institution. Finally, tumor-infiltrating lymphocytes have not been evaluated and there was no molecular analysis of the sample by gene expression profile or BRCA mutation testing. Some molecular subclassifications have predicted different patterns of response to NACT and survival outcomes.[40]

Conclusions

In summary, a timely and thorough evaluation of predictive and prognostic factors was carried out. Alcohol consumption and clinical stage III were determinants of lower response rate and worse survival. However, studies with better characterization of type, time, and dose of alcohol consumption are entirely necessary. The burden of post-NACT residual disease, represented by ypT and ypN, is likely to be usable as prognostic factors. Herein, pCR showed a strong association with better survival outcomes, being a potential surrogate for long-term outcomes.

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Author Contributions

JLdS and ACdM contributed to the concept and design of the study. LCST and ACdM contributed to supervision of the study. JLdS and BHRdP contributed to data collection and/or processing. JLdS, IAS, LCST, and ACdM contributed to analysis and/or interpretation of data. JLdS, BHRdP, IAS, LCST, and ACdM contributed to literature search. JLdS, BHRdP, IAS, LCST, and ACdM contributed to writing the manuscript. LCST and ACdM contributed to critical review of the manuscript.

Data Availability

Data used to support the findings of this study are available from the corresponding author. Upon request, may be released after review of the Institutional Review Board.

Ethical Approval

All procedures were in accordance with the ethical standards of the institutional and national research committee. The study was approved by the Ethics in Human Research Committee of INCA, Rio de Janeiro, Brazil, under number CAAE 61675516.9.0000.5274, and conducted in accordance with Good Clinical Practice guidelines.

Informed Consent

As this study has a retrospective observational design, the absence of the informed consent form was approved by the Institutional Review Boards.

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7.1.2 Artigo 2

Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Triple-Negative Breast Cancer: Assessing the Role of Immunohistochemical Biomarkers on Neoadjuvant Treatment

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Breast Cancer: Targets and Therapy

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Objective: This study aimed to investigate the influence of immunohistochemical (IHC) biomarkers in the response to neoadjuvant chemotherapy (NACT) and survival outcomes in the subset of locally advanced triple-negative breast cancer (TNBC).

Materials and Methods: The epidermal growth factor receptor (EGFR), androgen receptor (AR), cytokeratins (CK5/6, CK14 and CK17), Ki67 and p53 immunohistochemistry were evaluated on 171 cases of TNBC submitted to NACT and subsequently to surgery. Intensity and percentage of the expression of these biomarkers were combined to formulate a specific score, that was correlated with prognostic features and assessed for survival outcomes.

Results: Most patients had advanced clinical-stage tumors (stage III: 83.6%; cT3/T4: 85.9%; cN1-3: 71.3%). The predominant histological subtype was high-grade (67.3%) and invasive ductal carcinoma (93.6%). The residual cancer burden (RCB) 0–1 corresponded to 28.7% of cases and low-risk lymph node ratio (LNR) represented 77.2%. High Ki67 expression only showed a significant correlation with grade 3 tumors ($p = 0.0157$). CK5/6 was observed in 16% (27/169), CK14 was positive in 10.1% (17/169), CK17 in 91.1% (153/168), p53 in 52.6% (70/133), EGFR in 92.9% (157/169 cases), AR in 13% (22/169) and Ki67 index was scored ≥40% in 57.9% (95/165). No IHC biomarker significantly impacted response or survival. Regarding the analysis of the outcomes of event-free survival (EFS) and overall survival (OS), clinical stage ($p = 0.014$ and $p = 0.042$, respectively), RCB ($p < 0.0001$ and $p < 0.0001$, respectively) and LNR ($p < 0.0001$ and $p < 0.0001$, respectively) showed significant association.

Conclusion: No IHC biomarker evaluated showed a significant association with a response or survival outcomes in TNBC patients. Clinical stage, LNR and RCB stood out for strongly influencing survival.

Keywords: triple-negative breast cancer, neoadjuvant chemotherapy, biomarkers, residual burden cancer, lymph node ratio

Introduction

According to data from GLOBOCAN 2018, breast cancer stands out for being the second most commonly diagnosed malignancy for the general population, reaching over 11% of all new cancer cases. Indeed, it is the leading cause of cancer-specific death in women worldwide.[1] For Brazil, 66,280 new cases of breast cancer are estimated for each year in the period 2020–2022.[2]

Triple-negative breast cancer (TNBC) is defined by tumors that lack expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal

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growth factor receptor 2 (HER2).[3,4] This type of tumor behaves more aggressively and accounts for approximately 15% of breast cancers.[5] Treatment for TNBC has been challenging and tumor heterogeneity widely pointed out as the reason for different clinical outcomes, often leading to different patterns of response to neoadjuvant treatment, as well as discrepant survival.[6]

Some gene expression profile (GEP) testing has been proposed for molecular characterization of TNBC subgroups. In 2011, Lehmann et al[7] suggested a division of TNBCs into 7 molecular subtypes: immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR), unstable (UNS) subtype and two basal-like subtypes (BL1 and BL2). Thereafter, the subclassification refinement was revised to regroup into 4 subtypes (BL1, BL2, M and LAR) based on a retrospective analysis of some clinical trials dataset.[8] Although very promising, these assays are expensive and still inaccessible in many centers.

The GEP-based cluster analyses are intricately linked to the expression profiling of immunohistochemistry (IHC), which may represent a more accessible tool option for predicting response and survival outcomes. BL1 and BL2 subtypes usually express epidermal growth factor receptor (EGFR) and basal cytokeratins like CK5/6, CK14 and CK17. LAR usually has high androgen receptor (AR) expression. The high expression of p53 is likely to be an independent biomarker of shorter survival in some cohorts of patients with TNBC, as well as a high score of Ki67 proliferation index.[9]

The aim of this study was to investigate the influence of some IHC biomarkers, such as EGFR, AR, cytokeratins (CK5/6, CK14 and CK17), Ki67 and p53, in the response pattern to neoadjuvant chemotherapy (NACT) and survival outcomes in the subset of patients with locally advanced TNBC. This approach may help to obtain novel information for identifying specific high-risk subgroups in order to explore more effective treatments.

Materials and Methods

Study Design and Ethical Considerations

This is a cohort with retrospective data. The study was approved by the Ethics in Human Research Committee of the Brazilian National Cancer Institute (CEP-INCA), Rio de Janeiro, Brazil, under the number CAAE 61675516.9.0000.5274, and conducted in accordance with the Good Clinical Practice guidelines.

Patient Selection

Patients with newly diagnosed breast cancer enrolled at the Brazilian National Cancer Institute (INCA) between January 2010 and December 2014 were included if all the following criteria were met: a) women over 18 years old; b) confirmation of the histopathological diagnosis of TNBC (tumors with ER and PR score <1%, as well as HER-2 score 0/1 + or 2+ with negative FISH) by the INCA Pathology Department (DIPAT/INCA) according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines;[3,4] c) stage IIb-IIIc by the 7th American Joint Committee on Cancer – AJCC (T3-4NanyM0; TanyN1-3M0); d) submitted to NACT with anthracycline-taxane-based regimen followed by curative surgery at INCA. In its turn, patients with the second primary tumor, previously exposed to antineoplastic agents, with unresectable tumors, even when NACT was supplemented by complementary treatment with further chemotherapy and/or radiotherapy, were excluded.

Neoadjuvant Regimens

The NACT regimens were defined primarily as FAC-T (fluorouracil 500 mg/m2, doxorubicin 50 mg/m2 and cyclophosphamide 500 mg/m2, every 21 days for 3 cycles, followed by docetaxel 100 mg/m2 every 21 days for 3 cycles, intravenously) or AC-T (doxorubicin 60 mg/m2 and cyclophosphamide 600 mg/m2, every 21 days for 4 cycles, followed by docetaxel 100 mg/m2 every 21 days for 4 cycles, or followed by weekly paclitaxel 80 mg/m2 for 12 consecutive weeks without interval, intravenously).

Immunohistochemistry

Due to the scarcity of material, the core biopsy samples were analyzed in their whole tissue sections for all biomarkers of IHC. As for the surgical specimen samples, the tissue microarray (TMA) analysis was performed using standard procedures on 4-µm sections in the most representative areas of greatest tumor cellularity of formalin-fixed paraffin-embedded tissue specimens, and then stained for Ki67. For both specimens, the tumor cell staining was compared with that of negative and positive controls. Moreover, the slides were scored according to the percentage of positive cells versus the total cell number.

The immunostaining scores for ER, PR and HER2 were confirmed as negative according to ASCO/CAP guidelines.[10] Ki67 was assessed by nuclear staining using a mouse

monoclonal antibody (SP 6 clone, Cell Marque) at 1:500 dilution. The expression level of Ki67 was considered to be low if the percentage of nuclear staining was <40% and high if ≥40%.[11] For AR (clone SP107, Cell Marque; dilution 1:50),[12] CK5/6 (clone D5 & 16B4, Cell Marque; dilution 1:100), CK14 (clone D5 & 16B4, Cell Marque; dilution 1:1000) and CK17 (Clone EP98, Cell Marque; dilution 1:500) the cut-off value for the positive result was defined when the score was ≥1%.[13] EGFR (clone HPA018530, Sigma; dilution 1:300) and p53 (clone SP53, Cell Marque; dilution 1:500) were considered positive when ≥10% of positive cells were observed.[14] The representation of high and low expression of the markers are highlighted in Figure 1. The entire analysis was performed in duplicate by two experienced pathologists at the DIPAT-INCA.

Other Pathological and Clinical Variables

Patients were identified through the internal database and the data were collected from electronic hospital records and medical charts. The following clinical and pathological variables were evaluated: age at diagnosis, ethnicity (Caucasian or others according to national institutional statistical classifications, IBGE[15]), body mass index (BMI), schooling (≤8 years of formal education correspond to less than elementary level and >8 years correspond to more than elementary formal education), type of NACT (FAC-T or AC-T), clinical stage (II–III), clinical T stage (cT), clinical nodal stage (cN), residual cancer burden (RCB), histological type, Elston histological grade (1: low grade; 2: moderate grade; 3: high grade), type of surgery (radical or conservative, axillary approach). Regarding the lymph node ratio (LNR, the ratio of positive axillary lymph nodes to the total number of nodes examined), the patients were divided into low- (≤0.20), intermediate- (>0.20 and ≤0.65) and high-risk (>0.65) groups.[16]

Outcomes

The RCB score followed the standard four-level categorical variable (RCB "classes" 0, 1, 2, and 3).[17] The pathological complete response (pCR) was narrowly defined as no viable residual tumor in the breast or axilla (ypT0N0). Event-free survival (EFS) was calculated from the date of diagnosis to the earliest date of disease progression, death from any cause, or discontinuation of treatment for initiation of complementary treatment due to poor response to standard NACT. Overall survival (OS) was calculated from the date of diagnosis to death from any cause.

Statistical Analysis

Data were processed using R environment. All continuous variables were evaluated by the Shapiro–Wilk test of normality. Using the non-parametric Wilcoxon test, the value of Ki67 expression before and after NACT was correlated with response and survival outcomes. To assess the correlation of the Ki67 score with other clinical-pathological

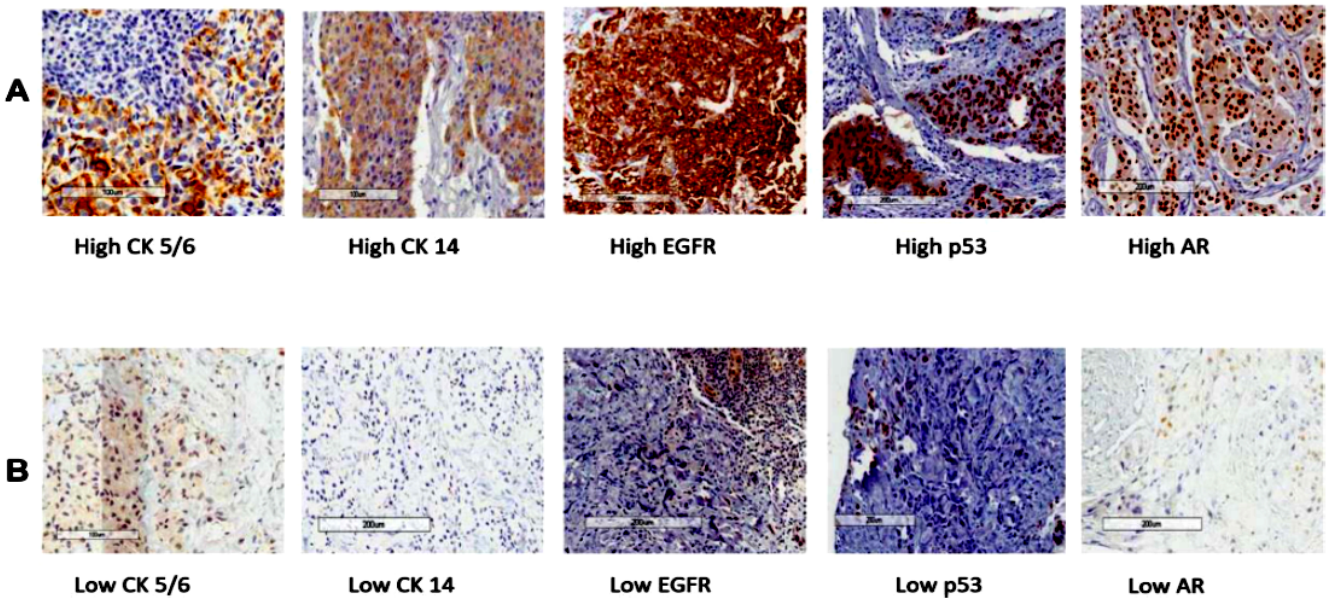

Figure 1 Triple-negative breast cancer. **(A)** Representative images of immunohistochemical staining of high CK 5/6, CK 14, EGFR, p53 and AR. **(B)** Representative images of immunohistochemical staining of low CK 5/6, CK 14, EGFR, p53 and AR. Original magnification: ×400 (×40 objective). **Abbreviation:** AR, androgen receptor.

variables, the Mann–Whitney *U*-test and, when necessary, the Spearman correlation test were used. For the RCB outcome, logistic regression was used for each variable assessed. Survival rates were calculated by Kaplan–Meier curves for each factor and were compared by Log-rank test. The crude Hazard Ratio (HR) for each factor was calculated by the Cox proportional hazards model. Regarding multivariate analysis, all variables with an association with survival outcomes at p-value <0.20 were included and the Akaike Information Criterion was used to pick the most suitable model for multiple Cox analysis. A p-value of less than 0.05 was statistically significant. The missing data were excluded from the analysis.

Results

A total of 235 patients were included according to the study criteria. After excluding patients with essential missing data, mainly due to scarce or unavailable tumor sample, 171 cases of women with locally advanced TNBC undergoing NACT followed by curative surgery were chosen for final analysis (Figure 2). The main characteristics of the patients are summarized in Table 1. The mean age was 50.5 years (standard deviation, SD 10.7). Furthermore, most women were non-Caucasian (53.8%) and the mean BMI was 28.5 kg/m2 (SD 5.9).

At diagnosis, most patients had outer quadrant (57.9%) and advanced clinical stage tumors (stage III: 83.6%; cT3/T4: 85.9%; cN1-3: 71.3%). In addition, the predominant histological subtype was high-grade (67.3%) and invasive ductal carcinoma (93.6%). At surgery, RCB 0–1 corresponded to 28.7% of cases and low-risk LNR represented 77.2%, followed by intermediate-risk LNR (15.2%) and low-risk LNR (7.6%) (Table 1). Among the variables evaluated, high Ki67 expression only showed a significant correlation with grade 3 tumors (p = 0.0157) (data not shown).

As for the systemic neoadjuvant treatment, 68.4% (117 cases) of the patients in the study received the AC-T

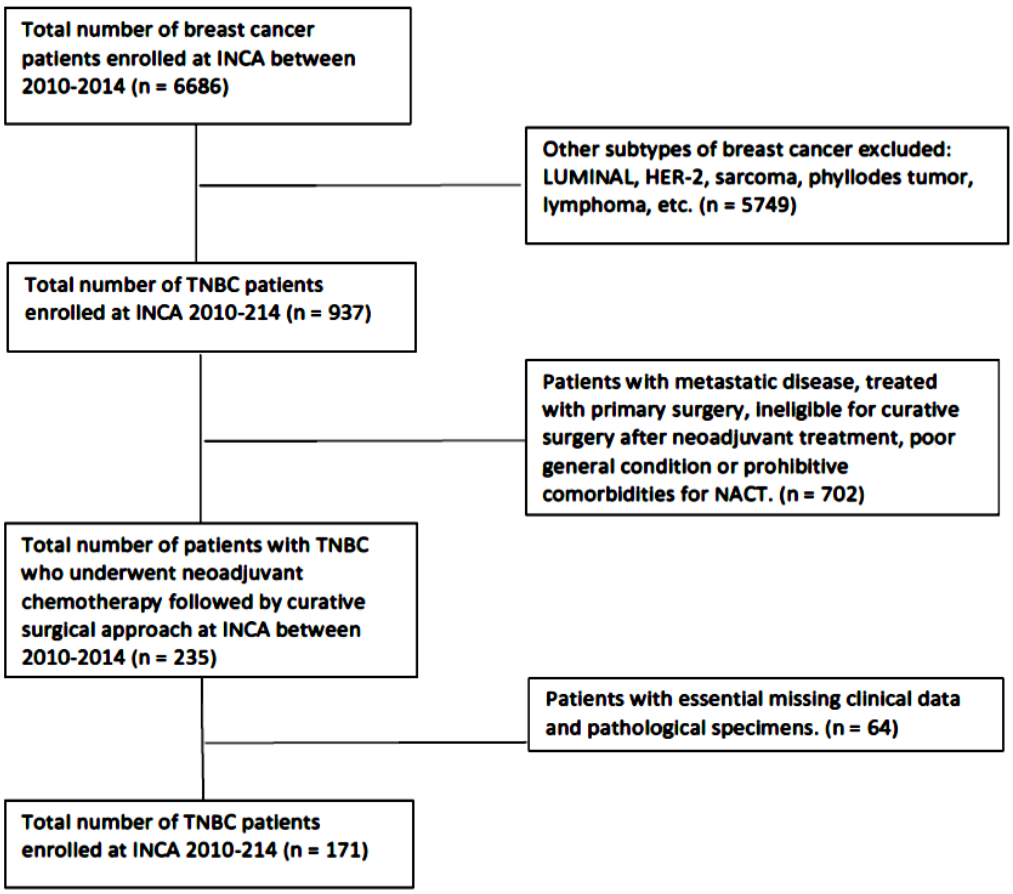

Figure 2 Study profile.

Table 1 Clinicopathologic Characteristics of Patients and Treatment Data

Clinical Variables	n = 171 (100%)
Mean age (SD)	50.5 (10.7)
Race/ethnicity White	78 (46.2%)
BMI mean Kg/m2 (SD)	28.5 (5.9)
Schooling ≥8years	90 (54.2%)
Clinical staging	
Stage II	28 (16.4%)
Stage III	143 (83.6%)
cTx	1 (0.6%)
cT2	23 (13.5%)
cT3	70 (40.9%)
cT4	77 (45%)
cN0	49 (28.7%)
cN1-N3	175 (71.3%)
Histological grade	
Grade 1	3 (1.8%)
Grade 2	53 (31%)
Grade 3	115 (67.3%)
Quadrant	
Inner	72 (42.1%)
Outer	99 (57.9%)
Histological type	
Metaplastic	11 (6.4%)
Non-special type invasive carcinoma	160 (93.6%)
NACT regimen	
AC-T	117 (68.4%)
FAC-T	54 (31.6%)
Time from diagnosis to NACT mean (SD)	104.9 (93.1)
Complete NACT treatment	149 (87.1%)
Type of surgery	
Breast conserving surgery	6 (3.5%)
Mastectomy	165 (96.5%)
Axillary approach	
Sentinel lymph node biopsy	10 (5.8%)
Axillary lymph node dissection	145 (86%)
Unknown	16 (8.2%)
RCB	
0	36 (21.1%)
1	13 (7.6%)
2	74 (43.3%)
3	48 (28.1%)
LNR	
Low risk (≤ 0.20)	132 (77.2%)
Intermediate risk (0.20–0.65)	26 (15.2%)
High risk (> 0.65)	13 (7.6%)

Abbreviations: SD, standard deviation; BMI, body mass index; NACT, neoadjuvant chemotherapy; AC-T, doxorubicin/cyclophosphamide followed by taxane; FAC-T, doxorubicin/cyclophosphamide/fluorouracil followed by taxane; RCB, residual cancer burden; LNR, lymph node ratio.

regimen and 87.1% (149 cases) completed the treatment. The average time from diagnosis to the start of NACT was 104.9 days (SD 93.1). The vast majority of patients underwent radical surgery (165 cases, 96.5%) and axillary dissection (145 cases, 86%) (Table 1).

As shown in Table 2, the positive expression of CK5/6 was observed in 16% (27 cases) of the total number of tested cases (169 cases). CK14 was positive in 10.1% (17 cases) of 169 cases. CK17 positive occurred in 91.1% (153 cases) of 168 cases tested. The p53 positivity was seen in 52.6% (70 out of 133 cases tested). EGFR was positive in 92.9% (157 cases) of the total number of tested cases (169 cases). AR was positive in 13% (22 cases) of 169 cases tested. And for the Ki67 index, 57.9% (95 cases) of core biopsy samples showed high expression, with a median of 40% (interquartile range, IR 55).

As for the outcome of RCB, clinical stage II was the only characteristic that significantly showed an association with the greater response ($p = 0.026$). The other variables and biomarkers did not show any association with the outcome of RCB, as highlighted in Table 2. Following the Akaike information criterion, the multivariate analysis of the final model chosen was formed by p53 ($p = 0.063$) and clinical stage ($p = 0.032$) (data not shown). In patients with residual disease, the variation in Ki67 expression pre- and post-NACT was not statistically significant in those who presented RCB1/2 versus RCB 3 (Figure 3).

The univariate analysis of clinical-pathological variables and biomarkers for risk of recurrence and risk of death is presented in detail in Table 3. With a median follow-up of 62.5 months (95% Confidence Interval, 95% CI 60.2–67.9), and considering that there were 83 events of death or recurrence, the estimate of patients alive without recurrence in 60 months was 57.42% (95% CI 50.11–65.78). As highlighted in Figures 4 and 5, regarding the analysis of variables and biomarkers for the outcomes of EFS and OS, by using the Log rank testing and Kaplan–Meier analysis, clinical stage ($p = 0.014$ and $p = 0.042$, respectively), RCB ($p < 0.0001$ and $p < 0.0001$, respectively) and LNR ($p < 0.0001$ and $p < 0.0001$, respectively) showed a significant association. The collinearity effect of the variables RCB, LNR and clinical stage observed in this cohort, as well as the negative results of immunohistochemical markers, hindered the multivariate analysis for EFS and OS.

Table 2 Correlation of Expression Profile of Biomarkers and Clinical-Pathological Characteristics with Residual Burden Cancer by Logistic Regression Through Univariate Analysis

Variables/Biomarkers	RCB 2/3	RCB 0/1	Crude p-value
	122 (71.4%)	**49 (28.7%)**	
Age mean (SD)	50.4 (10.4)	50.7 (12.2)	0.849
Ki67			0.127
Low expression	54 (78.3%)	15 (21.7%)	
High expression	64 (67.4%)	31 (32.6%)	
CK5/6			0.701
Negative	100 (70.4%)	42 (29.6%)	
Positive	20 (74.1%)	7 (25.9%)	
CK14			0.547
Negative	109 (71.7%)	43 (28.3%)	
Positive	11 (64.7%)	6 (35.3%)	
CK17			0.337
Negative	9 (60%)	6 (40%)	
Positive	110 (71.9%)	43 (28.1%)	
p53			0.057
Negative	50 (79.4%)	13 (20.6%)	
Positive	45 (64.3%)	25 (35.7%)	
EGFR			0.136
Negative	11 (91.7%)	1 (8.3%)	
Positive	109 (69.4%)	48 (30.6%)	
Androgen receptor			0.754
Negative	105 (71.4%)	42 (28.6%)	
Positive	15 (68.2%)	7 (31.8%)	
Quadrant			0.215
Inner	55 (76.4%)	17 (23.6%)	
Outer	67 (67.7%)	32 (32.3%)	
Clinical stage			0.026
II	15 (53.6%)	13 (46.4%)	
III	107 (74.8%)	36 (25.2%)	
NACT regimen			0.863
AC-T	83 (70.9%)	34 (29.1%)	
FAC-T	39 (72.2%)	15 (27.8%)	
Histological type			0.433
Non-special type IDC	113 (70.6%)	47 (29.4%)	
Metaplastic	9 (781.8%)	2 (18.2%)	
Grade			
1	2 (66.7%)	1 (33.3%)	
2	36 (67.9%)	17 (32.1%)	0.964
3	84 (73%)	31 (27%)	0.807

Note: Statistically significant results are in bold.

Abbreviations: RCB, residual cancer burden; SD, standard deviation; NACT, neoadjuvant chemotherapy; AC-T, doxorubicin/cyclophosphamide followed by taxane; FAC-T, doxorubicin/cyclophosphamide/fluorouracil; IDC, invasive ductal carcinoma.

Discussion

To date, this is one of the largest series in this subset of TNBC performed with Brazilian women. A thorough evaluation of clinical-pathological features and IHC biomarkers was carried out. INCA is the largest public tertiary cancer referral center in Brazil, and TNBCs account for approximately 14% (unpublished data) of all breast cancers enrolled at the institute, which is lower than the rates in other Western population. The results of this study suggested that there is no association of the IHC biomarkers tested with either response or survival outcomes. In contrast, it was shown that clinical stage III, RCB ≥2 and LNR >0.65 were associated with poorer survival outcomes.

The median age of 50.1 years is in line with those of other series already published, corroborating the idea that TNBC is likely to occur in younger women.[18] Other ominous features that also prevailed in the current study were high-grade tumors and locally advanced disease at diagnosis with axillary nodal involvement, which somehow explains the fact that almost all patients underwent radical surgery.[19] The mean time from diagnosis to treatment onset was quite long, over 100 days. Although not shown to be associated with survival outcomes in this cohort, it is highly suspected that delays in NACT onset can decrease the response rate and shorten survival.

In virtually all types of breast cancer, the higher expression of Ki67 has been associated with better response and increased risk of relapse to neoadjuvant chemotherapy. Based on a meta-analysis of 35 studies with 7716 patients enrolled,[11] a high Ki-67 expression ≥40% was strongly defined as a poor prognostic factor in resected TNBC, being associated with a greater risk of recurrence and death compared with lower expression rates. In the current cohort, more than half of the patients had Ki67 ≥40%, which is in accordance with previous studies.[20,21] In contrast to another series,[22] the Ki67 expression, as well as reduction of Ki67 expression more than 20% after NCT, was not associated with response rate or death.

Based on immunohistochemical staining for markers as CK5/6, CK14, CK17 and/or EGFR, basal-like breast cancer may be defined as a subset of the positive tumor for one or all the four markers.[23] Defined as an aggressive molecular subtype by the current classification proposed by Lehmann et al,[8] the survival outcomes for these tumors are likely to be poorer, although a higher pCR rate (41%) has been observed, suggesting greater chemosensitivity. Moreover, they are tumors that appear to be associated with higher histological grade, higher mitotic index and lower differentiation, and it is postulated that this is due to higher neovascularization level caused by vascular

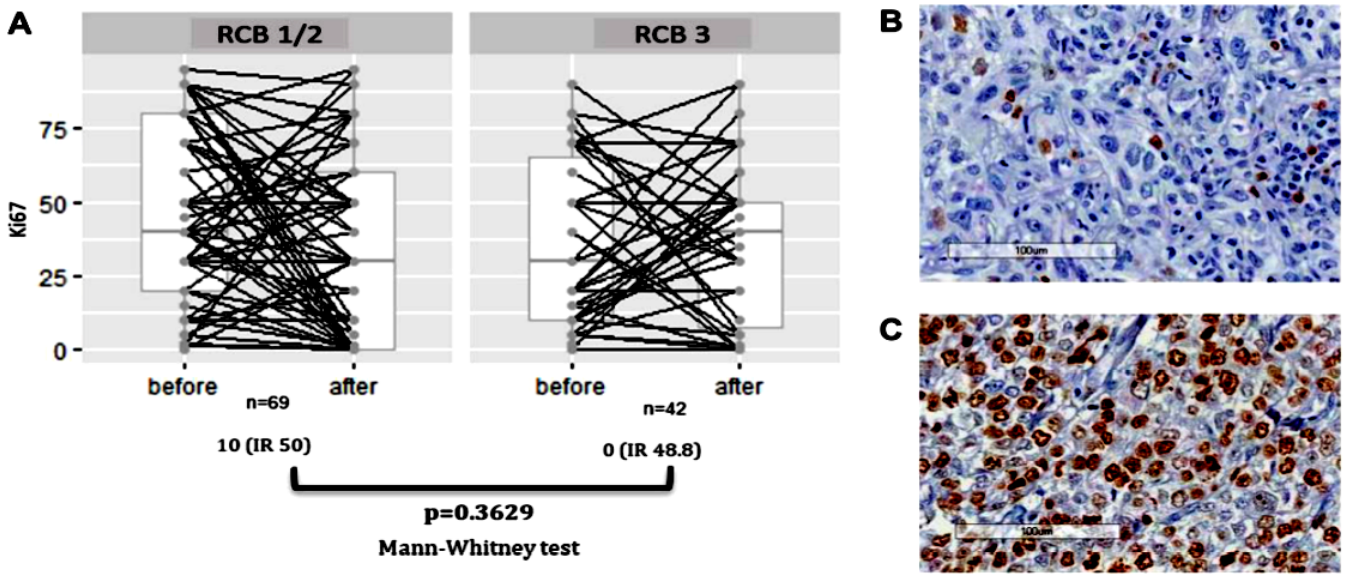

Figure 3 **(A)** The correlation between change in Ki67 value and RCB status in patients with residual disease. In such cases, the median absolute Ki67 reductions were 10% and 0% in patients with good response (RCB1/2) and poor response (RCB 3), respectively. **(B)** Immunohistochemical Ki67 staining in TNBC, representative image of Ki67 with low expression (magnification, x 200). **(C)** Representative image of Ki67 with high expression (magnification, x 200). **Abbreviations:** IR, interquartile range; RCB, residual cancer burden; TNBC, triple-negative breast cancer.

Table 3 Univariate Analysis According to Survival Outcome

Variables/Biomarkers	Crude HR for EFS (95% CI, p-value)	Crude HR for OS (95% CI, p-value)
Clinical stage		
II*		
III	2.56 (1.18–5.57, p = 0.017)	2.20 (1.01–4.79, p = 0.047)
RCB		
2/3*		
0/1	0.20 (0.10–0.41, p <0.001)	0.15 (0.07–0.35, p <0.001)
LNR		
Low risk*		
Intermediate risk	2.86 (1.71–4.78, p <0.001)	2.73 (1.59–4.69, p <0.001)
High risk	7.98 (4.14–15.38, p <0.001)	5.43 (2.81–10.47, p <0.001)
NACT regimen		
AC-T*		
FAC-T	1.35 (0.86–2.11, p = 0.188)	1.35 (0.85–2.15, p = 0.203)
CK14		
Negative*		
Positive	1.07 (0.53–2.14, p = 0.852)	0.92 (0.42–2.01, p = 0.836)
CK17		
Negative*		
Positive	1.06 (0.49–2.30, p = 0.884)	0.92 (0.42–2.01, p = 0.838)
CK5/6		
Negative*		
Positive	1.22 (0.69–2.18, p = 0.494)	1.26 (0.69–2.30, p = 0.451)
Pre-NACT Ki67 mean	1.00 (0.99–1.01, p = 0.589)	1.00 (0.99–1.00, p = 0.218)
Residual tumors		
Ki67 ≥40%*		
Ki67 <40%	0.82 (0.51–1.30, p = 0.398)	0.69 (0.42–1.13, p = 0.141)
Ki67 reduction		
≤20%*		
>20%	0.72 (0.45–1.17, p = 0.191)	0.65 (0.40–1.08, p = 0.101)
p53		
Negative*		
Positive	0.91 (0.55–1.51, p = 0.707)	1.06 (0.62–1.82, p = 0.827)
EGFR		
Negative*		
Positive	1.18 (0.48–2.91, p = 0.726)	0.98 (0.40–2.44, p = 0.969)
Androgen receptor		
Negative*		
Positive	0.84 (0.43–1.63, p = 0.609)	0.85 (0.42–1.70, p = 0.639)
Quadrant		
Inner*		
Outer	1.31 (0.84–2.05, p = 0.241)	1.34 (0.83–2.15, p = 0.227)

(Continued)

Table 3 (Continued).

Variables/Biomarkers	Crude HR for EFS (95% CI, p-value)	Crude HR for OS (95% CI, p-value)
Grade		
1		
2	1.19 (0.16–8.79, p = 0.865)	0.72 (0.10–5.35, p = 0.748)
3	1.25 (0.17–9.04, p = 0.825)	0.79 (0.11–5.74, p = 0.817)

Notes: *Reference. Statistically significant results are in bold.
Abbreviations: RCB, residual cancer burden; LNR, lymph node ratio; NACT, neoadjuvant chemotherapy; AC-T, doxorubicin/cyclophosphamide followed by taxane; FAC-T, doxorubicin/cyclophosphamide/fluorouracil followed by taxane.

endothelial growth factor (VEGF) overexpression. Approximately 75–80% of TNBC cases are estimated to be of the basal-like breast cancer subtype.[24]

The expression of the cytokeratins was quite variable. Through the individual analysis of each marker in this cohort, they were not confirmed as significant to influence response or survival. The grouped analysis of the markers to correlate with Lehmann's molecular subtypes was not possible due to the asymmetric distribution of the sample as well as the lack of molecular tests. For CK5/6 (16%) and CK14 (10.1%), there were few patients with positive expression, while for CK17 (91.1%) most of the patients were positive, which may suggest a different genomic profile of TNBC in the Brazilian population when compared to other populations. Probably due to technical issues, as the manual score might be impaired by the often weak and focal reactivity and intratumor heterogeneity, CK5/6 expression by IHC presents a significant variability amidst previous studies of TNBC, ranging from 24% to 72%.[25] The results of a cohort study with breast cancer patients performed by van de Rijn et al[26] have suggested that expression of CK 5/6 was associated with poor clinical outcomes in node-negative breast cancers, regardless of tumor size and tumor grade. Other authors also have suggested CK5/6 as a predictive and prognostic independent marker.[23,27,28]

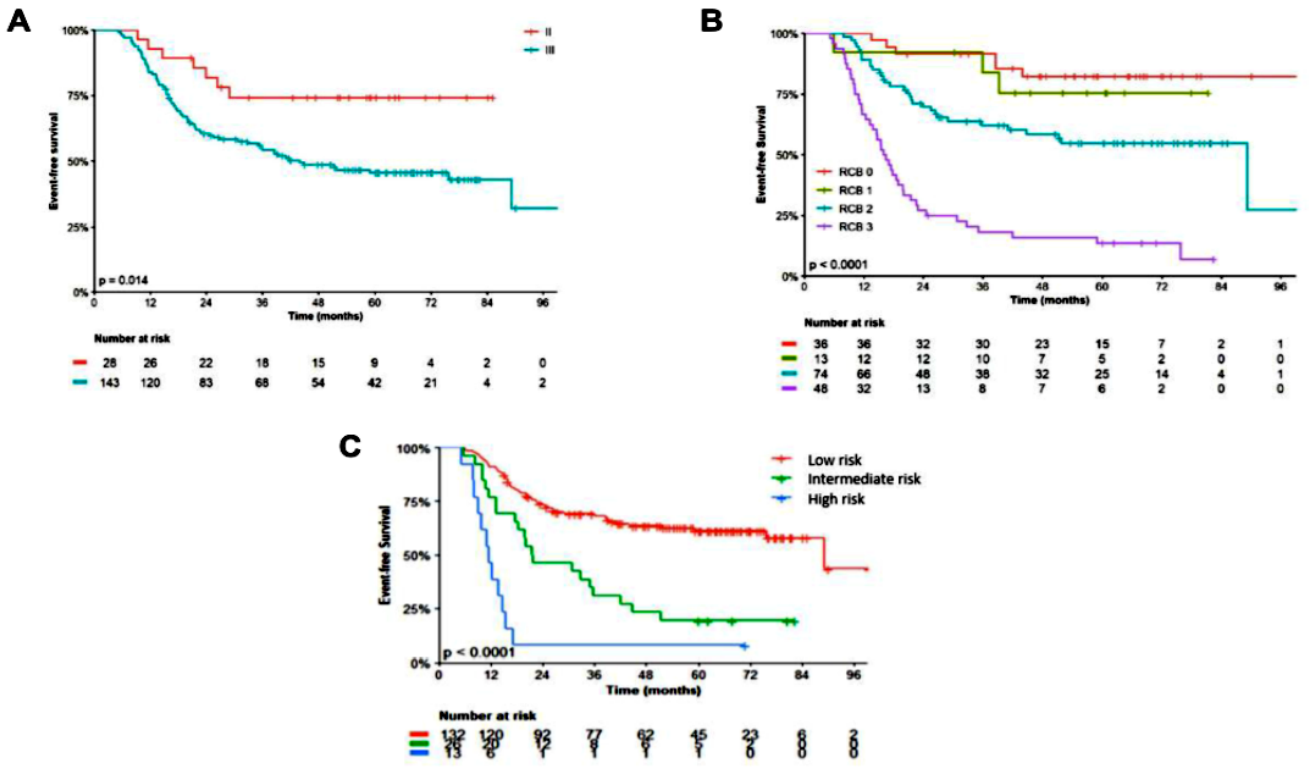

Figure 4 Kaplan–Meier event-free survival estimates according to: clinical stage (**A**), residual cancer burden (RCB) (**B**) and lymph node ratio (LNR) (**C**).

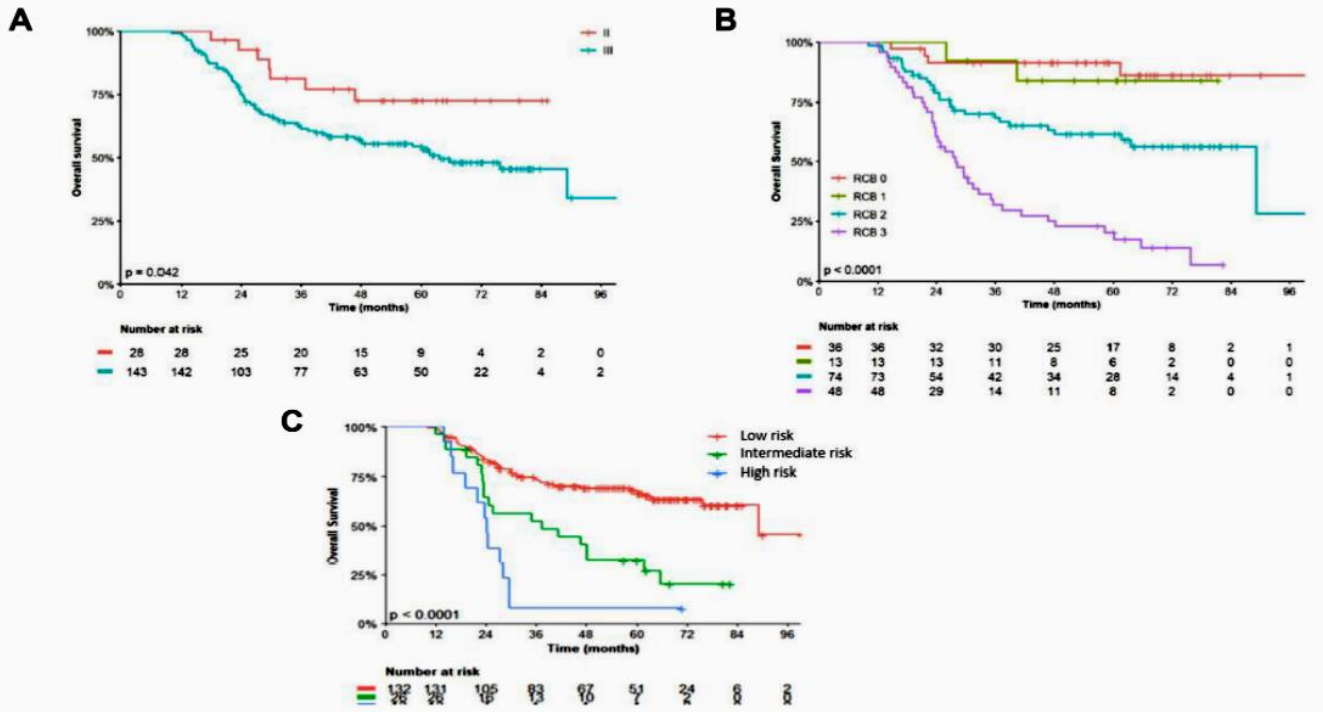

Figure 5 Kaplan–Meier overall survival estimates according to: clinical stage (**A**), residual cancer burden (RCB) (**B**) and lymph node ratio (LNR) (**C**).

Overall, the expression of CK14 and CK17 overlaps with CK5/6. In another Brazilian study of TNBC with less restrictive inclusion criteria, the prevalence of positive cases for CK14 was 26%.[29] In the study conducted at the Memorial Sloan-Kettering Cancer Center in patients with previously resected TNBC, CK14 positive cases corresponded to 46% of the sample and, unlike the current study, they were associated with worse disease-free survival (DFS, p = 0.003, 5-year DFS 69% vs 83%) and OS (p = 0.01, 5-year OS 71% vs 85%). Literature data on the prevalence of CK17 in patients with TNBC are very scarce. Some pooled analyzes with other cytokeratins have shown controversial results regarding the prognostic role of this marker.[26,30]

Another biomarker apparently less specific than CK5/6, used to define the basal-like subtype, is the immunoreactivity to EGFR.[7] The higher prevalence of patients with EGFR immunoreactivity (92.9%) in the present study may explain the lack of association with both response and survival outcomes. There is great variability in the prevalence of EGFR overexpression in TNBC cases among studies, ranging from 13% to 78%, due mainly to the lack of uniform IHC methods and to a considerable demographic variation.[31,32] Some previous data showed that higher EGFR gene copy number is likely to be associated with, higher tumor grade, axillary lymph node metastasis and poorer survival.[33] However, data from EGFR protein overexpression in triple-negative are controversial and apparently had no clinical relevance.[34,35]

With no impact on response or survival, the lower prevalence of AR in the present cohort (13%) may represent a regional characteristic. According to previous reports, there is a considerable variability in the immunohistochemical expression of AR in TNBC, ranging between 10% and 90%.[36–38] Two meta-analyses assessed the prognostic role of AR in cases with TNBC. The first assessed pooled data from 13 clinical trials that recruited 2826 patients with TNBC from 2007 to 2015. Herein, 24.4% of the cases were AR-positive, and it was associated with low tumor grade (40.8% of patients AR-positive), and post-menopausal status (26.9% of AR-positive patients) and lower risk of nodal involvement (28.8% of AR-positive patients).[39] In the second, with 521 TNBC patients, the odds ratio for DFS was 0.44 (p = 0.002).[40] However, it is important to mention that there was no correlation of AR status with OS outcome in any of these studies.

As a predictive marker, some evidence suggests that AR-positive patients are more likely to be chemo-resistant than AR-negative patients. Analysis of 637 core biopsy

samples from primary tumors of patients enrolled in the Gepartrio trial showed that pCR was 12.8% in AR-positive breast cancer compared to 25.4% in AR-negative ones (p <0.0001).[41] Similar results were observed in a Japanese retrospective cohort, in which AR-positive TNBCs presented a lower rate of pCR than AR-negative in a univariate analysis (HR 5.26; 95% CI 1.39–19.86, p = 0.014).[42]

Accounting for 52.6% of the patients tested in the present study, patients with p53 expression showed no significant association with response to NACT or survival. Similarly, a large South Korean retrospective study of 11,393 TNBC patients failed to show any association of p53 with survival outcomes or response to chemotherapy.-[43] Other studies that have shown some effect of p53 expression on response or survival to chemotherapy were limited by a small number of patients or heterogeneity. Indeed, another important limitation of these studies is that the IHC assay and the selected cutoff differ in each trial.[44–46] Generally, mutant proteins are more difficult to break down, being immunohistochemically detected. Conceptually, even p53 detection does not directly represent the mutation of p53, data from some studies suggest this correlation. Some data from the literature suggest that p53 mutant tumors respond better to chemotherapy than p53 wild tumors and other studies suggest a negative impact of the mutation on survival.[47–49]

Some limitations of the current study should be highlighted. The fact that it is a single-center study, as well as its retrospective design, gives a regional trait to the analyzed population, and the results can be influenced by marked geographic differences. Intratumoral heterogeneity may have compromised some results from the TMA analysis. There were also many losses due to scarce material in a core biopsy. In addition, it was not possible to perform any molecular analysis with the available samples.

Nevertheless, some strengths of the study should be mentioned. As it is a single-center study, the same diagnostic and treatment procedures were used for all patients during the study period, strengthening the internal validity of the data. In addition, the evaluation of the samples was carried out by two experienced pathologists and the TMAs were produced with strict quality control in order to decrease the risk of false results due to intratumoral heterogeneity and technical artifacts. Another strength was the robustness of the IHC panel of biomarkers analyzed in a specific subgroup of patients with strict exclusion criteria.

Conclusion

In conclusion, pre-NACT clinical-pathological characteristics already consolidated, such as tumor size, nodal status and clinical stage, were reaffirmed in the present study as prognostic and predictive factors in TNBC. Likewise, RCB and LNR, in the context of a pathological parameter of post-NACT response, also strongly influenced survival, and can therefore be considered important and low-cost prognostic factors to direct the best adjuvant approach.

TNBC is believed to be a heterogeneous disease comprising subtypes with diverse biological behaviors and clinical outcomes. Although several IHC biomarkers have been studied as prognostic factors in non-metastatic TNBC, few studies in the specific subset of patients with locally advanced TNBC undergoing NACT were performed, with controversial results.[50] Although negative, probably due to the technical limitations of a retrospective study, the data from the present cohort pave the way for further prospective studies in order to determine the real role of these factors in this scenario and correlate with expression profiling gene analyses. Lately, TNBC has been increasingly seen as a disease with intrinsic molecular and immunological heterogeneity, recognizing the variety of clinical phenotypes with diverse outcomes. Considering that NACT is insufficient in some cases, this new setting makes room for a demand for an urgent comprehensive subclassification that encompasses immune-molecular signatures to incorporate more targeted and effective treatment.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

The study was approved by the Ethics in Human Research Committee of INCA, Rio de Janeiro, Brazil, under registration number CAAE 61675516.9.0000.5274, and conducted in accordance with Good Clinical Practice guidelines.

Informed Consent

For this type of study with observational retrospective design with anonymized data analysis, the Institutional Review Board (Comitê de Ética em Pesquisa do Instituto Nacional de Câncer; CEP-INCA) decided in favor of waiving the consent form.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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7.1.3 Artigo 3

Title page

Title: Prognostic influence of residual tumor-infiltrating lymphocyte subtype after neoadjuvant chemotherapy in Triple-Negative Breast Cancer

Running title: Subtyping the tumor-infiltrating lymphocyte in Triple-Negative Breast Cancer

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Abstract

Objective: This study aimed to examine the prevalence and prognostic role of tumor microenvironment (TME) in triple-negative breast cancer (TNBC) after neoadjuvant chemotherapy (NACT) through immunohistochemical characterization.

Methods: The internal database of the Brazilian National Cancer Institute for women diagnosed with TNBC who underwent NACT and thereafter curative surgery between January 2010 and December 2014 was queried out. Core biopsy specimens and tissue microarrays containing surgical samples of TNBC from 171 and 134 women, respectively, were assessed by immunohistochemistry for CD3, CD4, CD8, CD 14, CD 56, CD 68, CD 117, FOXP3, PD-1, PD- L1, and PD-L2. Immune cell profiles were analyzed and correlated with response and survival.

Results: Mean age was 50.5 years, and most cases were clinical stage III [143 cases (83.6%)]. According to the multivariate analysis, only Ki67 and clinical stage significantly influenced the pattern of response to systemic treatment ($p = 0.019$ and $p = 0.033$, respectively). None of the pre-NACT IHC markers showed a significant association with event-free survival (EFS) or overall survival (OS). As for post-NACT markers, patients with high CD14 had significantly shorter EFS ($p = 0.015$), while patients with high CD3 ($p = 0.025$), CD4 ($p = 0.025$), CD8 ($p = 0.030$), FOXP3 ($p = 0.005$), high ratios CD4/FOXP3 ($p = 0.034$) and CD8/FOXP3 ($p = 0.008$), showed longer EFS. Only highly expressed post-NACT CD4 showed significantly influenced OS ($p = 0.038$).

Conclusion: The present study demonstrated that the post-NACT TILs subtype can be a determining factor in the prognosis of patients with TNBC.

Keywords: Triple-negative breast cancer; tumor-infiltrating lymphocyte; tumor microenvironment; neoadjuvant chemotherapy

1.0 Introduction

Triple-negative breast cancer (TNBC) is defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) overexpression[1,2]. Accounting for about 10-15% of breast cancer cases worldwide, this subtype amounts to over 300,000 new cases in women each year[3,4]. TNBC stands out for its aggressiveness,

invasiveness and high recurrence rate in the first three years after treatment, when compared to other types of breast cancer[5].

Recently, a growing trend has emerged toward treating patients with early-stage disease with neoadjuvant chemotherapy (NACT), supported by some plausible advantages: a significant increase in survival in patients with pathological complete response (pCR), the possibility of conversion to breast-conserving surgery, elimination of micrometastatic disease, as well as *in vivo* sensitivity test to chemotherapy[6,7].Tumor microenvironment heterogeneity, represented by tumor-infiltrating lymphocytes (TILs) and other types of immune cells in different proportions, has been widely cited as one of the main reasons for different clinical outcomes, including patterns of response to NACT and survival[8].

Several studies have been published suggesting a crucial role of the tumor microenvironment (TME) in carcinogenesis. In this context, some results suggest that TILs are mostly found in highly proliferative tumors, such as TNBC and HER2-positive breast cancers, influencing outcomes such as pathologic response to NACT as well as recurrence and survival[9–12].

More specifically, some interleukins (IL) secreted by specific infiltrated cell subtypes, such as IL-6 and IL-8, may exert a sustained stimulatory mechanism as loop feedback between the TME and cancer cells, impairing tumor growth by an immune attack. However, depending on the subtype of TILs, this effect can be inhibitory or stimulatory for breast cancer progression. T helper (CD4+) and cytotoxic (CD8+) lymphocytes, primary effector TILs subtypes, have been positively associated with a higher response rate to chemotherapy and with better overall survival (OS).

Conversely, infiltration by FOXP3+ regulatory T (Treg) cells is critical in maintaining immune tolerance and is likely to predict a worse prognosis by the so-called immune evasion. Likewise, strengthening this evasion of immune destruction, immune checkpoint proteins such as PD-1, PD-L1, and PD-L2 are likely to play an important role, not only in immunotherapy effectiveness but also in the anti-tumor effects of conventional anti-tumor drugs[13,14].The CD56 highly expressed, commonly related to tumor-infiltrating natural killer (NK) cells (CD56+ NK-TILs), has shown discrepant findings among different types of tumors. It is known that NK cells are effector lymphocytes, component of the innate immune system, and play an immunoregulatory part by inhibiting tumor growth and spread[15].

The predictive and prognostic function of immune biomarkers in TNBC remains unclear. This study aimed to investigate the influence of TILs, as well as PD-1, PD-L1 and PD-L2

expression, in the response pattern to NACT and survival outcomes in the subset of patients with locally advanced TNBC.

2.0 Materials and methods

2.1 Study design and ethical considerations

This is a cohort study with retrospective data. The study was approved by the Ethics in Human Research Committee of the Brazilian National Cancer Institute (CEP-INCA), Rio de Janeiro, Brazil, under the number CAAE 61675516.9.0000.5274, and conducted following the Good Clinical Practice guidelines.

2.2 Patient selection

Patients with newly diagnosed breast cancer enrolled at the Brazilian National Cancer Institute (INCA) between January 2010 and December 2014 were included if all the following criteria were met: a) women over 18 years old; b) confirmation of the histopathological diagnosis of TNBC by the INCA Division of Pathology(DIPAT-INCA) following the criteria of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines[1,2]: tumors with ER and PR score <1%, as well as HER2 score 0/1+ or 2+ with negative FISH; c) stage IIb-IIIc by the 7th American Joint Committee on Cancer - AJCC (T3-4NanyM0; TanyN1-3M0); d) submitted to NACT with anthracycline-taxane-based regimen (supplementary box 1) followed by curative surgery at INCA; e) the NACT was supplemented by complementary treatment with further chemotherapy and/or radiotherapy before surgery in some cases. On the other hand, the exclusion criteria comprised patients previously exposed to antineoplastic agents, with second primary or unresectable tumors after neoadjuvant treatment.

2.3 Immunohistochemistry

Due to the scarcity of material, the core biopsy samples were analyzed in their whole tissue sections for all biomarkers of IHC. As for the surgical specimen samples, the tissue microarray (TMA) analysis was performed using standard procedures on 4-μm sections in the most

representative areas of greatest tumor cellularity of formalin-fixed paraffin-embedded tissue specimens, and then stained for each biomarker. For both specimens, the tumor cell staining was compared with that of negative and positive controls.

Samples were immunostained for ER (clone EP1, Dako, prediluted), PR (clone PgR636, Dako, prediluted), HER2 (clone SP3, Cell Marque, diluted 1:500), CD3 (clone MRQ-39, Cell Marque, diluted 1:1000), CD4 (clone SP35, Cell Marque, diluted 1:400), CD8 (clone SP 16, Cell Marque, diluted 1:1000), CD14 (clone EPR3653, Cell Marque, diluted 1:200), CD56 (clone 123C3.D5, Cell Marque, diluted 1:800), CD68 (clone Kp-1, Cell Marque, diluted 1:1500), FOXP3 (clone 236A/E7, Abcam, diluted 1:50), PD-1 (clone NAT105, Cell Marque, diluted 1:100), PD-L1 (clone SP142, Ventana, prediluted) and PD-L2 (clone ab200377, Abcam, diluted 1:200).

The immunostaining scores for ER, PR and HER2 were confirmed as negative according to ASCO/CAP guidelines[16]. Ki67 was assessed by nuclear staining using a mouse monoclonal antibody (SP6 clone, Cell Marque) at 1:500 dilution. Intratumoral stromal immune markers were manually counted and scored by two experienced pathologists using a double-blind method as described hereafter. The tumor cell staining was compared with that of negative controls made from counterstaining with hematoxylin and of positive controls. In core biopsy and TMA specimens of surgical samples with residual tumor, for TILs subpopulations (CD3+, CD4+, CD8+ and FOXP3+), intratumoral stromal lymphocytes were counted semi-automatically and quantified as the average absolute number of immunolabeled lymphocytes at each of three selected field at 200x magnification.

In core biopsy specimens, for PD-1 and PD-L2, the slides were scored according to the percentage of positive cells divided by the number of fields to calculate the mean value for each case, determined at 200x magnification. The PD-L1 tumor proportion score (TPS) was defined as the percentage of viable tumor cells showing partial or complete membrane staining at any intensity. PD-L1 expression on immune cell (IC) was assessed as the proportion of tumor area occupied by PD-L1-positive IC of any intensity. The PD-L1 combined positive score (CPS) refers to the ratio between PD-L1-positive cells (tumor or immune cells) and the total number of tumor cells × 100, and was grouped here in negative (<1) or positive (≥ 1) status.

For statistical analysis, the cut-off points for most biomarkers were calculated using the surv_cutpoint function of the survminer R package to divide the scores into low and high-level

groups. In this context, considering the core biopsy markers and surgical specimen, the cut-off points were stated in supplementary tables1 and 2, respectively.

2.4 Other pathological and clinical variables

The data was collected from electronic hospital records and medical charts. The following clinical and pathological variables were retrieved: age at diagnosis, ethnicity (Caucasian or others according to Brazilian Institute of Geography and Statistics, IBGE)[17], body mass index (BMI), clinical stage (II-III), clinical T stage (cT), clinical nodal stage (cN), residual cancer burden (RCB), histological type, Elston histological grade (1: low grade; 2: moderate grade; 3: high grade), type of NACT (FAC-T or AC-T), complementary treatment, type of surgery (radical or conservative, axillary approach).

2.5 Statistical analysis

The RCB score followed the standard 4-level categorical variable (RCB "classes" 0, 1, 2, and 3)[18]. The pCR, similarly to RCB-0, was narrowly defined as no viable residual tumor in the breast or axilla (ypT0N0). Event-free survival (EFS) was calculated from the date of diagnosis to the earliest date of disease progression, death from any cause, or discontinuation of treatment for initiation of complementary treatment due to poor response to standard NACT. OS was calculated from the date of diagnosis to death from any cause or censored if the patient was known to be alive on the last day of data collection. All continuous variables were evaluated by the Shapiro-Wilk test of normality. For the RCB outcome, logistic regression was used for each variable assessed. The Kaplan-Meier method was used to estimate EFS and OS for each variable and was compared by the log-rank test. The Cox proportional hazards model was used to calculate the crude Hazard Ratio (HR) for each factor. Regarding multivariate analysis, all variables with an association with response and survival outcomes at p-value <0.20 were included, and the Akaike Information Criterion was used to pick the most suitable model for multiple Cox analysis. A p-value <0.05 was considered statistically significant. The missing data was excluded from the analysis. The statistical analyzes were conducted using R environment version 3.5.3.

3.0 Results

As shown in the study profile description in supplementary figure 1, one hundred and seventy-one women with TNBC treated with anthracycline-taxane-based NACT were included for evaluation. Detailed information regarding clinicopathological features and treatment data is highlighted in supplementary table 3. Mean age was 50.5 years (standard deviation, SD 10.7), most cases were clinical stage III [143 cases (83.6%)], and the mean BMI was 28.5 Kg/m² (SD 5.8). Invasive ductal carcinoma not otherwise specified (NOS) was the most prevalent histology [160 cases (93.6%)] and more than half of the cases were histological grade 3 [115 cases (67.3%)]. Neoadjuvant treatment predominantly consisted of AC-T [117 cases (68.4%)], and the pCR rate was 21% (36 cases). The vast majority of patients underwent mastectomy [165 cases (96.5%)] and axillary dissection [145 cases (93.5%)].

Median pre-NACT scores and cut-off points for IHC biomarkers for the general population are shown in supplementary table 1. CD3 [Median (IQR), cut-off point; 10 (29), 5.00] was the most predominant lymphomononuclear subpopulation, with lower values for CD14 [1 (4)] and CD 117 [0 (1)], while no CD56 subpopulation was found, which made it unviable perform the analyzes of this marker for previously determined outcomes. The numbers for the ratios were: CD4/FOXP3 [0.67 (2.90), 5.00], CD8/FOXP3 [0.50 (0.91), 3.27] and CD4/CD8 [1.00 (1.48), 5.50]. As for the PD1/PD-L1/PD-L2 axis immune checkpoint signaling, most of patients presented PD-1 low [144 cases (93.5%)], PD-L1 IC <1% [138 cases (85.7%)], PD-L1 TPS low [119 cases (70.4%)], PD-L1 CPS positive [100 cases (58.8%)] and PD-L2 low [105 cases (65.2%)].

According to the data shown in supplementary table 2, a total of 134 patients had surgical samples with residual tumor, and were evaluated for the IHC markers of selected TILs. CD14 was the most predominant marker [4.33 (12.67), 0.33], followed by CD 4 [2.67 (8.34), 4.00] and FOXP3 [2.67 (5.37), 0.67]. The results for ratios were observed as described: CD4/FOXP3 [1.31 (2.19), 0.72], CD8/FOXP3 [0.51 (0.71), 0.52] and CD4/CD8 [2.00 (4.28), 0.47].

The association of the clinical and pathological features with response to NACT was individually assessed and summarized in table 1. By multivariate analysis, only Ki67 showed a significant positive correlation with the pattern of response to systemic treatment ($p = 0.019$). On the other hand, stage III showed a significantly poorer lower rate of RCB 0/I as compared to stage II ($p = 0.033$).

The median follow-up time was 62.5 months (95% Confidence Interval, 95% CI 60.2 - 67.9). As highlighted in supplementary table 4 and 5, amid the IHC markers assessed in the core biopsy, none of them showed a significant association with the outcomes of EFS and OS. Figure 1 shows representative images of cases with high expression of IHC markers in core biopsies.

The analysis of post-NACT clinical and pathological features in patients with residual tumors is described in table 2 and table 3. By univariate analysis, patients with high CD14 had significantly shorter EFS ($p = 0.042$) than those with low expression of CD14. Conversely, high expression of CD3 ($p = 0.007$) and CD8 ($p = 0.013$), as well as high ratios CD4/FOXP3 ($p = 0.034$) and CD8/FOXP3 ($p = 0.008$), had significantly longer EFS. The Cox model chosen for multivariate analysis was comprised of six variables, five of which showed significant association for EFS: CD3 ($p = 0.025$), CD4 ($p = 0.025$), CD8 ($p = 0.030$), CD14 ($p = 0.015$) and FOXP3 ($p = 0.005$)(table 2).

Regarding the univariate analysis of post-NACT clinical and pathological features for OS, patients with high CD3 ($p = 0.025$) and CD8 ($p = 0.011$), as well as high ratios CD4/FOXP3 ($p = 0.025$) and CD8/FOXP3 ($p = 0.026$) had significantly greater survival than those with low expression. Herein, of the six variables that constituted the Cox model chosen for multivariate analysis of OS, only high CD4 ($p = 0.038$) was significantly associated with lower risk of death (table 3). Figures 2 and 3 show the Kaplan-Meier curves for EFS and OS, respectively, according to the evaluated post-NACT variables.

4.0 Discussion

In the TNBC subset, the influence of the tumor microenvironment in cancer cell proliferation, as well as in response to anti-cancer drugs, has been increasingly recognized over time. Furthermore, a growing body of evidence has pointed out that lymphomononuclear cells subtypes present in the TME are crucial driving factors of tumor progression and invasion. This is one of the few series to perform a thorough evaluation of TILs subtype in post-NACT residual tumors of women with TNBC. The main results suggested that the high expression of some markers in this subset might influence recurrence and death events.

The pCR (RCB-0) rate of 21.1% is similar to previous results from other published TBNC series using anthracycline and taxane-based NACT but can be considered a modest number when compared to data from treatment regimens that included new strategies such as dense-dose

regimens, PARP inhibitors, immunotherapy, antiangiogenic or platinum agents[19,20]. Amidst the IHC markers and clinicopathological features evaluated, only the clinical stage and Ki67 had a significant correlation with the pattern of response to NACT (p = 0.033 and 0.019, respectively) in the multivariate analysis. A phase II study conducted by Wang et al[21] enrolled 280 patients with stage II – III TNBC treated with neoadjuvant weekly paclitaxel and carboplatin showed that categorical and linear Ki-67 were independently correlated with pCR (p < 0.001). Likewise, a meta-analysis performed by Tao et al.[22] included 36 studies involving 6793 breast cancer patients, suggesting that pretherapeutic Ki-67 LI is associated with pCR in breast cancer patients undergoing NACT (p < 0.001). In the study conducted by Jamiyan et al[23] with patients with TNBC, high intratumoral TILs were found to be more expressed in patients with stage III over stage I/II (p = 0.006). The association of high CD4 expression with a higher rate of pCR in TNBC cases (p = 0.003) was observed in the cohort performed by García-Martínez et al[24].

The immune IHC markers assessed in core biopsy in the current study did not show a significant association with survival outcomes. Conversely, a meta-analysis performed by Gao et al[25] with 37 studies involving patients with TNBC showed that the upregulation of TILs predicted better disease-free survival (DFS) and OS, with pooled Hazard Ratios (HR) of 0.66 (95% CI, 0.57–0.76) and of 0.58 (95% CI, 0.48–0.71), respectively, for TILs level (high versus low). Specifically, the CD4+ TILs subgroup (high versus low) showed a better OS (HR 0.49, 95% CI 0.32–0.76) and DFS (HR 0.54, 95% CI 0.36–0.80), and the CD8 + TIL subgroup (high versus low) showed a better DFS only (HR 0.55, 95% CI 0.38–0.81). The FOXP3 + TIL subgroup (high versus low) also showed only better DFS (HR 0.50, 95% CI 0.33–0.75), with no statistical association with OS (HR 1.28, 95% CI 0.24–6.88). In a cohort of 150 breast cancer patients performed by Rathore et al[26], the intratumoral high CD4+ count (OR = 3.85, 95% CI = 3.28-16.71, p < 0.001), CD3+ (OR = 2.70, 95% CI = 1.76-8.30, p = 0.001) and CD8+ (OR = 2.58, 95% CI = 1.55-5.86, P = 0.001) showed better survival when compared to respective low counterparts.

In another cohort with 175 infiltrating ductal carcinomas of breast, although CD56+NK-TILs is highly expressed in 48.6% of cases, Rathore et al[27] suggested that this marker alone may not be sufficient for predicting the survival outcomes. To explore the tumor-associated macrophages (TAMs), Wang et al[28]evaluated the expression of CD68+ TILs in 48 samples of TNBC, showing upregulation in 71.4% of cases. Patients with high infiltration of CD68 had higher expression of inflammatory cytokines interleukin 6 (IL-6) and chemokine (C-C motif) ligand 5 (CCL-5) and

lower survival rates compared to the low infiltration group. As a marker related to TAMs, the infiltration of CD68+ cells is supposed to be positively related to tumor severity. A retrospective systematic review study conducted by Ni et al[29] reviewed the macrophage distribution in 1579 non-metastatic breast cancer specimens with anti-CD68 immunohistochemical staining. The data revealed that high density of CD68-TAMs was significantly related to ominous clinicopathological characteristics such as lymph node metastasis, high Ki67, poor histological grade and hormonal receptor negativity ($p < 0.001$for all comparisons).

Missense-specific mutations of *TP53* with loss of P53 protein function have been linked to increased expression of CD117 in some solid tumors, inhibiting cellular differentiation, proliferation, adhesion and apoptosis[30]. However, data regarding the prognostic impact of CD117 on TNBC are conflicting. Kashiwagi et al[31] and Luo et al[32] have suggested that CD117 protein is associated with recurrence and poor prognosis, on the other hand, other authors failed to find a significant association between CD117 and prognosis in breast cancer or TNBC[33,34].

The immune checkpoint receptor PD-1 has a crucial role in the tumor immune evasion process. The two ligands, PD-L1 and PD-L2, have distinct expression profiles depending on the tumor types[35]. Some previous studies have addressed an assessment of the influence of the PD-1/PD-L1/PD-L2 axis markers on the survival of patients with invasive breast cancer, more specifically TNBC, showing discrepant results. Mori et al.[36] demonstrated that the interaction between TILs and PD-L1 correlates with better survival outcome. In the study of Beckers et al[37], although PD-L1 is associated with a better outcome, the results failed to show an independent prognostic role in this subset of tumors. These conflicting results could be explained by different clinical outcomes along with various chemotherapy schedules, methods of evaluation of PD-L1 expression and definition of cut-offs. Asano et al[14] suggested that patients with low PD-1 and PD-L1 expressions in TNBC were associated with a higher pCR rate and significantly longer DFS, and low PD-L1 expression was an independent prognostic factor.

Some studies have used immune checkpoint inhibitors as a complement to the neoadjuvant treatment strategy in patients with locally advanced TNBC. Notably, initial studies like I-SPY 2[38] and the KEYNOTE-173[39] trials showed that the combination of pembrolizumab, an anti-PD-1 monoclonal antibody, with NACT significantly increased the pCR rate in early-stage TNBC. An interim analysis of the phase III KEYNOTE-522 trial reported a significantly higher pCR rate (64.8% versus 51.2%; $p < 0.001$) and better EFS (HR 0.63; 95% CI: 0.43 - 0.93) in the combination

group than in the NACT alone group, regardless of PD-L1 status though. EFS was significantly higher in the pembrolizumab group after a median follow-up of 15.5 months[40]. Some initial phase I/II trials suggest that the addition of durvalumab to NACT may increase the pCR to over 50%[41–43].

Some data has suggested that, in addition to the cytotoxic effect, the effectiveness of chemotherapy can also occur through the restoration of immunosurveillance inducing immunogenic cell death[44]. As shown in the results of the current cohort, some subtypes of TILs present in the residual tumor such as CD3, CD8 and CD4, as well as CD4/FOXP3 and CD8/FOXP3 ratios, influenced survival outcomes. A small series with 25 consecutive patients with breast cancer reported lymphocytes activation and attraction to tumor bed in 7 cases after NACT with a better prognosis in these cases[45]. Ladoire et al[46] evaluated surgical specimens from 111 patients with HER2 negative breast cancer, in which high CD8 and low FOXP3 cell infiltration after chemotherapy were significantly associated with improved RFS ($p = 0.02$) and OS ($p = 0.002$). The study conducted by Dieci et al[47], which evaluated TILs in patients with non-pCR TNBC after NACT, suggested that the treatment could convert a low TIL into a high TIL tumor and that this conversion could be associated with a longer 5 -year OS rate. García-Martínez et al[24] identified a specific pattern of TILs in the post-NACT residual TNBC, marked by the high infiltration of CD3 and CD68, which presented poorer DFS. This discrepant result might partially be explained by the predominant infiltration of CD68, a TAMs marker previously associated with poorer outcomes.

The finding that the upregulation of some subtypes of post-chemotherapy TILs could identify subgroups of patients with different prognosis pave the way for drug development and patient stratification, which could result in changes in the practical approach of patients in adjuvant treatment. Further data to unveil the mechanisms underlying the pattern of lymphomononuclear cells, as well as the changes after NACT may determine the development of new immune-targeted therapies for breast cancer in this setting, mainly in TNBC.

The strengths of this study rely mainly on the in-depth analysis of TME data after NACT by presenting the characteristics of the lymphomononuclear infiltrate and the consequent impact on survival. The study population is homogenous in that as only patients with locally advanced TNBC who underwent NACT followed by primary surgery were included. Moreover, all core biopsy and surgical samples were double-checked by blinded experienced pathologists. Lastly, a thorough descriptive presentation of clinicopathological variables was performed and multivariate analyzes reinforce the internal validity of the results.

The major limitation of the current study is its retrospective nature. So, some missing confounding factors may exist in the analysis. As a single-center study, some regional traits in the selected population may exist, and the results can be influenced by marked geographic differences. Intratumoral heterogeneity may have compromised some results from the TMA analysis. There were also many losses due to scarce material in the core biopsy. Also, it was not possible to perform any gene expression profile analysis with the available samples.

5.0 Conclusion

The present study demonstrated that the post-NACT TILs subtype could be a determining factor in the prognosis of patients with TNBC. Undoubtedly, considering that NACT may be insufficient to achieve pCR and ensure long survival in some cases, the composition of TME in post-NACT residual tumors of TNBC could be explored in the future to guide the extension of adjuvant treatment. Further studies with larger samples of TNBC patients are aimed to validate the findings of the current study.

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Competing interests: The authors declare no conflicts of interest.

Ethical approval: The study was approved by the Ethics in Human Research Committee of INCA, Rio de Janeiro, Brazil, under registration number CAAE 61675516.9.0000.5274, and conducted in accordance with Good Clinical Practice guidelines.

Informed consent: For this type of study with an observational retrospective design with anonymized data analysis, the Institutional Review Board (Comitê de Ética em Pesquisa do Instituto Nacional de Câncer; CEP-INCA) decided in favor of waiving the consent form.

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table 1. Correlation of clinical-pathological characteristics and expression profile of biomarkers with residual burden cancer by logistic regression through univariate analysis (n = 171).

Variables/biomarker	RCB 0/1	RCB 2/3	Crude p-value	Adjusted p-value
	50 (29.2%)	121 (70.8%)		

Age mean (SD)	51.0 (11.5)	50.3 (10.4)	0.849	
BMI Kg/m² mean (SD)	29.2 (7.2)	28.3 (5.2)		
Clinical stage			**0.026**	**0.033**
II	**13 (26%)**	**15 (12.4%)**		
III	**37 (74%)**	**106 (87.6%)**		
Grade			0.547	
1	1 (2.0%)	2 (1.7%)		
2	17 (34%)	36 (29.8%)		
3	32 (64%)	83 (68.6%)		
Histological type			0.433	
Invasive ductal carcinoma NOS	48 (96%)	112 (92.6%)		
Metaplastic	2 (4%)	9 (7.4%)		
NACT regimen			0.863	
AC-T	34 (68%)	83 (68.6%)		
FAC-T	16 (32%)	38 (31.4%)		
Ki 67 mean (SD)	51.6 (28.8)	42.5 (30.7)	0.127	**0.019**
CD3			0.907	
High	8 (16.7%)	18 (15.9%)		
Low	40 (83.3%)	95 (84.1%)		
CD4			0.046	0.147
High	37 (77.1%)	101 (89.4%)		
Low	11 (22.9%)	12 (10.6%)		
CD 8			0.581	
High	23 (47.9%)	59 (52.7%)		
Low	25 (52.1%)	53 (47.3%)		
CD 14			0.825	
High	7 (14.6%)	15 (13.3%)		
Low	41 (85.4%)	98 (86.7%)		
CD 68			0.571	
High	24 (50%)	62 (54.9%)		
Low	24 (50%)	51 (45.1%)		
CD117			0.437	
High	5 (10.4%)	17 (15%)		
Low	43 (89.6%)	96 (85%)		
FOXP3			0.340	
High	19 (39.6%)	54 (47.8%)		
Low	29 (60.4%)	59 (52.2%)		

PD-1			0.188	0.256
High	1 (2.2%)	9 (8.3%)		
Low	45 (97.8%)	99 (91.7%)		
PD-L1 TPS			0.238	
High	18 (36%)	32 (26.9%)		
Low	32 (64%)	87 (73.1%)		
PD-L1 IC			0.944	
High	7 (14.6%)	16 (14.2%)		
Low	41 (85.4%)	97 (85.8%)		
PD-L1 CPS			0.888	
Positive	29 (58%)	71 (59.2%)		
Negative	21 (42%)	49 (40.8%)		
PD-L2			0.121	
High	21 (43.8%)	35 (31%)		
Low	27 (56.2%)	78 (69%)		
CD4/FOXP3 ratio			0.210	
High	22 (45.8%)	64 (56.6%)		
Low	26 (54.2%)	49 (43.4%)		
CD8/FOXP3 ratio			0.841	
High	41 (85.4%)	97 (86.6%)		
Low	7 (14.6%)	15 (13.4%)		
CD4/CD8 ratio			0.540	
High	4 (8.3%)	13 (11.6%)		
Low	44 (91.7%)	99 (88.4%)		

RCB: residual cancer burden; SD: Standard deviation; BMI: Body mass index; NOS: not otherwise specified; NACT: neoadjuvant chemotherapy; AC-T: doxorubicin/cyclophosphamide followed by taxane; FAC-T: doxorubicin/cyclophosphamide/fluorouracil; CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; CD56: Cluster of Differentiation 56; CD68: Cluster of Differentiation 68; CD117: Cluster of Differentiation 117; FOXP3: Forkhead Box P3; PD-1: Programmed Cell Death Protein 1; PD-L1 TPS: Programmed Death-Ligand 1 tumor proportion scores; PD-L1 IC: Programmed Death-Ligand 1 tumor infiltrating immune cells; PD-L1 CPS: Programmed Death-Ligand 1 combined positive score; PD-L2: Programmed Death-Ligand 2.
Differences in absolute value correspond to missing data.
Statistically significant results are in bold.

Table 2. Post-NACT clinicopathological features and crude and adjusted Hazards Ratios for event-free survival (EFS) estimated by univariate analysis and multivariate analysis.

Post-NACT clinicopathological features	Univariate analysis		p-value	Multivariate analysis		p-value
	HR	95%CI		HR	95%CI	

Clinical stage (III versus II)	1.91	(0.88 - 4.16)	0.103	1.76	(0.75 - 4.14)	0.194
CD3 (high versus low)	**0.50**	**(0.30 - 0.82)**	**0.007**	**0.58**	**(0.30 - 1.10)**	**0.025**
CD4 (high versus low)	0.69	(0.42 - 1.13)	0.138	**0.52**	**(0.30 - 0.92)**	**0.025**
CD8 (high versus low)	**0.41**	**(0.20 - 0.83)**	**0.013**	**0.37**	**(0.15 - 0.91)**	**0.030**
CD14 (high versus low)	**2.58**	**(1.03 - 6.45)**	**0.042**	**3.66**	**(1.29 - 10.41)**	**0.015**
FOXP3 (high versus low)	1.53	(0.83 - 2.81)	0.170	**2.78**	**(1.35 - 5.75)**	**0.005**
CD4/FOXP3 ratio (high versus low)	**0.59**	**(0.36 - 0.96)**	**0.034**			
CD8/FOXP3 ratio (high versus low)	**0.51**	**(0.31 - 0.84)**	**0.008**			
CD4/CD8 ratio (high versus low)	2.31	(0.83 - 6.41)	0.107			

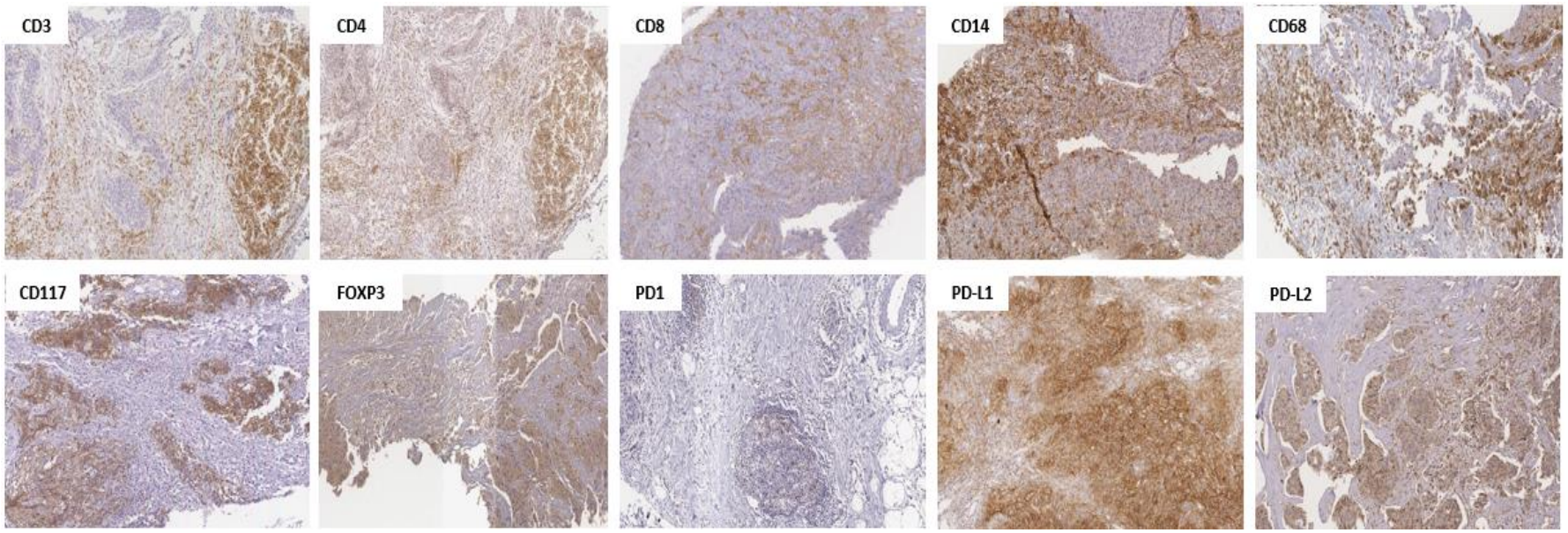
CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; CD68: Cluster of Differentiation 68; CD117: Cluster of Differentiation 117; FOXP3: Forkhead Box P3. Significant p-values are in bold.

Table 3. Post-NACT clinicopathological features and crude and adjusted Hazards Ratios for overall survival (OS) estimated by univariate analysis and multivariate analysis.

Post-NACT clinicopathological features	Univariate analysis		p-value	Multivariate analysis		p-value
	HR	95%CI		HR	95%CI	
Clinical stage (III versus II)	1.75	(0.80 - 3.83)	0.159	1.53	(0.65 - 3.60)	0.330
CD3 (high versus low)	**0.55**	**(0.32 - 0.93)**	**0.025**	0.77	(0.38 - 1.52)	0.449
CD4 (high versus low)	0.68	(0.41 - 1.14)	0.145	**0.52**	**(0.28 - 0.96)**	**0.038**
CD8 (high versus low)	**0.36**	**(0.16 - 0.79)**	**0.011**	0.40	(0.15 - 1.02)	0.055
CD14 (high versus low)	2.73	(0.99 - 7.57)	0.053	2.75	(0.95 - 7.98)	0.062
FOXP3 (high versus low)	1.63	(0.87 - 3.07)	0.130	2.05	(0.99 - 4.23)	0.051
CD4/FOXP3 ratio (high versus low)	**0.56**	**(0.34 - 0.93)**	**0.025**			
CD8/FOXP3 ratio (high versus low)	**0.56**	**(0.34 - 0.93)**	**0.026**			
CD4/CD8 ratio (high versus low)	1.96	(0.70 - 5.46)	0.199			

CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; CD68: Cluster of Differentiation 68; CD117: Cluster of Differentiation 117; FOXP3: Forkhead Box P3. Significant p-values are in bold.

Figure 1. Representative pictures of lymphocyte infiltration in triple-negative breast cancer core biopsy showing immunohistochemical staining of high CD3+, CD4+, CD8+, CD14+, CD68+, CD117+, FOXP3+, PD-1+, PD-L1+ and PD-L2+.The specimens are imaged at × 20 magnification.



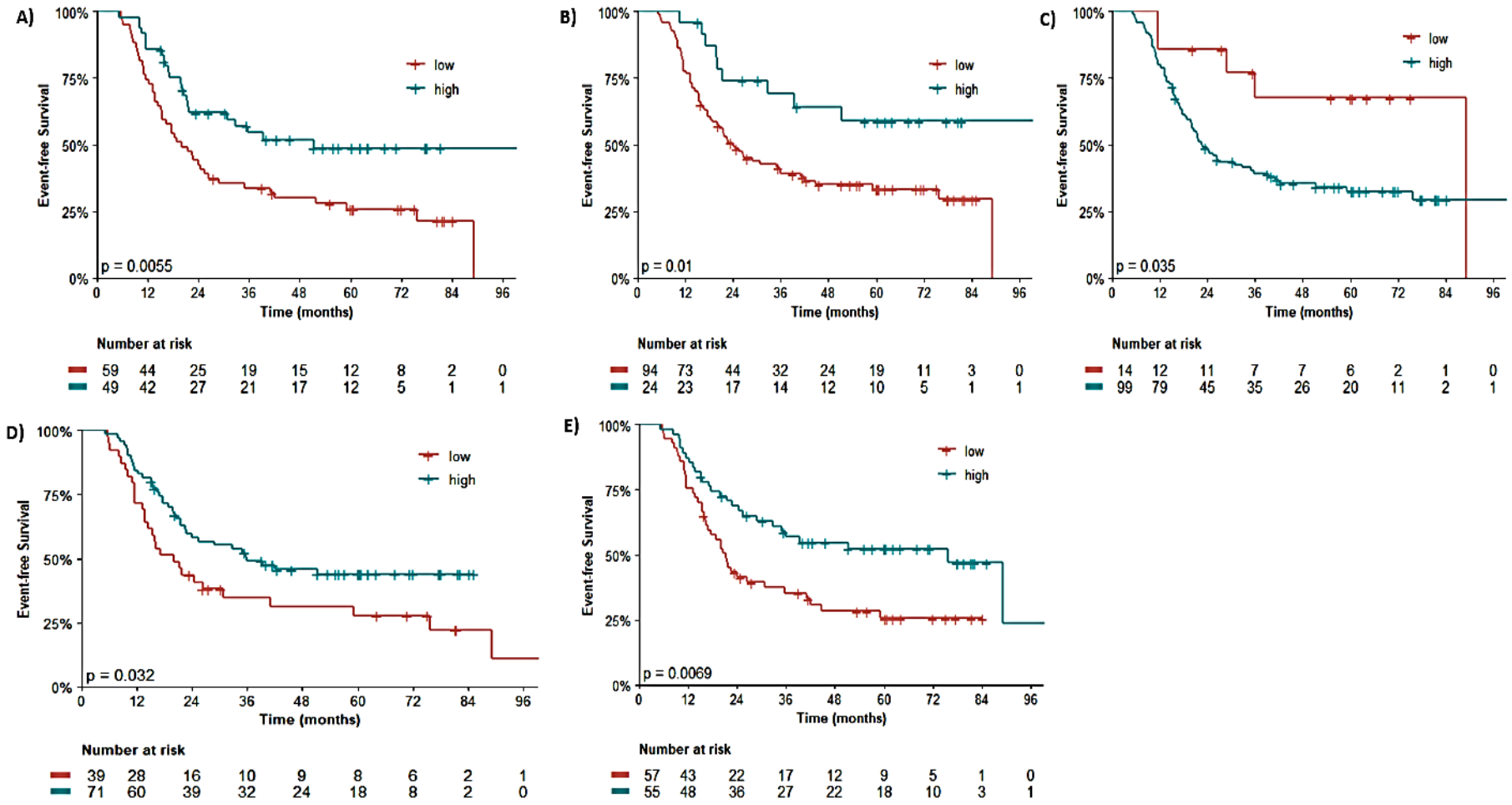


Figure 2. Event-free survival (EFS) by: A. CD3; B. CD8; C. CD14; D.CD4/FOXP3 ratio and E. CD8/FOXP3 ratio. Regarding the immunohistochemistry markers and the ratios, Kaplan-Meier curves for EFS were stratified according to the cut-off for prognostic evaluation and divided into low versus high subgroup for each variable subsets. The red solid line indicates patients with low values and the blue solid line high values. Tick marks indicate censored data.

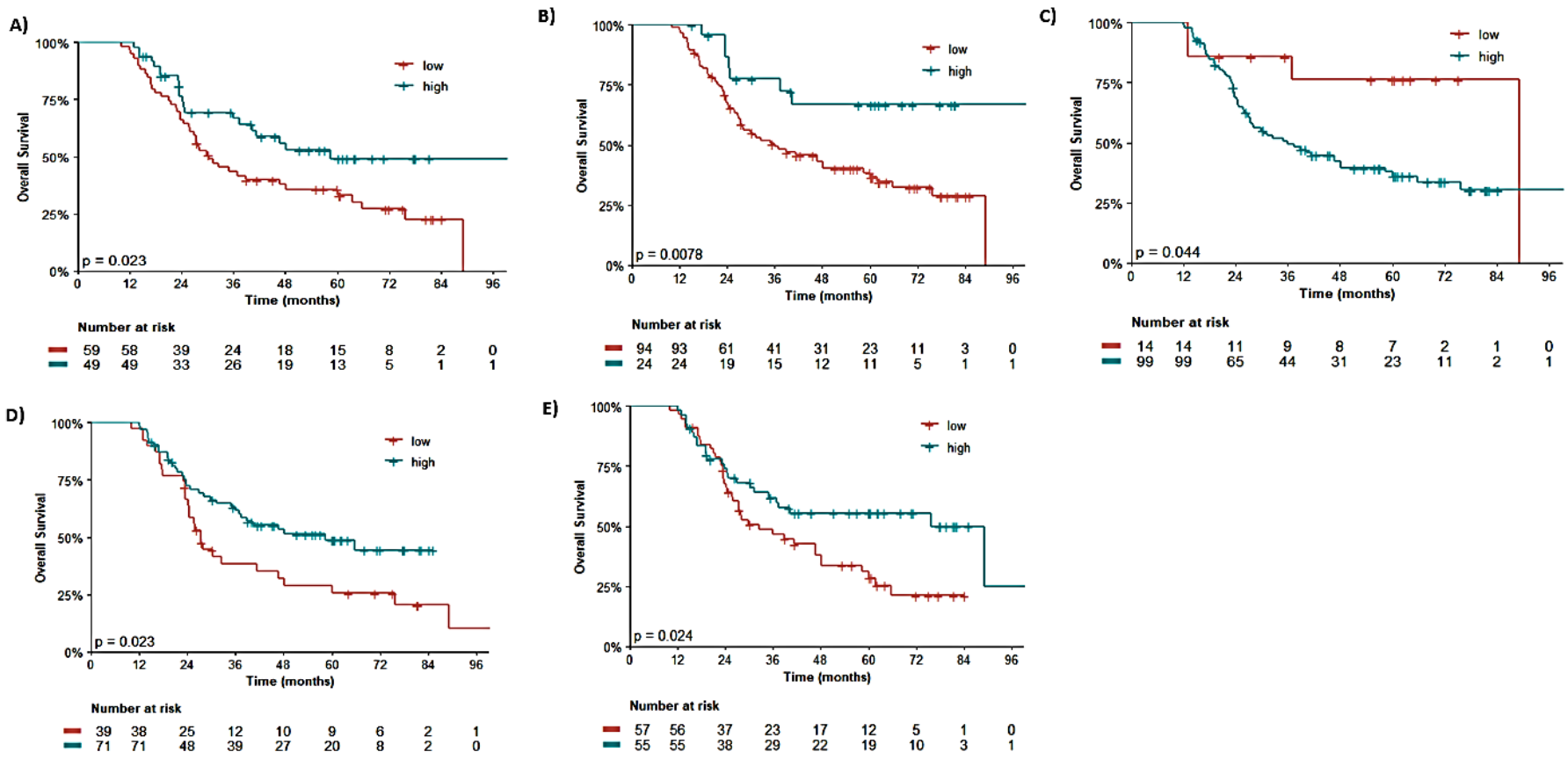


Figure 2. Overall survival (OS) by: A. CD3; B. CD8; C. CD14; D. CD4/FOXP3 ratio and E. CD8/FOXP3 ratio. Regarding the immunohistochemistry markers and the ratios, Kaplan-Meier curves for EFS were stratified according to the cut-off for prognostic evaluation and divided into low versus high subgroup for each variable subsets. The red solid line indicates patients with low values and the blue solid line high values. Tick marks indicate censored data.

Supplementary box 1. Neoadjuvant chemotherapy regimens

Standard NACT regimens	Dose/schedule
FAC-T	Fluorouracil 500 mg/m², doxorubicin 50 mg/m² and cyclophosphamide 500 mg/m², administered intravenously every 21 days for 3 cycles, followed by docetaxel 100 mg/m² every 21 days for 3 cycles.
AC-T	Doxorubicin 60 mg/m² and cyclophosphamide 600 mg/m², given intravenously every 21 days for 4 cycles, followed by docetaxel 100 mg/m² given intravenously every 21 days for 4 cycles, or followed by weekly paclitaxel 80 mg/m² given intravenously for 12 consecutive weeks without interval, defined here as a total of four 3-week cycles.
Complementary chemotherapy*	Dose/schedule
Cisplatin	75mg/m² administered every 21 days intravenously during radiotherapy.
Capecitabine	850 mg/m² orally twice daily for 14 days every 3 weeks concomitant with radiotherapy.

NACT: Neoadjuvant chemotherapy.

*Following the routine of the local oncology team, patients with tumors considered unresectable soon after NACT were exposed to complementary chemotherapy and/or salvage radiotherapy to achieve clinical response to enable the surgical approach.

Supplementary table 1. Pre-neoadjuvant chemotherapy immunohistochemistry markers (n = 171).

Marker	Median (IQR)	Cut-off point
CD3	10 (29)	5.00
High26 (16.1%)		
Low 135 (83.9%)		
CD4	1 (9)	0
High 138 (85.7%)		
Low 23 (14.3%)		
CD8	5 (10)	1.00
High 82 (51.2%)		
Low 78 (48.8%)		
CD14	1 (4)	5.00
High 22 (13.7%)		
Low 139 (86.3%)		
CD56	0 (0)	-
CD68	5 (9)	1.00
High 86 (53.4%)		
Low 75 (46.6%)		
CD117	0 (1)	3.00
High 22 (13.7%)		
Low 139 (86.3%)		
FOXP3	5 (39)	5.00
High 73 (45.3%)		
Low 88 (54.7%)		
PD-1	0 (0)	0
High 144 (93.5%)		
Low 10 (6.5%)		
PD-L1 TPS	0 (5)	1.00
High 50 (29.6%)		
Low119 (70.4%)		
PD-L1 IC	0 (0)	0
< 1% 138 (85.7%)		
1-5% 5 (3.1%)		
5-10% 10 (6.2%)		
> 10% 8 (5%)		
PD-L1 CPS	0 (1)	0
Positive 100 (58.8%)		
Negative 70 (41.2%)		
PD-L2	5 (49)	2.00
High 56 (34.8%)		
Low105 (65.2%)		
CD4/FOXP3 ratio	0.67 (2.90)	5.00
High86 (53.4%)		
Low75 (46.6%)		
CD8/FOXP3 ratio	0.50 (0.91)	3.27
High 138 (86.2%)		
Low22 (13.8%)		
CD4/CD8 ratio	1.00 (1.48)	5.50
High 17 (10.6%)		
Low 143 (89.4%)		

IQR: interquartile range; CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; CD56: Cluster of Differentiation 56; CD68: Cluster of Differentiation 68; CD117: Cluster of Differentiation 117; FOXP3: Forkhead Box P3; PD-1: Programmed Cell Death Protein 1; PD-L1 TPS: Programmed Death-Ligand 1 tumor proportion scores;PD-L1 IC: Programmed Death-Ligand 1 tumor infiltrating immune cells; PD-L1 CPS: Programmed Death-Ligand 1 combined positive score; PD-L2: Programmed Death-Ligand 2.
Differences in absolute value correspond to missing data.

Supplementary table 2. Post-neoadjuvant chemotherapy immunohistochemistry markers in residual tumors (n = 134).

Marker	Median (IQR)	Cut-off point
CD3	2 (5.70)	2.50
High 49 (45.4%)		
Low 59 (54.6%)		
CD4	2.67 (8.34)	4.00
High 49 (41.9%)		
Low 68 (58.1%)		
CD8	0.67 (2)	2.67
High 24 (20.3%)		
Low 94 (79.7%)		
CD14	4.33 (12.67)	0.33
High 99 (87.6%)		
Low 14 (12.4%)		
FOXP3	2.67 (5.37)	0.67
High 90 (78.9%)		
Low 4 (21.1%)		
CD4/FOXP3 ratio	1.31 (2.19)	0.72
High 71 (64.5%)		
Low 39 (35.5%)		
CD8/FOXP3 ratio	0.51 (0.71)	0.52
High 55 (49.1%)		
Low 57 (50.9%)		
CD4/CD8 ratio	2.00 (4.28)	0.47
High 104 (90.4%)		
Low 11 (9.6%)		

IQR: interquartile range; CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; FOXP3: Forkhead Box P3;
Differences in absolute value correspond to missing data.

Supplementary table 3. Clinical and pathological general features of eligible patients.

Features	n = 171
Age years mean (SD)	50.5 (10.7)
Race/ethnicity White	78 (45.6%)
BMI Kg/m² mean (SD)	28.5 (5.8)
Ki67 mean (SD)	45.1 (30.4)
Histological type	
Invasive ductal carcinoma NOS	160 (93.6%)
Metaplastic	11 (6.4%)
Clinical Staging	
II	28 (16.4%)
III	143 (83.6%)
Clinical T stage	
cTx	1 (0.6%)
cT2	23 (13.5%)
cT3	70 (40.9%)
cT4	77 (45%)
Clinical N stage	
N0	49 (28.7%)
N1-N3	122 (71.3%)
Histological grade	
Grade 1	3 (1.8%)
Grade 2	53 (31%)
Grade 3	115 (67.3%)
LVI	
Present	38 (26%)
Absent	108 (74%)
PI	
Present	21 (16.8%)
Absent	104 (83.2%)
NACT regimen	
AC-T	117 (68.4%)
FAC-T	54 (31.6%)
Type of surgery	
Breast-conserving surgery	6 (3.5%)
Mastectomy	165 (96.5%)
Axillary approach	
Sentinel lymph node biopsy	10 (6.5%)
Axillary lymph node dissection	145 (93.5%)
RCB	
0	36 (21.1%)
1	13 (7.6%)
2	74 (43.3%)
3	48 (28.1%)

SD: Standard deviation; BMI: Body mass index; NOS: not otherwise specified; LVI: lymphovascular invasion, PI: perivascular infiltration; NACT: neoadjuvant chemotherapy; AC-T: doxorubicin/cyclophosphamide followed by taxane; FAC-T: doxorubicin/cyclophosphamide/fluorouracil followed by taxane; RCB: residual cancer burden.
Differences in absolute value correspond to missing data.

Supplementary table 4. Pre-NACT clinicopathological features and Hazards Ratios for event-free survival (EFS) estimated by univariate analysis and multivariate analysis.

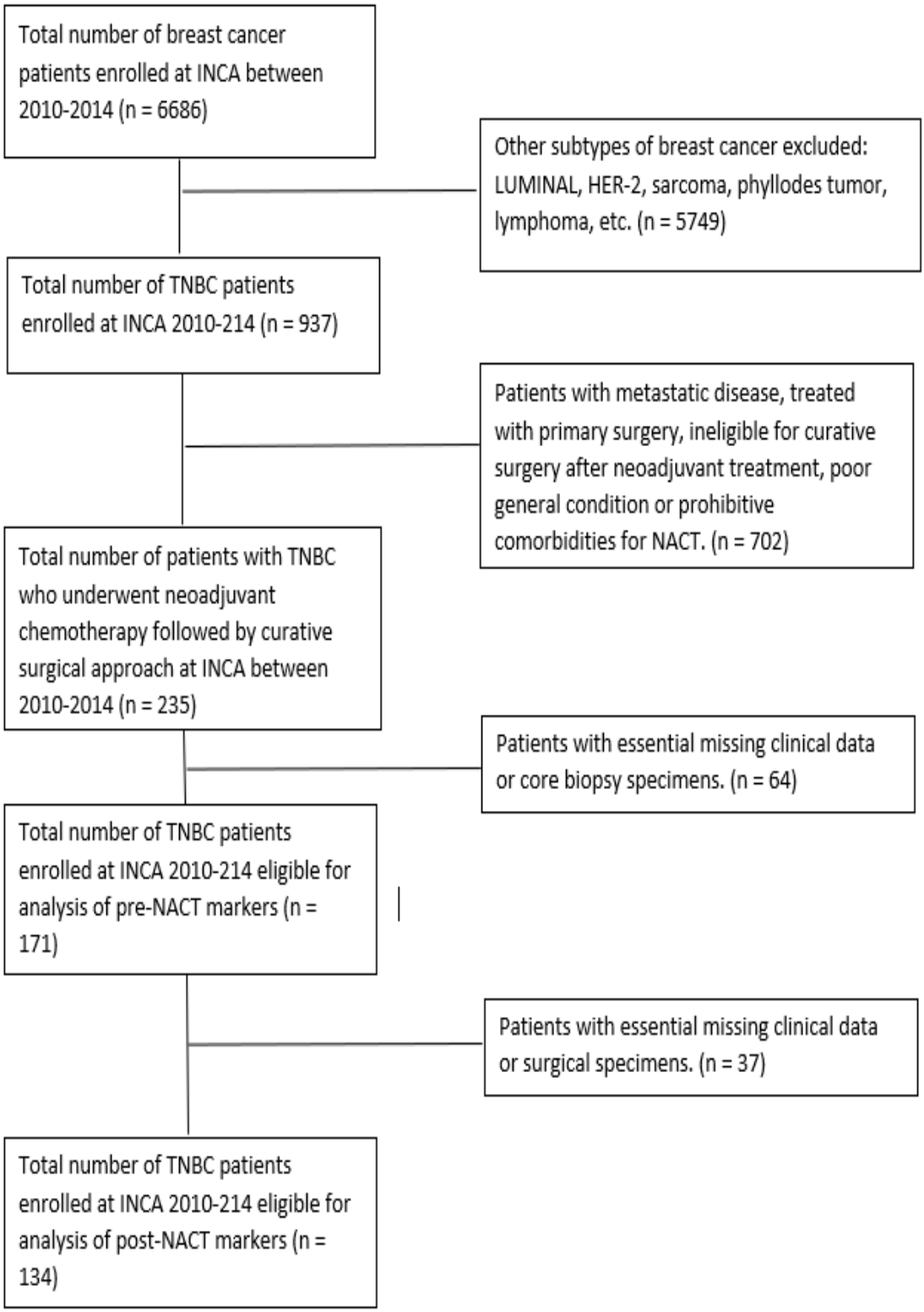
Pre-NACT clinicopathological features	Univariate analysis HR (95%CI)	p-value	Multivariate analysis HR (95%CI)	p-value
Age	0.99 (0.97 - 1.01)	0.285		
BMI	1.00 (0.96 - 1.03)	0.870		
Stage (III versus II)	**2.56 (1.18 - 5.57)**	**0.017**	**2.20(1.00 -4.86)**	**0.049**
CD3 (high versus low)	0.54 (0.26 - 1.13)	0.101	0.57 (0.26 - 1.29)	0.180
CD4 (high versus low)	2.28 (0.99 - 5.25)	0.053	2.51 (1.00 - 6.29)	0.050
CD8 (high versus low)	1.19 (0.76 - 1.87)	0.440		
CD14 (high versus low)	1.19 (0.63 - 2.25)	0.603		
CD68 (high versus low)	1.21 (0.77 - 1.89)	0.418		
CD117 (high versus low)	0.75 (0.36 - 1.57)	0.450		
FOXP3 (high versus low)	1.23 (0.78 - 1.92)	0.374		
PD-1 (high versus low)	1.41 (0.57 - 3.49)	0.462		
PD-L1 TPS (high versus low)	0.61 (0.36 - 1.03)	0.065	0.68 (0.39 - 1.17)	0.166
PD-L1 IC (high versus low)	1.48 (0.82 - 2.69)	0.197	1.63 (0.88 - 3.03)	0.118
PD-L1 CPS (positive versus negative)	0.81 (0.52 - 1.27)	0.364		
PD-L2 (high versus low)	1.14 (0.72 - 1.82)	0.575		
CD4/FOXP3 ratio (high versus low)	1.38 (0.88 - 2.18)	0.161		
CD8/FOXP3 ratio (high versus low)	1.31 (0.66 - 2.64)	0.442		
CD4/CD8 ratio (high versus low)	1.55 (0.82 - 2.93)	0.181		

CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; CD68: Cluster of Differentiation 68; CD117: Cluster of Differentiation 117; FOXP3: Forkhead Box P3; PD-1: Programmed Cell Death Protein 1; PD-L1 TPS: Programmed Death-Ligand 1 tumor proportion scores; PD-L1 IC: Programmed Death-Ligand 1 tumor infiltrating immune cells; PD-L1 CPS: Programmed Death-Ligand 1 combined positive score; PD-L2: Programmed Death-Ligand 2. Significant p-values are in bold.

Supplementary table 5. Pre-NACT clinicopathological features and Hazards Ratios for overall survival (OS) estimated by univariate analysis and multivariate analysis.

Pre-NACT clinicopathological features	Univariate analysis HR (95%CI)	p-value	Multivariate analysis HR (95%CI)	p-value
Age	1.01 (0.97 - 1.01)	0.241		
BMI	0.99 (0.95 - 1.03)	0.585		
Stage (III versus II)	**2.20 (1.01 - 4.97)**	**0.047**	2.03 (0.92 - 4.49)	0.080
CD3 (high versus low)	0.65 (0.31 - 1.36)	0.252		
CD4 (high versus low)	2.09 (0.91 - 4.84)	0.084	2.25 (0.90 - 5.62)	0.083
CD8 (high versus low)	1.09 (0.68 - 1.74)	0.732		
CD14 (high versus low)	1.21 (0.62 - 2.37)	0.578		
CD68 (high versus low)	1.14 (0.71 - 1.83)	0.599		
CD117 (high versus low)	0.92 (0.44 - 1.92)	0.821		
FOXP3 (high versus low)	1.31 (0.81 - 2.11)	0.268		
PD-1 (high versus low)	1.82 (0.73 - 4.55)	0.199		
PD-L1 TPS (high versus low)	0.67 (0.39 - 1.15)	0.144	0.67 (0.38 - 1.17)	0.161
PD-L1 IC (high versus low)	1.53 (0.82 - 2.85)	0.185	1.69 (0.90 - 3.20)	0.104
PD-L1 CPS (positive versus negative)	0.81 (0.50 - 1.30)	0.381		
PD-L2 (high versus low)	1.09 (0.66 - 1.79)	0.740		
CD4/FOXP3 ratio (high versus low)	1.19 (0.74 - 1.91)	0.476		
CD8/FOXP3 ratio (high versus low)	1.10 (0.55 - 2.22)	0.791		
CD4/CD8 ratio (high versus low)	1.23 (0.61 - 2.48)	0.567		

CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD14: Cluster of Differentiation 14; CD68: Cluster of Differentiation 68; CD117: Cluster of Differentiation 117; FOXP3: Forkhead Box P3; PD-1: Programmed Cell Death Protein 1; PD-L1 TPS: Programmed Death-Ligand 1 tumour proportion scores; PD-L1 IC: Programmed Death-Ligand 1 tumor infiltrating immune cells; PD-L1 CPS: Programmed Death-Ligand 1 combined positive score; PD-L2: Programmed Death-Ligand 2.
Significant p-values are in bold.



Supplementary figure 1. Flow chart of patient selection for the study

8 DISCUSSÃO

O presente estudo traz dados brasileiros envolvendo mulheres com CMTN localmente avançado submetidas à quimioterapia neoadjuvante. Pelos resultados encontrados no **artigo 1**, as 235 pacientes da coorte mostraram uma tendência a recidiva precoce, principalmente recidiva como metástase a distância. Os resultados mostraram que estádio clínico III e consumo de álcool estão associados com menor taxa de RPC e mediana de sobrevida mais curta. Entretanto, pacientes que conseguiram atingir RPC tiveram mediana de sobrevida consideravelmente mais longa.

Idade precoce ao diagnóstico e sobrepeso foram características presentes na maioria das pacientes do estudo, alinhando-se com dados mostrados em séries anteriores (PRAT *et al.*, 2010; MEI *et al.*, 2018). De modo similar, outras características de CMTN prevaleceram, como alto grau tumoral e doença avançada ao diagnóstico com envolvimento nodal, o que pode explicar, de certo modo, o fato de quase todas as pacientes terem sido submetidas a cirurgia radical (DENT *et al.*, 2007).

As taxas de consumo de álcool e de tabagismo foram bastante semelhantes a outras séries que incluíram mulheres com CMTN (GOU *et al.*, 2013; SCOCCIANTI *et al.*, 2014; BACCARO *et al.*, 2015; GOLDVASER *et al.*, 2017). Infelizmente, mais informações sobre a dose, duração e tipo de exposição ao álcool e ao tabaco não estavam disponíveis nos prontuários das pacientes para análise. Uma metanálise publicada em 2013 apontou que apenas uma ingestão mais regular e pesada de álcool, superior a 20 g/dia, estaria consistentemente associada ao aumento da mortalidade por câncer de mama e recorrência precoce (PARADA *et al.*, 2017). Aparentemente, pode haver alguma interferência do álcool na farmacocinética da quimioterapia, bem como implicações sociais que levem a uma menor adesão ao tratamento. Quanto ao tabagismo, não foi possível confirmá-lo neste estudo como fator preditivo para quimioterapia neoadjuvante, ou como fator prognóstico. Os dados da literatura são bastante controversos, com alguns resultados negativos contrastando com outros onde o tabagismo teve um impacto negativo na sobrevida. Curiosamente, parar de fumar após o diagnóstico de câncer de mama, pode reduzir o risco de mortalidade específica por câncer de mama (BILLE; SOLLIE, 2017).

Os dados de escolaridade foram semelhantes aos de outra coorte brasileira (TONELLOTTO *et al.*, 2019) e não mostraram associação com os desfechos de

resposta ou sobrevida. Uma coorte norueguesa (JACOBSEN; LUND, 1991) também não mostrou influência da escolaridade na sobrevida ou resposta. Por outro lado, outros resultados sugeriram que um maior nível de educação pode estar associado a uma sobrevida mais longa e melhor qualidade de vida (SHAHSAVARI *et al.*, 2015; LIU, Y. *et al.*, 2017). Quanto à avaliação do impacto da distância de casa ao centro de tratamento, não há muitos dados específicos na literatura. A grande variabilidade desse fator social entre os casos desta coorte pode explicar os resultados negativos.

O tempo médio de 90 dias do diagnóstico até o início do tratamento no presente estudo foi consideravelmente longo. Embora não tenha mostrado associação significativa com os desfechos de sobrevida nesta coorte, é altamente suspeito que atrasos no início da quimioterapia neoadjuvante possam influenciar negativamente os resultados do tratamento. Para evitar grandes atrasos no início do tratamento do câncer, o Governo Federal Brasileiro decretou a “Lei dos 60 dias” em 2012 (BRASIL, 2012). Tal lei foi instituída nacionalmente em 2013 e define o intervalo máximo que um paciente com câncer deve aguardar para iniciar o tratamento específico. No entanto, devido a problemas de infraestrutura do sistema público de saúde, essa meta ainda está longe de ser alcançada (PAULINO *et al.*, 2018).

Uma metanálise recente com análise individual de dados de pacientes (SPRING *et al.*, 2019) sugeriu que a RPC pode ser um potencial substituto dos desfechos de sobrevida. Na coorte atual, a taxa de RPC foi bastante semelhante à de outros estudos que usaram quimioterapia neoadjuvante a base de antraciclina-taxano. No entanto, essa taxa foi consideravelmente modesta quando comparada a estudos clínicos recentes, nos quais a RPC atingiu taxas com valores acima de 50%. Algumas razões possíveis para isso podem ser a nossa definição mais rigorosa de RPC (ypT0ypN0) e o número proporcionalmente maior de mulheres com tumores mais avançados no nosso estudo, bem como o uso de regimes de dose densa e a adição de novos medicamentos ao regime de quimioterapia neoadjuvante nos outros estudos, como inibidores de PARP, imunoterapia, e agentes antiangiogênicos (WU *et al.*, 2014).

A avaliação de marcadores tumorais por IHQ foi executada em amostras de core biópsia de 171 pacientes no **artigo 2**. Os resultados deste estudo sugeriram que não há associação dos biomarcadores IHQ testados com os resultados de resposta ou sobrevida. Entretanto, foi demonstrado que carga residual de câncer (RCB) ≥ 2 e razão de linfonodo (LNR) > 0,65 foram associados a piores resultados de sobrevida.

Em praticamente todos os subtipos de câncer de mama, a expressão mais alta de Ki67 foi associada a uma melhor resposta à quimioterapia neoadjuvante e, ao mesmo tempo, a um aumento do risco de recidiva. Baseado numa metanálise com 35 estudos e 7.716 pacientes incluídos (WU *et al.*, 2019), o cutoff de expressão de Ki-67 ≥40% foi fortemente sugerido como um fator de mau prognóstico em CMTN ressecado, estando associado a um maior risco de recorrência e morte quando comparado a escores mais baixos. Na presente coorte, mais da metade dos pacientes tinha Ki67 ≥40%, o que está de acordo com estudos anteriores (KEAM *et al.*, 2011; MATSUBARA *et al.*, 2014). Em contraste com outra série (WANG *et al.*, 2016), a expressão de Ki67, assim como a redução da expressão de Ki67 em mais de 20% após quimioterapia neoadjuvante, não influenciou a taxa de resposta ou a sobrevida.

Definido com base na marcação de imunohistoquímica para fatores como CK5/6, CK14, CK17 e/ou EGFR, o câncer de mama tipo *basal-like* pode englobar tumores positivos para um ou todos os quatro marcadores (NIELSEN *et al.*, 2004). Trata-se de um subtipo molecular mais agressivo pela classificação atual proposta por Lehmann et al (LEHMANN, B. D. *et al.*, 2016), sendo relatados piores resultados de sobrevida para esses tumores, embora uma taxa de RPC mais elevada (41%) tenha sido observada sugerindo maior sensibilidade a quimioterapia. Além disso, são tumores que parecem estar associados a maior índice mitótico/proliferação celular e menor diferenciação, o que provavelmente se deve ao maior nível de neovascularização causado pela superexpressão do fator de crescimento endotelial vascular (VEGF). Estima-se que aproximadamente 75–80% dos casos de CMTN sejam do subtipo de câncer de mama tipo basal-like (BADOWSKA-KOZAKIEWICZ; BUDZIK, 2016).

Nenhum dos marcadores analisados mostrou influência significativa nos desfechos resposta ou sobrevida. Não foi possível realizar a análise agrupada para tentar correlacionar com os subtipos moleculares de Lehmann devido à distribuição assimétrica da amostra, bem como à falta de testes moleculares em paralelo. A expressão das citoqueratinas foi bastante variável no presente estudo. Para CK5/6 (16%) e CK14 (10,1%), havia poucos pacientes com expressão positiva, enquanto para CK17 (91,1%) a maioria dos pacientes teve expressão positiva, o que pode sugerir um perfil genômico diferente do CMTN na população brasileira quando comparada a outras populações. Essa variabilidade também pode ser atribuída a problemas técnicos, pois o escore manual pode ter sido prejudicado pela reatividade

frequentemente fraca e focal, assim como heterogeneidade intratumoral. A expressão de CK5/6 por IHQ apresenta uma variabilidade significativa entre as séries anteriores de CMTN, variando de 24% a 72% (RYU *et al.*, 2011). Os resultados de um estudo de coorte com pacientes com câncer de mama realizado por Van de Rijn et al (2002a) sugeriram que a expressão de CK 5/6 estava associada com desfechos clínicos piores em cânceres de mama com axila negativa, independentemente do tamanho e do grau tumoral. Outros autores também sugeriram que CK5/6 pode ser um marcador preditivo e prognóstico independente (ABD EL-REHIM *et al.*, 2004; NIELSEN *et al.*, 2004; INANC *et al.*, 2014).

No geral, a expressão de CK14 e CK17 se sobrepõe à de CK5/6. Em outro estudo brasileiro de CMTN com critérios de inclusão menos rígidos, a prevalência de casos CK14+ foi de 26% (BROT et al., 2009).. No estudo realizado no *Memorial Sloan-Kettering Cancer Center* em pacientes com CMTN previamente ressecado, casos positivos de CK14 corresponderam a 46% da amostra e, ao contrário do estudo atual, foram associados a pior SLD (p = 0,003, SLD em 5 anos 69% versus 83%) e SG (p = 0,01, SG em 5 anos 71% versus 85%). Os dados da literatura sobre a prevalência de CK17 em pacientes com CMTN são muito escassos. Algumas análises combinadas com outras citoqueratinas mostraram resultados controversos em relação ao papel prognóstico desse marcador (VAN DE RIJN *et al.*, 2002ª; SABLE *et al.*, 2017).

Outro marcador aparentemente menos específico que CK5/6 usado para definir o subtipo *basal-like*, é o EGFR (LEHMANN *et al.*, 2011). A maior prevalência de pacientes com imunorreatividade ao EGFR no presente estudo (92,9%) pode explicar a falta de associação com os desfechos de resposta e de sobrevida. Existe também uma grande variabilidade na prevalência de superexpressão de EGFR em casos de CMTN entre as séries previamente publicadas, variando de 13% a 78%, devido principalmente à falta de métodos uniformes para os escores de IHQ e a variações demográficas (GLUZ *et al.*, 2009; GUMUSKAYA *et al.*, 2010). Alguns dados anteriores também sugerem que um número maior de cópias do gene EGFR pode estar associado com tumores menos diferenciados, comprometimento nodal axilar e pior sobrevida (PARK *et al.*, 2014). No entanto, os dados da superexpressão da proteína EGFR em CMTN são controversos e aparentemente não tiveram relevância clínica comprovada (LIU *et al.*, 2012; NAKAJIMA *et al.*, 2014).

Sem impacto significativo nos desfechos de resposta ou sobrevida, a menor prevalência de RA na presente coorte (13%) pode representar uma característica

demográfica regional. De acordo com relatos anteriores, há uma variabilidade considerável na expressão imunohistoquímica de RA no CMTN, variando entre 10% e 90% (NIEMEIER *et al.*, 2010; HE *et al.*, 2012). Duas metanálises avaliaram o papel prognóstico da RA em casos com CMTN. A primeira avaliou dados agrupados de 13 ensaios clínicos que recrutaram um total de 2.826 pacientes com CMTN de 2007 a 2015. Neste estudo, 24,4% dos casos eram RA+, estando esse grupo associado a tumores de baixo grau (40,8% dos pacientes RA+), a status pós-menopausal (26,9% das pacientes RA+) e a menor risco de envolvimento nodal (28,8% das pacientes RA+) (QU *et al.*, 2013). Na segunda metanálise, agrupando um total de 521 pacientes com CMTN, houve redução de 36% do risco de recidiva da doença (HR 0,44; p = 0,002) (KIM; JAE; YOON, 2015b). No entanto, é importante mencionar que não houve associação do status de RA com o desfecho de SG em nenhum desses estudos.

Como marcador preditivo, algumas evidências sugerem que pacientes com RA+ têm maior probabilidade de terem tumores resistentes a quimioterapia do que pacientes com RA-. A análise de 637 amostras de biópsia de tumores primários de pacientes inscritos no estudo Gepartrio mostrou que o a taxa de RPC foi de 12,8% nos pacientes com câncer de mama RA+ em comparação com a taxa de RPC de 25,4% nos RA- (p < 0,0001) (HILBORN *et al.*, 2016). Resultados semelhantes foram observados numa coorte retrospectiva japonesa, em que pacientes com CMTN RA+ tiveram uma menor taxa de RPC quando comparados a pacientes com CMTN RA- (HR 5,26; IC 95% 1,39-19,86, p = 0,014) (ASANO *et al.*, 2016).

Representando mais da metade dos pacientes testados no presente estudo, os pacientes com expressão de p53 não apresentaram associação significativa com resposta a quimioterapia neoadjuvante ou com sobrevida. De forma similar, um grande estudo retrospectivo sul-coreano de 11.393 pacientes com CMTN falhou em mostrar qualquer associação de p53 com resultados de sobrevida ou resposta à quimioterapia (BAE *et al.*, 2018). Outros estudos que mostraram algum efeito da expressão do p53 na resposta à quimioterapia ou na sobrevida foram limitados por um pequeno número de pacientes ou pela heterogeneidade. Outra limitação importante desses estudos é que a técnica de IHQ e o cutoff selecionado diferem em cada ensaio (COATES *et al.*, 2012; FERNÁNDEZ-CUESTA *et al.*, 2012; LARA *et al.*, 2011).

De forma geral, a proteína mutante P53 é mais difícil de se decompor, sendo detectadas por imunohistoquímica. Apesar de dados de alguns estudos sugerirem o

contrário, conceitualmente, a detecção dessa proteína por IHQ não está diretamente ligada a mutação do gene *p53*. Alguns dados de literatura mostram que os tumores p53-mutantes respondem melhor à quimioterapia do que os tumores p53-selvagens, enquanto outros estudos sugerem um impacto negativo da mutação na sobrevida (BLAGOSKLONNY, 1997; THOR *et al.*, 1992; PETITJEAN *et al.*, 2007).

No cenário de CMTN, a influência do microambiente tumoral na proliferação de células cancerosas, bem como em resposta a drogas anticâncer, tem sido cada vez mais reconhecida ao longo do tempo. Adicionalmente, um conjunto crescente de evidências apontou que os subtipos de células linfomononucleares presentes no microambiente tumoral são fatores fundamentais nos processos de invasão e crescimento tumoral. Essa associação foi bastante explorada no **artigo 3**. O presente estudo é uma das poucas séries a realizar uma avaliação minuciosa conjunta do subtipo de LITs em amostras de core biópsia e de tumores residuais pós-quimioterapia neoadjuvante de mulheres com CMTN. Os principais resultados sugeriram que a alta expressão de alguns marcadores neste cenário poderia influenciar eventos de recorrência de doença e óbito.

Os marcadores imunológicos de IHQ avaliados na core biópsia no presente estudo não mostraram associação significativa com os desfechos de resposta ou sobrevida. Por outro lado, uma metanálise realizada por Gao et al., (2020) com 37 estudos envolvendo pacientes com CMTN mostrou que a alta expressão dos LITs pode predizer melhor SLD e SG, com Hazard Ratios (HR) agrupados de 0,66 (IC95%, 0,57-0,76) e 0,58 (IC95%, 0,48-0,71), respectivamente, de acordo com a expressão de LITs (alto versus baixo). Especificamente, o subgrupo CD4+ LITs (alto versus baixo) apresentou um perfil melhor de SG (HR 0,49, IC 95% 0,32-0,76) e SLD (HR 0,54, IC 95% 0,36-0,80). Por outro lado, o subgrupo CD8 + LITs (alta versus baixa) apresentou melhor SLD apenas (HR 0,55, IC 95% 0,38-0,81) sem impacto em SG. O subgrupo FOXP3+ LITs (alta versus baixa) também apresentou melhor SLD (HR 0,50, IC 95% 0,33-0,75), sem associação significativa com SG (HR 1,28, IC 95% 0,24-6,88). Numa coorte com 150 pacientes com câncer de mama realizada por Rathore et al., (2014a), os grupos com marcadores intratumorais altos como CD4+ (OR=3,85, IC 95%=3,28-16,71, $p < 0,001$), CD3+ (OR=2,70, IC 95= 1,76-8,30, $p = 0,001$) e CD8+ (OR=2,58, IC 95%=1,55-5,86, $p = 0,001$) apresentaram melhor sobrevida quando comparados às respectivas contrapartes com expressão mais baixas.

Em outra coorte que avaliou 175 pacientes com câncer de mama, embora o marcador CD56+ Natural Killer (NK)-LITs tenha se mostrado altamente expresso em 48,6% dos casos, Rathore et al., (2014b) sugeriram que, isoladamente, ele pode não ser suficiente para influenciar os resultados de sobrevida. Para explorar os macrófagos associados ao tumor (TAMs), Wang et al., (2016) avaliaram a expressão de CD68+ LITs em 48 amostras de CMTN, mostrando alta expressão em 71,4% dos casos. Pacientes com alta expressão de CD68 apresentaram maior expressão das citocinas inflamatórias interleucina 6 (IL-6) e quimiocina (C-C) ligante 5 (CCL-5) e menores taxas de sobrevida em comparação com o grupo de baixa expressão. Como marcador relacionado aos TAMs, a infiltração de células CD68+ pode estar positivamente relacionada à gravidade do tumor. Uma revisão sistemática realizada por Ni et al., (2019) avaliou a prevalência de TAMs em 1579 amostras de mulheres com câncer de mama não metastático através do reagente anti-CD68. Os dados revelaram que a alta densidade de CD68-TAMs estava significativamente associada a características clinicopatológicas ominosas, como metástase axilar nodal, Ki67 alto, alto grau histológico e negatividade do receptor hormonal ($p < 0,001$ para todas as comparações).

Mutações específicas de *TP53* do tipo *Missense* com perda da função proteica de P53 têm sido associadas ao aumento da expressão do CD117 em alguns tumores sólidos, inibindo a diferenciação celular, proliferação, adesão e apoptose (LASOTA; MIETTINEN, 2008). No entanto, os dados relativos ao impacto prognóstico do CD117 no cenário de CMTN são conflitantes. Kashiwagi et al., (2013) e Luo et al., (2018b) sugeriram que a proteína CD117 está associada a maior recidiva e a prognóstico ruim. Por outro lado, outros autores não conseguiram encontrar uma associação significativa entre CD117 e prognóstico no cenário de câncer de mama (MEDINGER *et al.*, 2010; JANSSON *et al.*, 2014).

O receptor de checkpoint imune PD-1 tem um papel crucial no processo de evasão imune durante a tumorigênese. Os dois ligantes, PD-L1 e PD-L2, possuem perfis de expressão distintos a depender do sítio tumoral (ISHIDA *et al.*, 1992b). Alguns estudos prévios avaliaram a influência dos marcadores do eixo PD-1/PD-L1/PD-L2 em sobrevida de pacientes com câncer de mama invasivo, mais especificamente CMTN, mostrando resultados pouco significativos. Mori et al., (2017) demonstraram que a interação entre LITs e PD-L1 pode estar associado com melhores desfechos de sobrevida. No estudo de Beckers et a., (2016b), embora o PD-

L1 estivesse associado a melhor prognóstico, a análise multivariada não mostrou um papel prognóstico independente no subgrupo de alto expressores. Esses resultados conflitantes podem ser explicados pelo uso de diferentes desfechos clínicos nos estudos, juntamente com regimes diversos de quimioterapia, métodos diversos de avaliação da expressão PD-L1, resultando em escores diferentes. Asano et al., (2018) sugeriram que pacientes com CMTN com baixa expressão de PD-1 e PD-L1 apresentavam maior taxa de RPC e mediana de SLD significativamente maior, sendo PD-L1 um fator prognóstico independente na análise multivariada.

Alguns estudos têm usado inibidores de checkpoint como um complemento à estratégia de tratamento neoadjuvante em pacientes com CMTN localmente avançado. Notavelmente, estudos iniciais como i-SPY 2 em Nanda *et al.,* (2020) e o KEYNOTE-173 em Schimid *et al.,* (2020) mostraram que a combinação de pembrolizumabe, um anticorpo monoclonal anti-PD-1, com a quimioterapia neoadjuvante padrão aumentou significativamente a taxa de RPC nas pacientes com CMTN em estadios mais iniciais. Uma análise interina do estudo de fase III KEYNOTE-522 mostrou uma taxa de RPC significativamente maior (64,8% versus 51,2%; $p < 0,001$) e melhor SLE (HR 0,63; IC 95% : 0,43 - 0,93) no grupo da combinação em comparação com o grupo que recebeu apenas quimioterapia neoadjuvante, independentemente do status PD-L1. A SLE manteve-se significativamente maior no grupo pembrolizumabe após um seguimento mediano de 15,5 meses (SCHMID, Peter *et al.*, 2020).

Os resultados ensaio clínico de fase III IMpassion031 sugeriram que a adição do anti-PD1 atezolizumabe à quimioterapia neoadjuvante (nab-paclitaxel semanal seguido de doxorrubicina e ciclofosfamida) aumentou a taxa de RPC geral de 41% para 58%, independente do status de PD-L1, sem associação com desfechos de sobrevida (MITTENDORF et al., 2020). Por outro lado, os resultados do estudo NeoTRIPaPDL1 não demonstraram um aumento estatisticamente significativo nas taxas de RPC com a adição de atezolizumabe à quimioterapia neoadjuvante (nab-paclitaxel e carboplatina administrados nos dias 1 e 8 a cada 21 dias por oito ciclos) (GIANNI et al., 2020). O aumento da taxa de RPC pela adição de anti-PDL1 durvalumabe no estudo de fase II Gepar Nuevo do German Breast Group não atingiu a estatística de significância (LOIBL et al., 2019).

Alguns dados sugerem que, além do efeito citotóxico, a eficácia da quimioterapia também pode ocorrer através da restauração da “imunovigilância”

induzindo a morte celular por mecanismos imunogênicos (GREEN *et al.*, 2009). Como mostrado nos resultados do presente estudo, alguns subtipos de LITs presentes no tumor residual como CD3, CD8 e CD4, bem como as razões CD4/FOXP3 e CD8/FOXP3, podem influenciar os desfechos de sobrevida. Uma pequena série com 25 pacientes com câncer de mama sugeriu a ativação de linfócitos com atração para o leito tumoral em 7 casos como efeito da quimioterapia neoadjuvante, podendo garantir um melhor prognóstico nesses casos (DEMARIA *et al.*, 2001).

Ladoire *et al.*, (LADOIRE *et al*., 2011) avaliaram amostras cirúrgicas de 111 pacientes com câncer de mama HER2 negativo, em que a alta expressão de CD8 e baixa expressão de FOXP3 após quimioterapia foram significativamente associadas à melhora de SLR (p = 0,02) e OS (p = 0,002). O estudo realizado por Dieci et al., (2014a), que avaliou LITs em amostras de tumor residual de pacientes com CMTN após quimioterapia neoadjuvante, sugeriu que o tratamento poderia converter grupos de baixa expressão em grupos de alta expressão de LITs, e que essa conversão poderia estar associada a uma taxa de SG de 5 anos mais longa. García-Martínez et al., (2014) identificaram um padrão específico de LITs nos tumores residuais de paciente com CMTN, marcados pela alta infiltração de CD3 e CD68, apresentando SLD mais curta. Este resultado pode ser parcialmente explicado pela infiltração predominante do CD68, um marcador da TAM que pode estar associado a piores desfechos de sobrevida.

Através de 110 amostras pareadas de core biópsia e peça cirúrgica, os resultados de um trabalho publicado recentemente por Park et al. (PARK et al., 2020) sugerem que a quimioterapia neoadjuvante em pacientes com câncer de mama localmente avançado podem induzir alterações dinâmicas no microambiente imunológico do tumor, assim como regulação positiva de assinaturas inflamatórias, que variam por subtipo e resposta tumoral. Os aumentos nas proporções de TILs e células T CD8+ em resposta a NAC estão independentemente associados à RPC.

Os pontos fortes desta coorte baseiam-se principalmente na análise aprofundada dos marcadores tumorais na core biópsia, bem como nos dados após quimioterapia neoadjuvante, apresentando as características do infiltrado linfomononuclear e o seu possível impacto na sobrevida. A população do estudo é homogênea, pois os critérios de inclusão foram muito rígidos. Além disso, todas as biópsias e amostras cirúrgicas foram duplamente verificadas por patologistas experientes e cegados. Por fim, foi realizada uma apresentação descritiva minuciosa

das variáveis clínico-patológicas e análises multivariadas, reforçando a validade interna dos resultados.

A principal limitação do presente estudo é seu desenho retrospectivo. Portanto, alguns fatores de confusão ausentes podem existir na análise. Como um estudo de centro único, algumas características regionais na população selecionada podem existir e os resultados podem ser influenciados por diferenças geográficas marcantes. A heterogeneidade intratumoral pode ter comprometido alguns resultados da análise TMA na core biópsia. Houve também muitas perdas devido à escassez de material no DIPAT/INCA, assim como artefatos de análise. Não foram realizados esquemas densos e nem adicionados novos agentes antineoplásicos já consolidados, seja o uso de platina no tratamento neoadjuvante, seja o tratamento adjuvante de manutenção com capecitabina, já que não são intervenções disponíveis na rotina institucional. O estadiamento inicial por imagem das pacientes pode ter sido comprometido pela disponibilidade do exame em um Centro do SUS. Além disso, não foi possível realizar nenhuma análise de perfil de expressão gênica com as amostras disponíveis.

9 CONSIDERAÇÕES FINAIS

Uma avaliação oportuna e detalhada dos fatores preditivos e prognósticos que possam influenciar desfechos de resposta e sobrevida de pacientes com CMTN localmente avançados submetidos à quimioterapia neoadjuvante foi realizada no presente estudo Características clínico-patológicas ao diagnóstico já consolidadas, como tamanho do tumor, status nodal axilar e estadiamento clínico foram reafirmadas no presente estudo como fatores preditivos e prognósticos. A RPC mostrou uma forte associação com melhores resultados de sobrevida, configurando-se como um substituto potencial para desfechos de sobrevida de longo prazo. Da mesma forma, RCB e LNR, no contexto de avaliação patológica de resposta pós-quimioterapia neoadjuvante, também influenciaram fortemente a sobrevida, podendo, portanto, ser considerados fatores prognósticos importantes e de baixo custo para direcionar a melhor abordagem adjuvante. O consumo alcoólico foi determinante social de menor taxa de resposta e pior sobrevida. Entretanto, estudos com melhor caracterização do tipo, tempo e dose de consumo de álcool são absolutamente necessários.

Acredita-se que o CMTN seja uma doença heterogênea que compreende subtipos com diversos comportamentos biológicos e desfechos clínicos. No presente

estudo, a prevalência dos marcadores tumorais de IHQ avaliados apresentou grande variabilidade em relação ao que é relatado em literatura em outras populações. Há dados muitos escassos e conflitantes de marcadores tumorais de IHQ no contexto específico de pacientes com CMTN localmente avançado submetidos a quimioterapia neoadjuvante.

A constatação de que a alta expressão de alguns subtipos de LITs pós-quimioterapia neoadjuvante poderia identificar subgrupos de pacientes com prognósticos diferentes abre caminho para melhor estratificação de risco e desenvolvimento de novas drogas-alvo, o que poderia resultar em mudanças na dinâmica de abordagem prática no tratamento adjuvante. Mais dados são necessários para desvendar os mecanismos subjacentes ao padrão de infiltração tumoral por LITs, bem como as mudanças desse padrão após a quimioterapia neoadjuvante. Isso pode facilitar o desenvolvimento de novas terapias imuno-direcionadas para o câncer de mama neste cenário, principalmente no cenário de CMTN.

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ANEXOS

Anexo A

Artigo publicado sobre a influência de biomarcadores em câncer de mama triplo-negativo (*Triple negative breast cancer: A thorough review of biomarkers*).

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Triple negative breast cancer: A thorough review of biomarkers

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ABSTRACT

Triple-negative breast cancer (TNBC) is defined as a type of breast cancer with lack of expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 protein. The tumorigenesis is not likely to be driven by hormonal or HER2 pathway. In comparison to other types of breast cancer, TNBC stands out for its aggressive behavior, more prone to early recurrence. Historically, TNBC has been considered a disease with poor response to molecular target therapy, requiring better validation of biomarkers. Recent issues related to tumor heterogeneity have been widely discussed suggesting the subdivision of TNBC into different molecular subtypes. Through a complete research on the main published trials databases and platforms of ongoing clinical studies, the current manuscript was carried out in order to present a critical view of the role of immunohistochemical and molecular biomarkers for the prognosis and response prediction of TNBC to traditional therapy and new molecular target agents.

1. Introduction

Within the spectrum of breast cancer, triple negative breast cancer (TNBC) is known as a type of breast cancer in which there is a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) (Ryu et al., 2011). According to Globocan, the estimated incidence of breast cancer for 2018 was 2,088,849 new cases worldwide, considered the most incident tumor among women, excluding non-melanoma skin cancer (Changavi et al., 2015; Bray et al., 2018).

More common among specific ethnicities, such as Latin, African and African American women, TNBC accounts for approximately 10%–15% of all breast cancers (Jo et al., 2009). These tumors are also characterized by aggressive behavior, with trend to early relapse and metastatic spread to the lung, liver and central nervous system, as well as poorer survival. At diagnosis, patients usually present with clinically positive axillary lymph node, larger primary tumor size, pushing borders and poorer Nottingham prognostic index, calculated using pathological criteria and used to determine prognosis following surgery for breast cancer (Dent et al., 2007).

Tumor heterogeneity of TNBC has been widely pointed as the reason for different clinical outcomes, with diverse response rates either to traditional treatments or to new targeted therapies, often leading to discrepant times of survival. Some authors (Prat et al., 2010; Sørlie et al., 2001; Burstein et al., 2015; Lehmann et al., 2011) have used genomic expression profile (GEP) assays for molecular characterization of TNBC subgroups, defining their "molecular fingerprints". As shown in Fig. 1, intersections may occur between the proposed subclassification groups.

The most comprehensive and used subclassification was proposed by Lehmann et al. Lehmann et al. (2011), through a division of TNBCs into 7 molecular subtypes: immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR), unstable (UNS) subtype and two basal-like subtypes (BL1 and BL2). Thereafter, a subclassification refinement was performed to be defined in only 4 groups (BL1, BL2, M and LAR) based on a retrospective analysis of some clinical trials dataset (Lehmann et al., 2016). Based on this cluster analysis from both GEP and some additional information of immunohistochemistry (IHC), this subclassification can be used as prognostic and predictive tool and determine specifications for proof of concept that involves discovery of new drugs and design of clinical trials with better patient selection for personalized treatment to

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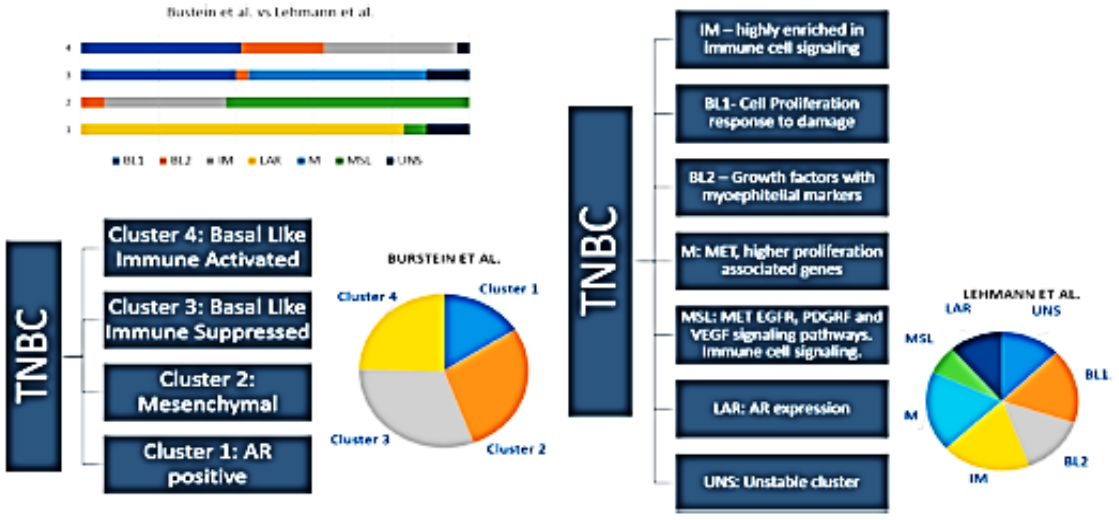

Fig. 1. Intersections of the subclassifications of Lehmann et al. and Burstein et al.
Lehmann et al. in 2011 proposed a division of TNBCs into 7 molecular subtypes: immunomodulatory (IM), mesenchymal (MI), mesenchymal stem-like (MSL): luminal androgen receptor (LAR), unstable (UNS) subtype and two basal-like subtypes (BL1 and BL2). in 2015: Burstein et al. used DNA profiling to identify TNBC subtypes: Cluster 1: luminal AR (LAR), cluster 2: mesenchymal (MES), cluster 3: basal-like immunosuppressed (BLIS), and cluster 4: basal-like immune-activated (BLIA). Comparing the two classifications, cluster 1 contains all of Lehmann's LAR tumors and cluster 2 contains most of Lehmann's mesenchymal stem—like. Lehmann's basal-like 1 and basal-like 2 tumors are split between clusters 3 and 4, mesenchymal tumors reside in cluster 3, whereas the immunomodulatory tumors are distributed across clusters 2 and 4: which express common signaling pathways]

improve response and survival outcomes.

Biomarkers are defined as reproducibly quantifiable biological variables. In clinical oncology practice, they can be measured as parameters to predict survival or even response to a therapeutic intervention, as defined by the National Institutes of Health (FDA-NIH Biomarker Working Group, 2016). When used in translational research discussions, they can also refer to factors used for early diagnosis, monitoring of treatment, as well as to provide "personalized" drug information. The integration of biomarkers into clinical practice depends on laboratory and clinical validations through well-designed clinical trials.

A complete search of relevant literature was undertaken to identify suitable published papers from peer-reviewed journals that assessed reliable predictive and prognostic biomarkers in TNBC. From immunohistochemical testing to attempts to genomic expression profiling, several recent studies involving TNBC patients have identified new biological tumor factors as potential biomarkers, some with promising results while others with conflicting data. The present paper provides a critical overview of the vast universe of these biomarkers and their subclassifications.

1.1. *TP53 gene, p53 protein and Ki-67*

The tumor protein 53 gene (*TP53*) is located on chromosome 17 (17p13.1) and encodes the p53 protein, a transcription factor that suppresses tumor growth and is essential in the process of cellular response to DNA damage. When DNA damage occurs, there is increase in p53 transcription, causing cell cycle arrest and DNA repair or cell death. This is effectively established by interaction with targets such as p21, cycling dependent kinase (CDK), repair proteins (PARP, BRCA) and PTEN (Girardini et al., 2011; Walerych et al., 2012), (Di Agostino et al., 2006).

Literature data suggest that *TP53* mutation is most frequently found in malignant tumors, occurring in approximately 30% of cases of breast cancer and in 75%–80% of TNBCs, according to the Cancer Genome Atlas project (Cancer Genome Atlas Network, 2012). The mutant p53 protein may exert aberrant functions, interacting differently with downstream targets, upregulating CDK-1 and PI3K/AKT/mTOR pathway, downregulating tumor suppressing proteins such as p63 and p73, stimulating cell proliferation and thereafter evading apoptosis (Turner et al., 2013). The p53 protein expression in TNBC tumor tissues may vary according to the type of mutation occurring in the *TP53* gene: patients with missense mutations tend to show high p53 protein expression, since they normally produce a more stable full-length protein, in contrast to patients with deletion mutations, which do not express the protein (Yemelyanova et al., 2011).

Several studies have attempted to determine the impact of *TP53* mutation on TNBC patient prognosis. In a study with 174 TNBC tumors harboring a *TP53* mutation, low mRNA expression was associated with poor prognosis in patients with missense mutation, 5-year distant recurrence-free survival (5-years-DRFS: low vs high, 50.0% vs 87.8%; p = 0.009), however no significant association was observed in patients with deletion mutations (Kim et al., 2016). Analyzing p53 protein expression, data from a retrospective Kenyan population study revealed that women with node-negative TNBC with p53 expression by IHC presented significantly higher overall survival (OS) than patients without p53 expression (p53+ vs. p53-; Hazard Ratio [HR] 2.8; 95% confidence interval [CI]: 1.1–7.1, p = 0.022) (Breast cancer, 2016).

As an option for personalized therapies, although formerly p53 has been regarded as "undruggable", recent studies have emerged with compounds that can selectively target the p53 mutant protein and restore its wild-type properties in breast cancer cells, such as PRIMA-1 and PRIMA-1^{Met} (p53 reactivation and induction of massive apoptosis 1) (Synnott et al., 2017). As PRIMA-1 and PRIMA-1^{Met} compounds have shown anticancer activity in TNBC cell lines of animal models and have not demonstrated evidence of major toxicities in a dose-finding phase I trial (Lehmann et al., 2012), they may be considered an attractive p53-targeted therapy for evaluation in larger clinical studies of patients with TNBC.

Over the years, research has developed several molecular techniques to measure cell proliferation rates. One of which is the quantification of proliferation-related membrane antigens by IHC. Ki-67, a protein encoded by the *MKI67* gene (marker of proliferation Ki-67 gene), is the most commonly used cell membrane antigen to determine cell proliferation, and is therefore considered a prognostic biomarker in breast cancer, although with an uncertain value in the context of

patients with TNBC (Viale et al., 2008; Blows et al., 2010). These tumors tend to have increased expression of Ki-67, with studies showing prevalence of 44.7%–53.4% of tumors with Ki-67 expression over 20% (Nakagawa et al., 2011).

Recently, results from a retrospective cohort that evaluated 363 women with operable early TBNCs through multivariate analysis have suggested that higher Ki-67 score is an independent risk factor for disease-free survival (DFS; RR 2.83, 95% CI: 1.58–5.06, $p < 0.001$) and OS (RR 3.18, 95% CI: 1.48–6.79, $p = 0.003$) (Wang et al., 2016a). In another cohort, Ki-67 was significantly correlated with the TNBC phenotype. The mean value was 44.7% and 22.2% in TNBC and non-TNBC patients, respectively, and higher scores were also associated with advanced stage ($p = 0.004$) and nodal involvement ($p = 0.033$) (Ilie et al., 2018).

1.2. – Epidermal growth factor receptor, c-KIT and cytokeratins (CK 5/6, CK 14, CK 17, CK 56)

Epidermal growth factor receptor (EGFR) is part of a transmembrane glycoprotein family with a tyrosine kinase domain that activate signal transduction pathways, playing an important role in cell proliferation and apoptosis inhibition (Lehmann et al., 2011). The prevalence of EGFR overexpression in TNBC cases is quite variable among studies, ranging from 13 to 78% (Gluz et al., 2009; Gumuskaya et al., 2010), due to lack of standardized measurement of IHC results and to a wide demographic variation. Although some results suggest a strong association of higher *EGFR* gene copy number with poor survival, higher tumor grade and axillary lymph node metastasis, data from EGFR protein overexpression in triple-negative is controversial and it has not been confirmed as a prognostic biomarker (Park et al., 2014; Nakajima and Ishikawa, 2012; Liu et al., 2012).

In a cohort with 287 patients with TNBC, 36.2% were positive for EGFR, and multivariate analysis indicated that EGFR was a significant independent prognostic factor in terms of DFS ($p = 0.011$) with poorer outcomes in all patients (Liu et al., 2012). Although EGFR overexpression is common in metastatic TNBC, phase 2 studies evaluating the efficacy of EGFR inhibitors such as tyrosine kinase inhibitors (TKIs, gefitinib, afatinib and erlotinib) and monoclonal antibodies (cetuximab and panitumumab) have not shown any effective results (Carey et al., 2012; Albanell et al., 2019; Dickler et al., 2009).

Signaling by c-KIT (CD117) is likely to play an important role in cell transformation and differentiation. The c-KIT protein expression is detected in approximately half of TNBC tumor tissues (Jansson et al., 2014). The aberrant activation of the *c-KIT* gene is part of the process of carcinogenesis and metastatic mechanisms of various human malignancies. Studying hyperactivation and alterations of the c-KIT pathway is highly interesting because it is potentially amenable to TKI treatment with imatinib, which is already traditionally used in the treatment of some onco-hematological diseases such as chronic myeloid leukemia as well as in solid tumors such as Gastrointestinal Stromal Tumors (GISTs) and dermatofibrosarcoma protuberans (Shams and Shams). In a retrospective study of 58 patients with TNBCs, the positive status of c-KIT by IHC was associated with *TP53* missense mutations ($p = 0.031$), vascular invasion, recurrence and higher Ki-67 proliferation index. The presence of c-KIT protein expression and *TP53* missense mutations together in the primary tumors was an independent prognostic factor for worse survival (Luo et al., 2019). In another cohort with 653 TNBCs, c-KIT positive staining was accompanied by decreased OS ($p = 0.036$) (Thike et al., 2010).

Basal like breast cancer is considered an aggressive subtype of TNBC and usually express basal cytokeratins like CK 5/6, CK 14 and CK 17 (Cheang et al., 2008). CK 5/6 expression ranges from 24% to 72% in TNBC (Ryu et al., 2011; Gokoz et al., 2010). Some results suggested that CK 5/6 positive TNBC have poorer prognosis independent of well-known clinical-pathological features (Nielsen et al., 1989; van de Rijn et al., 2002). Other results reported that CK 5/6 also have a positive correlation with nodal metastasis and tumor size (Inanc et al., 2014). On the other hand, a cohort with 150 patients failed to determine a correlation of CK 5/6 expression with ominous clinicopathological features, probably due to the low expression of CK 5/6 in that sample (Hashmi et al., 2018). Overall, the expression of CK 14 and CK 17 overlaps with CK 5/6. The CK 17 alone was not identified as a reliable prognostic biomarker42]. In a cross-sectional study of 150 patients with TNBC, CK 14 expression was positive in 50.8% of the cases(Lesar et al., 2016), whereas in a study with Asian women it was around 39.6% (YANXI et al., 2016).

1.3. - Vascular endothelial growth factor (VEGF)

The signaling of angiogenesis, mediated by vascular endothelial growth factor (VEGF), is crucial in the process of growth and tumor spreading. VEGF comprises a family of 6 proteins: VEGF-A, B, C, D, E and placental growth factor. The alternative splicing of mRNA creates 4 isoforms, the most common being VEGF165. The mediators of gene expression are hypoxia, growth factor, nitric oxide, oncogenes, HER2 and tumor suppressor genes (Holmes and Zachary, 2005; Gerwins et al., 2000). VEGF is highly expressed in around 30–60% of patients with TNBC (Linderholm et al., 2009). Along with VEGF, the scoring of microvascular density by IHC has been used as a prognostic biomarker in TNBC. Conceptually, a high mean vascular density in breast cancer has been associated with dismal prognosis and worsening survival (Chanana et al., 2014; Ali et al., 2011).

In a retrospective cohort evaluating Swedish women with operable breast cancer, the mean serum VEGF level of 87 patients with TNBC was statistically higher than that of non-TNBC patients. Comparing the two groups, 62% of patients with TNBC had a VEGF expression higher than the median value, while only 47% non-TNBC patients had a higher expression of the biomarker ($p = 0.036$) (Linderholm et al., 2009). Likewise, in a small clinical trial, 60 patients with metastatic breast cancer were treated with a low-dose metronomic therapy with capecitabine and cyclophosphamide. In the patients with partial and completed response, the VEGF levels was declined after 2 and 6 months of treatment ($p = 0.001$) (El-Arab et al., 2012). In another study that recruited 41 Egyptian women with metastatic TNBC, the VEGF-A presented higher level in patients with larger tumors compared to those with smaller ones ($p = 0.053$), as well as in cases of progressive disease compared to those who had partial response or stable disease ($p = 0.016$) (Taha et al., 2009).

VEGF has been widely studied as a target for treatment of TNBC in several studies evaluating efficacy of bevacizumab, the humanized monoclonal antibody of VEGF-A. Among 663 patients with triple-negative tumors enrolled in a clinical trial, the addition of bevacizumab to neoadjuvant chemotherapy significantly increased the rate of pathological complete response (pCR) from 27.9%–39.3% ($p = 0.003$) (Bucherini et al., 2012). Likewise, 46 patients with metastatic TNBC enrolled in a phase 2 trial had overall response rate (ORR) of 65.2% (95% CI: 52.9 %–80.4 %) (Saloustros et al., 2018). Despite showing increased progression-free survival (PFS), three phase 3 studies (Miller et al., 2007; Pivot et al., 2010; Robert et al., 2011) failed to show a statistically significant improvement in OS. Also, regarding adjuvant setting, bevacizumab did not show any benefit in survival outcomes (Cameron et al., 2013). Other therapies with monoclonal antibodies or anti-VEGF TKIs have not demonstrated benefit in this setting (Ribatti et al., 2016).

1.4. – Androgen receptor

Androgen receptor (AR) is part of a complex of steroidal hormone receptors that modulate transcription factors, controlling gene expression in different cellular processes, sometimes in a dualistic way. AR can both stimulate proliferation and dedifferentiation and induce apoptosis and cell death, depending on the simultaneously activated

signaling pathways. Although early studies have suggested a negative prognostic effect of AR in TNBC, the latest data have reaffirmed that patients with AR-positive TBNC have a more favorable outcome. The immunohistochemical expression of AR in TNBC may vary widely between 10–90 % according to the cohort (Niemeier et al., 2010; He et al., 2012; Galal et al., 2013; Gucalp and Traina, 2016).

A large systematic review with meta-analysis assessed pooled data from 13 clinical trials that recruited 2826 patients with TNBC from 2007 to 2015. Herein, 24.4% of the cases were AR-positive, and it was associated with low tumor grade (40.8% of patients AR-positive), and post-menopausal status (26.9% of AR-positive patients) and lower risk of nodal involvement (28.8% of AR-positive patients) (Wang et al., 2016b). Likewise, other data suggested that patients with more aggressive TNBC were negative for AR, whereas cases with higher AR expression were associated with early-clinical staging disease, low Ki-67 and low nuclear grade (Gasparini et al., 2014; McNamara et al., 2013; Maeda et al., 2016). As for the pooled analysis of 12 studies grouping 5270 women with TNBC, AR-positive group had 48% reduction of risk of progression or death compared to AR-negative patients (DFS HR 0.52; 95% CI: 0.43-0.64) (Qu et al., 2013). Similarly, in other meta-analysis with 521 TNBC patients, the odds ratio for DFS was 0.44 ($p = 0.002$) (Kim et al., 2015). However, there was no correlation of AR status with OS outcome in any of these studies.

Regarding the predictive value of AR status in TNBC, there are some evidence that AR-positive patients are more likely to be chemo-resistant than AR-negative patients. Analysis of 637 core biopsy samples from primary tumors of patients enrolled in Gepartrio trial showed that pCR was 12.8% in AR-positive breast cancer compared to 25.4% in AR-negative ones ($p < 0.0001$) (Hilborn et al., 2016). Similar results were observed in a Japanese retrospective cohort, in which AR-positive TNBCs presented lower rate of pCR than AR-negative in a univariate analysis (HR 5.26; 95% CI: 1.39–19.86, $p = 0.014$) (Asano et al., 2016).

Some clinical trials have evaluated the efficacy and safety of anti-androgens in patients with advanced or metastatic TNBC. A single-arm study with bicalutamide as monotherapy in heavily treated patients showed a clinical benefit rate (CBR) of 19% with a median PFS of 12 weeks (range 6.25–57.5 months) (Gucalp and Traina, 2016). Furthermore, a phase 2 trial with patients treated with single-agent enzalutamide AR-positive TNBC showed a CBR at 16 and 24 weeks of 35% and 29%, respectively (Traina et al., 2018). And finally, in a phase 2 study with monotherapy abiraterone, the CBR was 20% and median PFS was 2.8 months with manageable adverse events (Bonnefoi et al., 2016).

1.5. - Homologous recombination deficiency (HRD) and BRCA 1/2 mutations

All cells in the human body undergo constant external aggressions to the DNA apparatus. However, they rely on efficient DNA damage response (DDR) machinery. Double-strands breaks are severe forms of damage and are repaired by two main pathways: error-free homologous recombination and nonhomologous end-joining (NHEJ) (Jasin and Rothstein, 2013; Anon, 2019a). Initially described in patients with *BRCA1 and BRCA2* gene mutations, homologous recombination deficiency (HRD) can occur in sporadic cancers through genetic and epigenetic inactivation of other components (PALB2, BARD1, BRIP1, RAD51B, RAD51C, RAD51D, ATM, FAAP20, CHEK2, FAN1, FANCE, FANCM, and POLQ), a condition defined as "BRCAness" (Lord and Ashworth, 2016). Homologous recombination-deficient (HRd) tumors is likely to be more sensitive to platinum chemotherapy as well as to inhibitors of the DNA repair enzyme poly-ADP ribose polymerase 1 (PARP1) (Tan et al., 2008; Underhill et al., 2011).

Germline *BRCA 1/2* mutations are present in approximately 14–20 % of TNBCs, but a larger proportion of patients have been reported to harbor HRD (Couch et al., 2015; Sharma et al., 2014; Telli et al., 2016). In a retrospective study of 45 patients with TNBC, among *BRCA1/2* wild-type patients, HRd patients were more likely to achieve pCR (OR 16; 95% CI: 1.65–160.41, $p = 0.0041$) compared with non-HRd patients (Telli et al., 2018). Likewise, in a cohort of 77 patients with TNBC, 19.5% were *BRCA* mutated (*BRCA*m), and the 5-year recurrence-free survival (RFS) estimates were 51.7% for *BRCA* wild-type (*BRCA*wt) versus 86.2% for *BRCA*m, ($p = 0.031$) and 5-year OS estimates were 52.8% and 73.3% ($p = 0.225$) for *BRCA*wt and *BRCA*m, respectively (Gonzalez-Angulo et al., 2011).

A phase 3 study evaluated the role of the addition of the PARP inhibitor veliparib plus carboplatin to standard neoadjuvant chemotherapy in patients with TNBC. The pCR rate was significantly higher in the paclitaxel, carboplatin, and veliparib group than in patients receiving paclitaxel alone (53% of 316 patients vs 31% of 158, $p < 00,001$), but not when compared with the population that receiving paclitaxel plus carboplatin without the PARP inhibitor (58% of 160 patients, $p = 0,36$). (Loibl et al., 2018). Recently, a phase 3 trial compared olaparib monotherapy with standard therapy in patients with germline *BRCA*m HER2-negative metastatic breast cancer previously treated with two or more chemotherapy regimens. Of the 150 patients with TNBC, the HR for PFS was 0.43 (95% CI: 0.29-0.63) in favor of olaparib monotherapy (Robson et al., 2017).

In addition to deleterious mutations of BRCA and HRD profile, the genomic instability has also been assessed by techniques such as loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST) (Abkevich et al., 2012; Birkbak et al., 2012; Sastre-Garau et al., 2012). A phase 2 trial evaluated the efficacy of gemcitabine, carboplatin, and iniparib as neoadjuvant therapy for BRCAm patients with TNBC. The overall pCR rate in the intention-to-treat population ($n = 80$) was 36% (90% CI: 27–46) and the mean HRD-LOH scores were higher in responders compared with non-responders ($p = 0.02$) (Telli et al., 2015).

1.6. - Tumor-infiltrating lymphocytes and PD-L1/PD-1 expression

More recent studies have focused on the tumor microenvironment as a determinant of survival, invasiveness and metastasis in cases of TNBC. Normal breast tissue generally does not contain immune cells, but breast tumor tissue and surrounding stroma may contain higher levels of immune cell infiltrates (Degnim et al., 2014). There is growing evidence of the role of tumor lymphocytic immune infiltrates in TNBC. The immunoassay concept hypothesizes that host immunity, depending on peritumoral and intra-tumoral composition, can either stimulate tumor growth or eradicate the disease, grounding the definition of immune evasion and immunogenicity, respectively. Following this idea, tumor cells are initially rejected by the immune system, then remaining surviving tumor cells persist in a state of dormancy and, after upregulating pro-survival pathways, express molecules that promote immune suppression and angiogenesis. Herein, elimination, equilibrium and escape phases make up the three stages of immunoediting (Cancer Immunoediting, 2019).

CD4 and CD8 T-helper lymphocytes are part of the pro-inflammatory complex of type 1 immunity needed to eliminate tumor cells. Both innate immune system (neutrophils, monocytes, macrophages, antigen-presenting cells) and adaptive system cells (B and T-lymphocytes) are critical to prompt recognition and response to pathogens as well as non-self-cells or tumor antigens. Many antigens present in the cell membrane of breast cancer can activate and stimulate T-cells, inducing regulatory immune response. However, the ability of immune suppression is critical for the survival of normal cells (Society and Clinical, 2015).

Tumor-infiltrating lymphocytes (TILs) are highly expressed in approximately 20% of TNBC cases. Some studies have suggested that TILs in breast cancer may be a surrogate biomarker for adaptive immune response, especially for the TNBC subtype, considered to be one of the most immunogenic. There is a consensus that the cytotoxic effect of chemotherapy is partially influenced by the immune reaction against

tumor cells. In the same way, chemotherapy may provide a better immune response by modifying the microenvironment, as well as increasing tumor immunogenicity, leading to tumor shrinkage (DeNardo and Coussens, 2007; Schmidt et al., 2008).

In a neoadjuvant anthracycline and taxane-based studies, the pCR rates in lymphocyte-predominant breast cancer were greater than in tumors without any TILs, 42% and 3%, respectively (Denkert et al., 2010). Also in a meta-analysis that included neoadjuvant chemotherapy studies, TNBC with higher CD8+ and lower FoxP3+ T-lymphocyte levels was associated with better pCR rates (OR 2.49; 95% CI: 1.16–3.83) (Mao et al., 2014). For 278 cases of TNBC operated on with residual disease following neoadjuvant treatment evaluated in a multicenter restrictive cohort, the 5-year OS rate was 91% (95% CI: 68 %–97 %) for high-TIL patients (n = 27) and 55% (95% CI: 48 %–61 %) for low-TIL patients (HR 0.19; 95% CI: 0.06-0.61, p = 0.0017) (Dieci et al., 2014). Similarly, in a pooled analysis of the TNBC cases of two phase III randomized adjuvant breast cancer trials (ECOG 2197 and ECOG 1199), for every 10% increase in TILs, a 14% reduction of risk of recurrence or death (p = 0.02), 18% reduction of risk of distant recurrence (p = 0.04), and 19% reduction of risk of death (p = 0.01) were observed (Adams et al., 2014).

The anti-tumor activity of checkpoint immune inhibitors has been extensively studied in TNBC. Programmed cell death protein 1 (PD-1) is an immune checkpoint receptor that limits the action of T-cell effector within tissues, playing a crucial role in the tumor immune evasion process. The two ligands of PD-1, with distinct expression profiles in tumor types, are PD-L1 and PD-L2 (Ishida et al., 1992). The regulation of PD-L1 can occur by several processes: response to IFN-gamma action, oncogenic signaling, deletion or silencing of PTEN with consequent overexpression of the PI3K pathway (Castaneda et al., 2016a).

Immunotherapy with PD-1 and PD-L1 inhibitors results in the activation of T-cells, restoring the host's anti-tumor immune activity, demonstrating long-lasting activity and increased survival in selected tumors. Due to differences in the methods of detection, sampling and tumor size, the expression rate of PD-1/PD-L1 is variable in several studies (Ghebeh et al., 2006). Through analysis by IHC in patients with TNBC, PD-L1 have been reported between 15.8% and 30% of cases (Ghebeh et al., 2006; Castaneda et al., 2016b; Beckers et al., 2016). In this setting, PD-L1 scoring has been strongly used as a predictive biomarker and can be measured on tumor cells (TC) or on tumor infiltrating immune cells (IC). The in situ mRNA hybridization has been detected in PD-L1 mRNA in 55%–60 % of tissue microarrays (Schalper et al., 2014).

In a large retrospective cohort involving patients with breast cancer, PD-L1 upregulation, detected through mRNA analysis, was associated with poor prognostic features (large tumor size, ER-negative, PR-negative, HER2-positive status, high proliferation, basal and HER2-enriched subtypes), with a higher pCR (50% vs 21%) and a 5-year metastasis-free survival rate of 61% (95% CI: 0.58-0.64) (Sabatier et al., 2015). Similarly, another retrospective study showed a positive significant association between the PD-L1 ≥ 25% status and the achievement of pCR (p = 0.024) (Cerbelli et al., 2017).

In a phase 1 trial of patients with TNBC treated with single-agent atezolizumab, the overall response rate (ORR) for patients with PD-L1 ≥ 5% of IC (as also called IC2/3) were 17% versus 8% for PD-L1 < 5% (defined as IC0/1). The group with over 10% of TILs score or with more CD8 + cells in primary tumor was also more likely to have higher ORR and longer OS (Schmid et al., 2017). Recently, the combination atezolizumab and nab-paclitaxel was evaluated as first-line therapy for TNBC in a large phase 3 study. Among patients with PD-L1 positive tumors (defined as PD-L1 ≥ 1% on IC), the median PFS was 7.5 months vs 5 months in PD-L1 negative (HR 0.62; 95% CI: 0.49-0.78; p < 0.001) and the median OS was 25.5 and 15.5 months, respectively (HR 0.62; 95% CI: 0.45-0.86) (Emens et al., 2018; IMpassion130 Substudy, 20192019). Table 1 presents the main studies with TNBC immunotherapy.

Table 1
Summary of phase 3 trials of immunotherapy in TNBC.

Trial name	Line of therapy and population	Intervention	Control	Comments
IMpassion 130	First-line therapy - previous (neo)adjuvant chemotherapy allowed only if treatment was completed ≥ 12 months (mo) before randomization	Nab-paclitaxel plus atezolizumab	Nab-paclitaxel plus placebo	Published 2018 – median PFS ITT pts 7.2 mo vs 5.5 mo (HR 0.8, p = 0.002); in the PD-L1+ pts: 7.5 mo vs 5.0 mo (HR 0.62, p < 0.001). ASCO 2019 update median OS in PD-L1+ pts with atezolizumab + nP 25.0 mo vs placebo + nP 18.0 mo (HR 0.71, 95% CI: 0.54 - 0.93) (IMpassion130 Substudy, 2019; van Schoonenveld et al., 2015).
IMpassion 131	First Line treatment	Paclitaxel plus atezolizumab	Paclitaxel plus placebo	Estimated Completion date 2021. (NCT 03125902)
IMpassion 132	First Line early recurrence <12mo after curative treatment	Chemotherapy plus atezolizumab	Chemotherapy plus placebo	Estimated completion data 2021. (NCT 03371017)
KEYNOTE 119	Previously treated second or third line	Pembrolizumab	Physician's choice	Estimated completion data 2019. (NCT 02555657)
KEYNOTE 355	First Line	Pembrolizumab plus chemotherapy	Placebo plus Chemotherapy	Estimated completion data 2019. (NCT 02819518)

Table 2
Summary of prognostic biomarkers in triple negative breast cancer.

Molecular Biomarker	% of TNBC with expression/mutations	Main Function	Prognostic significance	Targeted therapies	References
TP53 gene	75-80% (somatic mutations)	Apoptosis	Low gene expression in TP53 missense mutations correlate with poor prognosis (worst DFS, but conflicting data).	NA	Kim et al., 2016; (Kim et al., 2016); Synnott et al., 2017; (Synnott et al., 2017)
KI-67	45-53% (high expression, > 20%)	Cell proliferation	High index and high expression correlate with shorter DFS and OS.	NA	Wang et al., 2016a,2016b;(Wang et al., 2016a) ; Ilie et al., 2018; (Ilie et al., 2018)
EGFR	13–78 %	Cellular growth	Increased expression associates with worst DFS.	Erlotinib, gefitinib, afatinib	Gumuskaya et al., 2016 (Gumuskaya et al., 2016); Gluz et al., 2009; (Porta et al., 2014)[10]
c-KIT	50%	Cell transformation and differentiation	Predictor of poor cancer-specific survival in patients with TNBC	Imatinib	Kashiwagi et al., 2013 (Sarlie et al., 2001); Jansson et al., 2014; (Jansson et al., 2014)
VEGF	32 % – 62 %	Angiogenesis	High levels associate with disease progression and metastasis rates.	Bevacizumab	Lindeholm et al., 2009; (Ali et al., 2011); Ali et al., 2011; (El-Arab et al., 2012)
Androgen receptor	10-55%	Cell proliferation and dedifferentiation	Positive expression correlates with higher DFS. May be associated with chemo-resistance.	Bicalutamide, enzalutamide, abiraterone	Niemeier et al., 2010; (He et al., 2012)[10]; He et al., 2012; (Gallo et al., 2013); Gucalp and Traina, 2016; (Wang et al., 2016b)
BRCA1 and *BRCA2* genes	14–20 % (germline mutations)	DNA-double strand break repair	Mutated status correlates with increased DFS.	PARP inhibitors - Olaparib	Gonzalez-Ángulo et al., 2012; (Loibl et al., 2018); Robson et al., 2019(Abkevich et al., 2012)
PD-L1 protein	15-30%	Tumor immune evasion process	High expression correlates with a higher survival rates in trials with checkpoint inhibitors	Immune Checkpoint Inhibitor - atezolizumab	Castaneda et al., 2016a,2016b (Ghebeh et al., 2006); Ghebeh et al., 2006; (Castaneda et al., 2016b); Beckers et al., 2016; (Schalper et al., 2014);
Notch pathway	~10%	Cell Proliferation and differentiation	Potential target under development	AL101	Speiser et al., 2013(Mathe et al., 2016); Broner et al., 2019; (Pineda et al., 2018)
PI3-kinase pathway	~25%	Cell Proliferation	Multiple genomic alterations lead to activated PI3-Kinase pathway, including activation in PIK3CA, AKT and mTOR or inactivation in tumor suppressor genes such as PTEN,	PI3K inhibitor - alpelisib AKT inhibitors – ipatasertib, capivasertib	Baselga, 2011 (Speiser et al., 2013); Pascual and Turner, 2019(Baselga, 2011); Kim et al., 2017 (Porta et al., 2014)

NA: not applicable; DFS: disease free survival; OS: overall survival.

1.7. – microRNAs and long non-coding RNAs

MicroRNA (miRNA; miR) expression signatures have been studied for both early diagnosis and treatment monitoring, through intense search for reliable early-stage blood biomarkers in TNBC. Defined as small noncoding RNA molecules of 17–27 nucleotides in length, miRNAs exhibit regulatory function in the expression of multiple genes through the stimulation or degradation of mRNA targets. Noncoding RNAs are functional RNA molecules that are transcribed from the DNA, but not classified as protein-coding due to the lack of the long open reading frames (ORFs), and these include the miRNAs, the small interfering RNAs (siRNAs), the piwi-interacting RNAs (piRNAs) and the long non-coding RNAs (lncRNAs). Due to its stability, miRNAs may be advantageously studied in non-invasive samples, such as blood, serum and urine (van Schooneveld et al., 2015).

Specific miRNAs could serve as potential prognostic biomarkers in TNBC. In a recent meta-analysis with 21 relevant studies, reduced expression of miR-155 and higher miR-21 expression was associated with poorer OS (crude HR 1.49; 95% CI: 1.26–1.72 and crude HR 2.50; 95% CI: 1.56–4.01, respectively) (Lü et al., 2017). A pilot study with blood-borne miRNA signatures from 21 basal-like TNBC cases treated with neoadjuvant therapy highlighted 321 miRNAs that were deregulated when comparing expressions pre and post-treatment (among them, miR-34a, with p-value <00,001), and also found that complete responders showed a tendency to have higher miRNA levels after platinum-based neoadjuvant chemotherapy, suggesting that changes in miRNA expression during treatment may have predictive value in pCR (Kahraman et al., 2018).

The miR-34 family members miR-34a, miR-34b and miR-34c have been among the most studied miRNAs in TNBC and have shown multiple roles as biomarkers (Malla et al., 2019). Mir-34a have been associated with attenuation of tumor growth in TNBC and miR-34c with worse prognosis (Adams et al., 2016; Anon, 2019b). As several studies have shown that miR-34a acts as a tumor suppressor, inducing cell cycle arrest, apoptosis and senescence in cancer cell lines, multiple authors have investigated its potential as a target for therapy and, recently, a liposomal miR-34a mimic formulation called MRX34 have been evaluated in patients with advanced solid tumors (Imani et al., 2018). By comprehensive analyses of miRNAs expression data from TCGA database, a study suggested that higher levels of miR-135b-5p, miR-9-3p and miR-135b-3p have favorable prognosis, despite high expression of miR- 455-5p exhibited poor prognosis in TNBC (Bao et al., 2019).

LncRNAs are transcripts with lengths exceeding 200 nucleotides that may not be translated into proteins. They modulate transcription of protein-coding genes by association with proteins to regulate their functions and control RNA maturation and transport (Zhang et al., 2017). Like miRNAs, they also perform regulatory functions in various hallmarks of cancer biology. LncRNAs are disordered in many cancer types, including TNBC (Matouk et al., 2009). Considered as presumed biomarkers, a set of lncRNA are pointed out in some recent studies as consistently aberrantly expressed in TNBC, and may be involved in both carcinogenic process and progression (Augoff et al., 2012).

A lncRNA, known as long non-coding RNA in non-homologous end joining pathway 1 (LINP1), was found to be overexpressed in TNBC. LINP1 enhances double-strand DNA break repair by serving as a scaffold to form a synaptic complex joining the broken DNA ends and promoting its repair. LINP1 blockade may therefore increase the sensitivity of TNBC to radiotherapy (Sakthianandeswaren et al., 2018). Another lncRNA, termed long intergenic noncoding RNA for regulator of reprogramming (lincRNA-RoR), is overexpressed in TNBC and serves as a competitive endogenous RNA for miR-145, a regulator of embryonic stem cell renewal, suggesting that lincRNA-RoR may be a critical factor for TNBC metastasis and could serve as a biomarkers or therapeutic target for TNBC therapy, as well as miR-145 (Eades et al., 2014). The lncRNA known as highly up-regulated in liver cancer (HULC) is also upregulated in TNBC tissues and cell lines and correlates with poorer clinical outcomes and might be a potential therapeutic target in TNBC (Shi et al., 2016).

The metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a highly conserved lncRNA, known to regulate gene expression by modulating transcription and posttranscriptional RNA processing. Recent pre-clinical studies have pointed out MALAT1 as a potential biomarker in TNBC, helping to predict prognosis and metastasis (Zhao et al., 2014). Long intergenic noncoding RNA for kinase activation (LINK-A) is critical for growth factor-induced normoxic HIF1α signaling pathway, also being a promising therapeutic target in TNBC (Lin et al., 2016). The HOX transcript antisense intergenic lncRNA (HOTAIR) has significant role in tumorigenesis with increased expression in TNBC. Combination treatment with lapatinib and imatinib repressed HOTAIR expression in TNBC cells through inhibition of β-catenin and inhibited cell growth (Wang et al., 2015).

1.8. Promising molecular biomarkers

Tumor heterogeneity, biopsy sampling, tissue processing and storage are serious limitations of the histopathological analysis by IHC that may impair the subtyping and even the diagnostic accuracy, leading up to poorer outcomes. Therefore, some molecular biomarkers have been explored to guide treatment and improve the approach of patients with TNBC (Andreopoulou et al., 2017; Srinivasan et al., 2002). Plasma circulating tumor DNA (ctDNA), which is obtained through "liquid

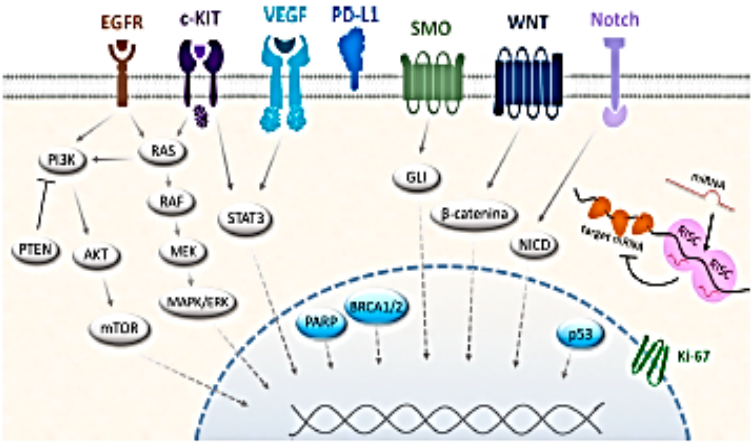

Fig. 2. Key signal transduction mechanisms in TNBC tumorigenesis.

biopsy" by blood sampling, provides genetic information not only from the primary tumor but also from the metastatic disease (Diaz and Bardelli, 2014). Besides being a non-invasive method, the ctDNA can potentially provide timely and comprehensively information, laying the groundwork for real-time disease monitoring, enabling more accurate prognostics evaluation and even early therapy modification (Olsson et al., 2015; Iqbal et al., 2015).

The prognostic value of ctDNA is still under evaluation in solid tumors and its validation in TNBC is urgently needed. In a small study of 36 TNBC patients using digital PCR for detection of *TP53* mutations by liquid biopsy, one patient with rising ctDNA levels experienced tumor progression during neoadjuvant chemotherapy (Riva et al., 2017). Next-generation sequencing was used in matched tumors in other study of 38 patients with early-stage TNBC. Patients with detectable ctDNA had poorer disease free survival (Chen et al., 2017). Other study was negative in proving the prognostic and predictive value of ctDNA for metastatic TNBC (J. M et al., 2015).

The prognostic utility of circulating tumor cells (CTC) for stratification of patients with stage metastatic disease was evaluated in the large multicenter cohort with 358 TNBCs. Patients with score <5 CTC/7.5 ml had longer median overall survival than those with score ≥ 5 CTC/7.5 ml (23.8 months vs. 9.0 months, $p < 0.0001$) (Cristofanilli et al., 2019). CTC resistant to anoikis, a type of apoptosis in which triggering occurs after the cell detaches from the native extracellular matrix, can predispose to metastasis, particularly in TNBC. Some studies point to the manipulation of some genes and molecules such as miRs may advantageously influence the sensitivity to anoikis (Tajbakhsh et al., 2019).

DNA methylation loci is another strong candidate to biomarker for TNBC, since they are more stable than RNA and is readily detectable in tissue samples and blood (How Kit et al., 2012). Many patterns of methylation have been assessed in different subtypes of breast cancer. In a small cohort of 39 patients, the presence of the BRCA1 promoter methylation was associated with poorer OS and RFS in TNBC (Sharma et al., 2014). Using whole genome DNA methylation analysis, another study proposed signatures that divided TNBC into three prognostic subgroups with different methylated regions (DMRs) (Stirzaker et al., 2015). Other cohort with 23 primary TNBC samples highlighted the important role that DNA methylation plays in the altered gene expression of TNBC-specific genes and lymph node metastases (Mathe et al., 2016). Using pre-treatment samples in an attempt to identify an epigenomic signature that could predict higher pCR rates after neoadjuvant chemotherapy in patients with TNBC, one study validated a hypermethylation pattern of 2 specific genes that accurately can predict response in TNBC (Pineda et al., 2018). However, further validation in prospective trials is warranted.

Notch signaling may be a potential target for the treatment of breast cancer. In the mammalian system, there are four Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4). Notch activates many genes associated with differentiation and/or survival, including, transcription factors, cyclin D1 and c-Myc. Notch-1 and Notch-4 expression has particularly been associated with the triple negative subtype of breast cancer (Speiser et al., 2013). This motivated the development of Notch inhibitors, including AL101, a pan-Notch gamma secretase inhibitor, with preclinical data with interesting results supporting the design of clinical trials of AL101 as a targeted therapy for TNBC with a hyperactivated Notch pathway (Broner et al., 2019).

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR)-dependent pathway is one of the most important pathways associated with cell metabolism, proliferation, differentiation, and survival. When deregulated, they play a crucial role during the tumorigenesis process, interacting with other signaling processes, also causing resistance to therapies. These pathways may be aberrantly expressed in breast cancer, and mutations may occur in up to 25% of cases (Baselga, 2011). Phosphatase and tensin homologate (PTEN), Inositol polyphosphate 4-phosphatase type II (INPP4B) and others phosphatases are key regulators of this pathway (Porta et al., 2014). Multiple genomic alterations can potentially lead up to hyperactivated PI3K pathway, including activating events in the oncogenes *PIK3CA*, *AKT* and *MTOR* or inactivating events in tumor suppressor genes such as PTEN (Pascual and Turner, 2019).

Different subtypes of TNBC have specific PI3K pathway mutations/alterations. For example, PIK3CA and AKT1 mutations are more likely to be found in androgen receptor-positive TNBC and INPP4B is frequently inactivated in basal-like TNBC[146(p)]. Some data have suggested that the AKT activation can be used as a potential predictive biomarker to AKT inhibitors. There is evidence from a phase 2 trial suggesting that this pathway may be successfully targeted in TNBC. The addition of ipatasertib, an AKT inhibitor, to paclitaxel as first-line therapy for metastatic TNBC improved progression-free survival over placebo (stratified HR 0.59; 95% CI: 0.26–1.32, $p = 0.018$) (Kim et al., 2017). Phase III trials with PI3K inhibitors are ongoing.

Fig. 2 summarizes the main signal transduction pathways that play a fundamental role in tumorigenesis processes as well as in the mechanisms of therapeutic resistance in cases of TNBC. And Table 2 shows the main biomarkers with their impacts on prognosis or survival.

2. Conclusion

Undoubtedly, recent advances have been made in understanding TNBC as a disease with intrinsic molecular and immunological heterogeneity, recognizing the variety of clinical phenotypes. This new scenario demands an urgent comprehensive subclassification that incorporate immune-molecular signatures for a more targeted and effective treatment. Although PARP inhibitors and checkpoint inhibitors have been recently incorporated in some settings, cytotoxic chemotherapy remains as the mainstay of therapy against TNBC, resulting in different outcomes for patients with similar clinicopathologic features.

The role of a more complete accessible panel of immunohistochemical biomarkers has improved decisions in the treatment of TNBC. Additionally, new biomarkers have been proposed to predict survival and response to chemotherapy in many cases, allowing for personalized approaches such as the need for dose escalation as well as incorporation of new antitumor agents into the standard regimen. On the other hand, more modern NGS-based biomarkers still need to be better validated through reliable prospective studies and to become more accessible to daily practice.

Formerly considered an unattainable disease by molecular therapy, the TNBC has recently been the center of successful investigations for the incorporation of new targeted therapy, due to improvements in response predictions. Considering the proposed subtypes with their molecular variations as defined by specific biomarkers, the incorporation of platinum agents, checkpoint inhibitors and PARP inhibitors, great advances have been achieved in both neoadjuvant treatment and in the metastatic disease approach.

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Anexo B

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REVIEW ARTICLE

Cancer immunotherapy: the art of targeting the tumor immune microenvironment

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Abstract
For many decades, cancer treatment has been strongly directed toward the development of cytotoxic and cytostatic drugs, quite often leading to disappointing results due to the inter- and intra-tumoral heterogeneity. Lately, this intra-cellular look has given way to the understanding of the tumor microenvironment, thus enabling modification of the immunological dynamics between tumor cells and their host. An era of new drugs aiming to unlock the host immune system against tumor cells is steadily increasing. Strategies involving adoptive cell therapy, therapeutic vaccines, immune checkpoint inhibitors and so on have provided spectacular clinical responses and increased survival in previously refractory settings and "hard-to-treat" cancers. Based on a comprehensive search in the main scientific databases, annals of recent renowned oncology congresses and platforms of ongoing trials, the clinical pharmacology characteristics of the main classes of immunotherapeutic agents, as well as the new treatment strategies related to immunotherapy in solid tumors, are carefully discussed throughout this review.

Keywords Immunotherapy · Immune microenvironment · Adoptive cell therapy · Therapeutic vaccines · Immune checkpoints inhibitors

Abbreviations

NK	Natural killers cells
ACT	Adoptive cell transfer therapy
CR	Complete response
CARs	Chimeric antigen receptors
TCR	Linked to T cell receptor
MHC	Major histocompatibility complex
TILs	Tumor-infiltrating lymphocytes
SD	Stable disease
EBViNT	EBV-induced natural T cell
APCs	Antigen-presenting cells
FDA	Food and drugs administration
HR	Hazard ratio
DC	Dendritic cell
RR	Response rate
CTLA-4	T lymphocyte-associated antigen 4
PD-1/PD-L1	Programmed cell death protein 1 pathway
iRAEs	Immune-related adverse events
irRC	Immune-related response criteria
PD	Progressive disease
OS	Overall survival
ITT	Intetion-to-treat
PR	Partial response
MSI-H	Microsatellite instability-high
dMMR	Mismatch repair deficient
IDO	Indoleamine 2,3 dioxygenase

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Introduction

Initially, in 1909 some scientists suggested that the immune system could have some role in cancer response [1]. Thereafter, in 1957 other colleagues postulated a new theory about cancer immunosurveillance indicating that the immune system could have the ability to perceive the abnormal cells, destroying them and consequently preventing tumor growth [2]. Unlike former cancer therapies that directly target malignant cells, immunotherapeutic agents stimulate the body's immune system to target and

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attack the tumor, which is otherwise invisible or resistant to the immune response. However, the definitive evidence that different types of immunotherapy can have therapeutic benefits in cancer treatment has been acquired only recently.

Cancer immunotherapy involves the use of a wide variety of therapeutic modalities such as cytokines, vaccines, cell therapies and transfection agents that stimulate the host's antitumor response by increasing the effector cells, as well as other agents that decrease the host's suppressor mechanisms by modifying tumor environment through the modulation of immune checkpoints. Therefore, immunotherapy comprises treatments that enhance the innate power of the immune system to combat cancer [3].

In other words, cancer immune treatment benefits the host by inhibiting tumor progression as well as shaping the microenvironment composition of the emerging tumor. Initially described by Schreiber et al. [4] as an "immunoediting" process, the interaction dynamics of host and tumor cells evolves sequentially through the phases of elimination, equilibrium and escape, resulting in deep modification of innate and adaptive immune responses throughout the tumorigenesis. Firstly, emerging transformed cells are inhibited by immune effector cells of the innate immune response, natural killer (NK) cells, and by the host effector molecules, such as IFN-γ perforin, Fas/FasL, and TRAIL. Secondly, this process leads to immune selection and immune sculpting that, subsequently, induce tumor variants with low immunogenicity and resistance to immune effector cells in the equilibrium phase. However, the molecular mechanisms that trigger this phase remain poorly understood. Lastly, tumors perform several strategies to avoid recognition by the immune system, enabling them to grow and spread undetectably. This phase of tumor cell escape can involve reduced immune recognition, increased resistance, cell survival and the release of soluble factors that foster an immunosuppressive tumor microenvironment [5].

Tumor development and progression are usually accompanied by a large burden of mutations and re-expression of embryonic genes leading to the translation into neoantigens that can be recognized by T cells [6]. Shankaran et al. [7] consistently reported the process of recognition and control of tumor growth by T cells. Through an experiment involving a comparative analysis of the behavior of carcinogen-induced tumors in immunodeficient and wild-type mice, the T cell reactivity was crucial for the immunogenicity of mature tumors. More recently, these definitions have been reaffirmed and recognized as a hallmark of cancer [8]. However, this mechanism is not always effective in the eradication of cancer cells during the tumor development. Low mutational rates or improper antigen presentation might result in unsatisfactory interactions between tumor antigen and specific T cells. Moreover, the tumor antigen-specific T cell pairs are not always capable of spreading homogenously through the tumor microenvironment [9].

Based on a favorable modification of the tumor microenvironment with promising clinical results, immunotherapy has become a critical tool for approaching cancer in several sites. Through a comprehensive search in the main scientific databases, annals of recent renowned oncology congresses and platforms of ongoing trials, the main objective of the current study is to carry out a broad and updated review of the main classes of immunotherapeutic agents used in cancer management as well as to discuss the new trends of translational research in progress in solid tumors. For further understanding, Table 1 summarizes the key studies addressed to the main classes of immunotherapeutic agents, while Table 2 outlines the year some new drugs have been approved by the Food and Drug Administration (FDA) and their respective indications for a wide variety of settings. Moreover, a diagram with an overview on the new challenges and future directions in this field is wisely presented in Fig. 1.

Adoptive cell therapy

Adoptive cell transfer therapy (ACT) functionally modifies T lymphocytes, leading them objectively to recognize and attack a broad spectrum of specific cell targets, setting it up as a powerful therapy in the context of advanced cancer. The main approaches to cell therapy involve autologous tumor-infiltrating lymphocytes (TILs) selected for their antitumor reactivity or autologous T cells genetically engineered with TCRs or chimeric antigen receptors (CARs), as well as the emerging natural killer cells engineered with CARs. As a promising therapeutic tool in development, some interesting but still conflicting results using ACT have been published in small reports for some types of solid tumors. And in this context, some challenges have been faced in the use of ACT in solid tumors, since they often present with primary sites with difficult infiltration of infused T cells, are composed of a microenvironment known as immunosuppressive and have heterogeneous expression of antigens [10].

Preliminary data have shown clinical benefit of ACT based on autologous tumor-infiltrating lymphocytes (TILs) for patients with advanced solid tumors as a highly personalized treatment. T cells are obtained from autologous fresh tumor tissues and, after ex vivo activation and extensive expansion, are reinfused to patients, enabling the successful trafficking of T cell to the tumor microenvironment [11]. Although some early phase I/II studies in a few specialized care centers have consistently shown significant responses, mainly in melanoma, performing complete durable regressions in over 20% of patients, the production of TILs remains very costly and complex [12]. As for non-melanoma tumors,

Table 1 Key studies with immunotherapy

Agents	Indication	Design/study	Regimen	Results	References
ACT	Melanoma	Phase II	TILs + chemotherapy	ORR 50%	[21]
Vaccines	Castration-resistant prostate cancer	Phase I	Sipuleucel-T vs placebo	OS 25.8 vs 21.7 months (HR 0.73; $p=0.001$)	[27]
Immune checkpoints blockade					
Anti-CTLA-4	Melanoma	Phase III	Ipilimumab 3 mg/kg vs gp100	OS 10.1 vs 6.4 months (HR 0.68; $p<0.001$)	[43]
	Melanoma	Phase III/EORTC 18071	Ipilimumab 10 mg/kg vs placebo	5 year RFS 40.8% vs 30.3% (HR 0.76; $p<0.001$)	[44]
	Melanoma	Phase III	Tremelimumab 15 mg/kg vs chemotherapy	OS 12.6 vs 10.7 months (HR 0.88; $p<0.127$)	[49]
Anti-PD1	Melanoma	CHECKMATE-238 (phase III)	Nivolumab vs ipilimumab	12 months rate RFS 70.5% vs 60.8% (HR 0.65; $p<0.001$)	[53]
		KEYNOTE-006 (phase III)	Pembrolizumab vs ipilimumab	OS NR vs 16 months (HR 0.68; $p=0.0009$)	[45]
	NSCLC	KEYNOTE-024 (phase III)	Pembrolizumab vs docetaxel	PFS 10.3 vs 6.0 months (HR 0.50; $p=0.005$)	[96]
		KEYNOTE-021 (phase I/II)	Chemotherapy ± pembrolizumab	ORR 55% vs 29%	[62]
		KEYNOTE-189 (phase III)	Pembrolizumab ± chemotherapy	PFS 8.8 vs 4.9 months (HR 0.52; $p<0.001$)	[63]
		KEYNOTE-042 (PD-L1 ≥ 1%; phase III)	Pembrolizumab vs chemotherapy	OS 16.7 vs 12.1 months (HR 0.81; $p=0.0018$)	[97]
	Renal cell carcinoma	Phase III	Nivolumab vs everolimus	OS 25.0 vs 19.6 months (HR 0.73)	[54]
	Platino-refractory SCCHN	CHECKMATE-141 (phase III)	Nivolumab vs chemotherapy	OS 7.5 vs 5.1 months (HR 0.7; $p=0.01$)	[55]
		KEYNOTE-012 (phase Ib)	Single-arm pembrolizumab	ORR 16.0% (95% CI 11–22)	[98]
	Urothelial carcinoma	KEYNOTE-052 (phase II)	Single-arm pembrolizumab	ORR 29.0% (95% CI 24–34)	[66]
		CHECKMATE-275 (phase II)	Single-arm nivolumab	ORR 19.6% (95% CI 15.1–24.9; 53/270)	[56]
	Colorectal cancer	CHECKMATE-142 (phase II)	Single-arm nivolumab	ORR 28% (95% CI 20.8–42.9)	[57]
	HCC	CHECKMATE-040 (phase I/II)	Single-arm nivolumab	ORR 20% (95% CI 15–26).	[58]
	Cervical cancer	KEYNOTE-158 (phase II)	Single-arm pembrolizumab	ORR 14.3% (95% CI 7.4–24.1)	[67]
		KEYNOTE-028 (phase Ib)	Single-arm pembrolizumab	ORR 17.0% (95% CI 5–37)	[99]
	Gastric cancer	KEYNOTE-059 (phase II)	Single-arm pembrolizumab	13.3% (95% CI 8.2–20.0)	[68]
Anti-PDL1	NSCLC	OAK (phase III)	Atezolizumab vs docetaxel 75 mg/m^2	OS 13.8 vs 9.6 months (HR 0.73; $p<0.001$)	[71]
		IMpower 150 (phase III)	Carboplatin/paclitaxel/bevacizumab ± atezolizumab	OS 19.2 vs 14.7 months (HR0.78; $p=0.016$)	[72]
		PACIFIC (phase III)	Adjuvant chemoradiotherapy ± durvalumab	PFS 16.8 vs 5.6 months (HR 0.52; $p<0.001$)	[84]
	Urothelial carcinoma	IMvigor211 (phase III)	Atezolizumab vs chemotherapy	OS 11.1 vs 10 months (HR 0.87; $p=0.41$)	[75]
	Merkel cell carcinoma	JAVELIN Merkel 200 part B (phase II)	Single-arm avelumab	ORR 62.1%	[78]

Table 1 (continued)

Agents	Indication	Design/study	Regimen	Results	References
Combination					
	Melanoma	CHECKMATE-069 (phase II)	Ipilimumab/nivolumab vs ipilimumab	NR vs 4.4 months (HR 0.40; $p<0.001$)	[47]
	Renal cell carcinoma	CHECKMATE-214 (phase III)	Ipilimumab/nivolumab vs sunitinib	NR vs 26.0 (HR0.63; $p<0.001$)	[48]
IDO inhibitors	Melanoma	ECHO-202/KEY-NOTE-037 (phase I/II)	Epacadostat + nivolumab	PFS 12.4 months; ORR 56%	[86]
		ECHO-301/KEY-NOTE-252 (phase III)	Epacadostat + pembrolizumab vs placebo + pembrolizmab	PFS 4.7 vs 4.9 months (HR 1.0; $p=0.517$)	[87]
	Urothelial carcinoma	ECHO-202/KEY-NOTE-037 (phase I/II)	Pembrolizumab + epacadostat	ORR 35%	[89]
	Renal cell carcinoma	Phase II	Indoximod + checkpoint inhibitor	ORR 55.7%	[90]

ORR objective response rate, *NR* not reached, *OS* overall survival, *PFS* progression-free survival, *NSCLC* non-small-cell lung carcinoma, *EORTC* European Organisation for Research and Treatment of Cancer, *SCCHN* squamous cell carcinoma of the head and neck, *HCC* hepatocellular carcinoma, *IDO* indoleamine 2,3-dioxygenase-1

Table 2 FDA approval of immunotherapy over the years

	2011	2014	2015	2016	2017	2018	2019
Ipilumumab	Melanoma		Stage III melanoma				
Nivolumab		Melanoma	mNSCLC mRCC	Hodgkin lymphoma mHNC mRCC	Stage III melanoma HCC MSI-h colon cancer Urothelial cancer	mSCLC	
Ipilimumab + nivolumabe			Melanoma			mRCC MSI-h mCRC	
Pembrolizumab		Melanoma	mNSCLC	mNSCLC first line mHNC	Hodkgin lymphoma mNSCLC first line plus chemotherapy Urothelial cancer MSI-h tumors Gastric cancer	Cervical cancer HCC Merkel cell carcinoma mNSCLC first line plus chemotherapy Mediastinal large B-cell lymphoma	mRCC plus axitinib Melanoma adjuvant
Atezolizumab				Bladder cancer mNSCLC		mNSCLC plus chemotherapy	mSCLC plus chemotherapy first line mTN breast cancer plus chemotherapy
Durvalumab					Urothelial cancer	Stage III NSCLC	
Avelumab					Merkel cell carcinoma Urothelial cancer		

mNSCLC metastatic non-small cell lung cancer, *mHNC* metastatic head and neck carcinoma, *mRCC* metastatic renal cell carcinoma, *MSI-h* microsatellite instability-high, *mCRC* metastatic colorectal cancer, *HCC* hepatocellular carcinoma, *mSCLC* metastatic small cell lung cancer, *mTN* metastatic triple negative

Fig. 1 Immuno-oncology: new challenges and future directions

Zacharakis et al. [13] reported a successful case of refractory breast cancer with complete durable regression using adoptive transfer of autologous lymphocytes reactive against mutant versions of four proteins-SLC3A2, KIAA0368, CADPS2 and CTSB. In a small clinical trial that recruited patients with metastatic cervical cancer for treatment with human papillomavirus-targeted tumor-infiltrating T cells, one third of patients had objective response [14].

Chromosomal replication can lead to telomere loss or shortening by 50–100 base pairs of DNA per cell division. This phenomenon has been described in T cells during the process of expansion and differentiation from naive to memory T cells that undergo extensive expansion, causing induction of cell death. However, this process is compensated by the action of telomerase, which has decreased activity with repeated antigen stimulation, leading to telomere shortening [15]. Telomere length of TILs correlates positively with clinical response to therapy as well as suggested as a marker of proliferative potential of the transferred T cell [16]. Similarly, CD27, expressed by naive and memory T cells but downregulated in late stage effector, has been assessed in studies of TILs therapy [17]. Higher numbers of infused CD8+/CD27+T cells are positively associated with clinical response to TILs, pointing to proliferative potential as a determinant of effective ACT in humans. However, these data are still inconsistent and there is considerable overlap in both telomere length and CD27 expression among responding and non-responding patients, which precludes the use of these markers to better select patients for ACT based on TILs [18].

Current efforts are focusing, not only on improving TILs therapy, but also on expanding other cell therapies for distinct malignancies. One of the ACT's strategies is to transfer genetically modified T cells expressing tumor-associated antigens (TAAs). Recently, the use of engineered T cells has taken place at the TIL extraction site since it is a more easily

performed procedure by enabling the acquisition of a highly specific T cell from the peripheral blood replacing the need for tumor extraction and expansion of TILs, regardless of the amount of lymphocyte infiltration into the tumor. The engineered T cells have a highly avid T cell receptors to the TAAs and may be modified for other costimulatory activities to improve clinical response. Mostly, they are chimeric antigen receptors (CARs), defined as single-chain antibodies, composed by variable fragments and linked to T cell receptor (TCR) and T cell costimulatory receptor signaling domains. These compounds connect with the cell-surface antigens in a non-major histocompatibility (MHC) restricted way, or even the traditional $\alpha\beta$ TCRs that recognize epitopes of intracelular antigens presented by MHC molecules [19–21]. Unfortunately, the results of studies with CAR T cells in solid tumors still remain disappointing [22].

Differently from haematological malignancies, the major challenge for the use of CAR T cell in solid tumors lies in finding a specific tumor antigen with no expression in normal tissues. Unacceptable rates of on-target, off-tumor toxicities in patients treated with CD19 and B cell maturation antigen (BCMA)-specific CARs, mainly severe haematological toxicities such as B cell and plasma cell aplasia, have been described in clinical studies [23]. The antigen density in tumor cells, coupled with activation and cytokine production, has been pointed out as a possible marker of CAR T cell functionality [24]. Since systemic administration has been shown to be harmful, another important challenge for CAR T cell use is to establish efficient trafficking to and expansion at the tumor site. One of the solutions can be by refining the chemotaxis of T cells to the tumor site and reginal delivery with a better understanding of the tumor microenvironment as an active agent [25].

Therapeutic vaccines

The therapeutic cancer vaccines do not have the limitation of benefiting a specific group of cancer patients because it has a less restrictive mechanism of action. As mentioned before, solid tumors are heterogeneous with regard to the expression of cell membrane antigens, which makes them more resistant to checkpoint blockade and ACT. The naive T cells are activated by antigen-presenting cells (APCs), such as dendritic cells, which are essential for the effective action of vaccines. Therefore, the active components of the therapeutic vaccines would be: formulations, delivery vehicles, tumor antigens and immune adjuvants [26]. On the other hand, minimal clinical effectiveness could be attributed to the poor pharmacokinetic properties resulting in rapid clearance.

Whole-cell vaccines are composed of autologous and allogeneic groups of modified tumor cells from the patient and non-self cells. The FDA approved an autologous vaccine composed of immune system cells for oncology use. Sipuleucel-T has shown reduction of 22% in the risk of death as compared with the placebo group (hazard ratio, HR 0.78, 95% CI 0.61–0.98, $p=0.03$) [27]. Several allogenic cancer cell vaccines are being tested, including vaccines to treat several solid tumors of different sites [28]. However, none have proved effective enough to be licensed.

One of the main difficulties for the creation of effective personalized therapeutic cancer vaccines (peptide and genetic) is the identification of the most suitable antigens to use. Tumor neoantigens result from somatic mutations and are highly immunogenic. Although already identified as excellent antigenic targets, their identification was not possible until the recent availability of the next generation sequencing. Some research strands, such as The Cancer Genome Atlas (TCGA), based on RNA-sequencing data from thousands of samples across solid tumor, indicate that the number of neoantigens is directly proportional to expression gene activity signature of T cells [29]. The tumor antigen mRNA-transfected dendritic cell (DC) vaccines have been shown as the major focus of research involving DC vaccines, in which antigenic response is induced by T cells [30].

Therapeutic cancer vaccines as monotherapy have not shown consistent advantages. Despite appropriate antigen selection and vaccination platform, many agents failed due to lack of comprehension of immune-suppressive microenvironment and tumor cell-intrinsic mechanisms. Combinations of therapeutic vaccines with other immunotherapeutic agents have been shown to be synergistic and more effective. In a phase I/II study performed by Gibney et al. [31], a response rate (RR) of 30% and a median duration of response of 14.6 months were achieved in 92 ipilimumab-refractory patients with melanoma submitted to nivolumab and multipeptide vaccine.

Immune checkpoints

Immune checkpoint pathways operate at different levels of the immune response. Allowing the immune system to distinguish from self to non-self, these pathways generate an immune response to antigens although managing to prevent autoimmunity and maintaining a normal immunologic homeostasis. As previously said, the ability to evade the immune system is one of the hallmarks of cancer through specific inhibitory signaling pathways, such as the T lymphocyte-associated antigen 4 (CTLA-4), which is likely to occur peripherally in the lymph nodes by inhibiting T cell proliferation early in the immune response, and programmed cell death protein 1 pathway (PD-1/PD-L1), performing inhibitory processes in different sites of tumor [32, 33].

The CTLA-4 blockade conventionally modulates the early T cell response by a standard of regulatory feedback

inhibition. Naive T cell activation in the secondary lymphoid organs occurs simultaneously with costimulatory signals, such as the binding of the CD28 receptor from the T cell to the CD80 (also known as B7-1) and CD86 (or B7-2) from the APC. But, the CTLA-4 is also expressed on the surface of T cells and, thereafter, the inhibitory signal is bound by binding with B7-1 and B7-2 receptors, with higher affinity than the CD28. The main objective of the CTLA-4 pathway is to confer immune tolerance. PD-1, unlike CTLA-4, is an inhibitory regulator of effector T cell activity in peripheral tissues and tumor environment [34]. PD-1 interaction with PD-L1 (also known as B7-H1) and PD-L2 downregulates the antigen receptor signaling leading to immune cell activation [35]. PD-L1 is induced hematopoietic cells through the IFN-gamma produced by activated T and NK cells, whereas PD-L2 is more selectively expressed by dendritic cells and macrophages fundamentally induced by IL-4 [36]. Besides, not all circulating T cells do express the PD-1 receptor, being induced by T cell receptor (TCR) complex stimulation or exposure to cytokines such as IL-2, IL-7, IL-15, IL-21, and transforming growth factor (TGF)-β [37].

The immunologic checkpoint blockade with anti-CTLA-4 and anti-PD-1/PD-L1 antibodies have proven efficacy in several types of cancer. They activate T cells maintaining the proliferation and production of cytokines in the tumor microenvironment and, therefore, immune response to tumor antigens. However, immune-related adverse events (iRAEs) are likely to occur through nonspecific immunologic activation, including dermatologic, gastrointestinal, hepatic, endocrine, and other less common inflammatory events. The treatment of these conditions usually involves temporary and careful immunosuppression with corticosteroid, which may negatively influence the efficacy of immunotherapy when used continuously and for long term, tumor necrosis factor α antagonists, mycophenolate mofetil or other agents [38].

Response evaluation criteria in solid tumors (RECIST) v 1.1, very efficient for cytotoxic chemotherapy, are not a reliable tool for use in the evaluation of response to immunotherapy [39]. In this setting, the immune-related response criteria (irRC), with better refinement, avoiding misleading tumor pseudoprogression, has become an option. By the new rules, new lesions are included in the total burden assessment without immediately being considered progressive disease (PD) and require confirmation of apparent initial disease progression on a subsequent radiographic assessment. The irRC was developed from experience with anti-CTLA-4 therapy trials, but some patients treated with PD-1 agents have similarly shown the same immune-related patterns of response. Some weaknesses noted are the higher interobserver variability, the time-consuming measurement and the fact that irRC was based on malignant melanoma specifically treated with anti-CTLA-4 or anti-PD-1/PD-L1 monoclonal antibodies (mAbs), which may differ from the effect of new drugs under development against different targets with different effect patterns [40, 41].

Anti-CTLA-4

Before immunotherapy, the standard treatment for patients with advanced melanoma was chemotherapy and median overall survival (OS) of patients was quite shorter, less than one year [42]. The CTLA-4 was the first checkpoint receptor to be clinically targeted. In an era lacking effective therapies in the treatment of melanoma, ipilimumab was firstly approved by FDA in 2010 for newly diagnosed or previously treated metastatic/unresectable melanoma based on a pivotal phase **III** study, reaching 10 months median OS with 46% survival rate at 1 year [43]. Thereafter, it was approved by FDA in 2015 for patients with stage **III** melanoma completely resected as adjuvant treatment based on the phase **III** study EORTC 18071, in which 40.8% were free of relapse ($p<0.001$) and 65.4% were alive after a follow-up of 5.3 years ($p=0.001$) [44]. Later on, PD-1 inhibitors (nivolumab or pembrolizumab) demonstrated survival gain and improved toxicity profile compared to ipilimumab, becoming the new first-line treatment standard for advanced melanoma [45, 46].

But, still in 2015, the combination of ipilimumab and nivolumab (anti-PD1 inhibitor) became the new standard treatment for newly diagnosed unresectable/metastatic melanoma without mutation of *BRAF*, approved by the FDA based on the phase **II** CHECKMATE-069, in which the new drug combination reduced the risk of progression or death by 60% compared with ipilimumab alone (HR 0.40, 95% CI 0.22–0.71, $p<0.002$) [47]. Likewise, in April 2018, this same combination was approved as first-line treatment for intermediate or poor risk advanced renal cell carcinoma based on CHECKMATE-214, a randomized open-label phase **III** trial that reduced the risk of death by 37% over sunitinib (HR 0.63, $p<0.001$) [48]. However, the already consolidated synergistic benefits of the combination ipilimumab and nivolumab, due to the chance of simultaneous inhibition of different immune checkpoints, are counterbalanced by the challenge of overcoming the higher immune-mediated toxicity rate as well as relative costs in use in lower–middle income economies.

As for the new CTLA-4 inhibitor, tremelimumab, Ribas et al. [49], through a large phase **III** study, failed to demonstrate statistically significant increase in survival over standard-of-care chemotherapy (HR 0.88, $p=0.127$). The negative results were probably due to the selection of patients with a better prognostic profile and the fact that the patients in the comparator group were treated on the progression with ipilimumab, which was already widely available during the study. Tremelimumab as monotherapy or in combination was also tested for metastatic renal cell

carcinoma, metastatic colorectal cancer, and advanced gastric and esophageal adenocarcinoma; however, no clinically significant benefit was reported [50, 51].

Anti-PD-1

Nivolumab, a PD-1 inhibitor, was initially approved by the FDA in December 2014 for patients with non-mutated *BRAF* unresectable/metastatic melanoma refractory to ipilimumab. Right after, in March 2015, it was expanded for the treatment of patients with metastatic squamous non-small cell lung cancer (NSCLC) with progression on or after platinum-based chemotherapy. In the trial designed by Brahmer et al. [52], the risk of death was 41% lower with nivolumab than with docetaxel ($p < 0.001$). Thereafter, in 2017, nivolumab approval for use as adjuvant treatment for patients with node-positive completely resected melanoma was supported by the CHECKMATE-238, in which nivolumab reduced the risk of recurrence by 35% over the standard ipilimumab ($p < 0.0001$) [53].

As for other solid tumors, nivolumab is now a great option for treatment of advanced renal cell carcinoma, previously exposed to antiangiogenic therapy, based on a randomized trial that showed an increase of about 6 months in the median OS compared with the group control treated with everolimus ($p = 0.002$), regardless of PD-L1 expression [54]. Thereafter, some pivotal trials were driven toward the assessment of efficacy and safety of the use of nivolumab for head and neck cancer. In November 2016, the FDA granted nivolumab approval for patients with recurrent or metastatic platinum-resistant squamous cell carcinoma of the head and neck based on the positive results of the phase III CHECKMATE-141 trial, in which median OS was statistically significantly improved with nivolumab over single-agent chemotherapy (HR 0.70, 95% CI 0.53–0.92, $p = 0.0101$) [55].

For the context of platinum-refractory locally advanced or metastatic urothelial carcinoma setting, in May 2017, nivolumab confirmed its FDA approval for use through the CHECKMATE-275 trial, in which impressively 19.6% (95% CI 15.1–24.9) of patients responded to treatment with nivolumab, including 7 patients with complete response [56]. In the same year, FDA approved nivolumab for the treatment of adult and pediatric (12 years and older) patients with microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer (mCRC) that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan supported by the results of CHECKMATE-142 trial that reported 31.1% (95% CI 20.8–42.9) of ORR [57]. In June 2017, nivolumab also emerged as a new option for patients with sorafenib-resistant hepatocellular carcinoma, based on the results of phase I/II CHECKMATE-040 trial that showed 20% (95% CI 15–26) of ORR in the dose-expansion phase [58].

As for the anti-PD-1 agent pembrolizumab, the first FDA approval was for use in patients with advanced unresectable recurrent melanoma based on the positive results of KEYNOTE-006 trial, which showed a 32% reduction in the long-term risk of death over the control group with ipilimumab ($p = 0.0009$) [59]. Subsequently, the use of pembrolizumab as monotherapy in patients with previously untreated advanced NSCLC with PD-L1 expression greater than 50% of tumor cells was supported by the impressive results of two phase III trials, prolonging the median OS in more than 15 months over the use of cisplatin-based chemotherapy [60] and decreasing the risk of death by 31% [61]. With some preclinical evidence that chemotherapy modifies favorably the immunogenic tumor status, the combination of pembrolizumab with platinum-based chemotherapy for NSCLC without sensitizing *EGFR* or *ALK* mutations as first-line treatment has become a standard, regardless of PD-L1 status, virtually doubling the ORR in an early clinical trial [62]. These positive results were recently confirmed by large published phase III studies for nonsquamous NSCLC [63] and squamous cell NSCLC [64].

Further indications of pembrolizumab for other solid tumors have been based on the results from pivotal clinical trials with promising results, some cases with traditionally "hard-to-treat" tumors. KEYNOTE-012, which showed responses of 6 months or longer observed in 82% ($n = 23/28$) of the responding patients [65]. As for the first-line setting in cisplatin-ineligible advanced urothelial cancer with PD-L1-expression cutoff of 10%, a single-arm phase II trial presented 38% of ORR (95% CI 29–48) [66]. The results of KEYNOTE-158, the multi-cohort trial, led to the approval of pembrolizumab for use in patients with recurrent or metastatic cervical cancer expressing PD-L1 platinum refractory, in which ORR was 14.3% (95% CI 7.4–24.1) and the median duration of response for responders has not yet been reached (95% CI 4.1–18.6+) [67]. Other tumor sites, such as PD-L1 positive recurrent metastatic gastric or gastroesophageal junction adenocarcinoma [68], and MSI-H solid tumors refractory to standard cytotoxic treatments without consolidated treatment options [69, 70], were also approved for the use of pembrolizumab as monotherapy.

Anti-PD-L1

Atezolizumab, an IgG1 antagonist antibody to PD-L1, was approved by the FDA for the treatment of metastatic NSCLC with progressive disease after platinum-based chemotherapy and/or anti-EGFR/ALK therapy. In the phase III OAK trial, patients with recurrent advanced NSCLC, regardless of *EGFR/ALK* mutation and PD-L1 status, had an increase in median OS in more than 4 months ($p < 0.001$) [71]. In the IMpower 150 trial, PD-L1 unselected advanced nonsquamous NSCLC had an improvement in the risk of death

by 22% when exposed to the combination of atezolizumab with chemotherapy alone ($p=0.016$). This benefit was also surprisingly observed in patients with *EGFR* and *ALK* mutation [72].

Atezolizumab is additionally indicated for the treatment of recurrent platinum-refractory advanced urothelial carcinoma, based on the results of clinical trials that demonstrated durable activity and good toxicity profile in this population [73–75]. The use as first-line therapy for patients with cisplatin-ineligible advanced or metastatic urothelial carcinoma was based on a single-arm phase II trial, in which the ORR was 23% (95% CI 16–31) and the CR rate was 9% ($n=11$), and 19 of 27 responses were ongoing at 17.2 months' median follow-up [76]. For other tumor types such as breast cancer, renal carcinoma, mesothelioma, melanoma, and other malignancies, clinical trials with atezolizumab, combined with chemotherapy/monotherapy, are still ongoing and will provide more information about immunotherapy benefits in these settings.

In May 2017, FDA granted conditional approval for avelumab, another anti-PD-L1 agent, for the treatment of advanced urothelial carcinoma, based on the results of the combined analysis of two phase I expansion cohorts that showed an ORR of 17% (95% CI 11–24), including 6% of CR, presenting a more unfavorable toxicity profile compared to other checkpoint inhibitors [77]. Another prospective clinical study with some cohorts of patients with different settings of advanced urothelial cancer demonstrated ORR ranging from 33 to 62.1% [78]. More data on the use of avelumab are currently being evaluated in ongoing clinical trials for different indications, as for renal carcinoma in association with axitinib, for solid tumors in association with chemotherapy or immunotherapy, for head and neck cancer [79–82].

Durvalumab, a human immunoglobulin G1 kappa (IgG1κ) monoclonal antibody that blocks the interaction of programmed cell death ligand 1 (PD-L1) with the PD-1 and CD80 (B7.1), was approved in May 2017 for metastatic urothelial carcinoma after platinum-based chemotherapy, based on a phase I/II, in which durvalumab demonstrated favorable clinical activity with 17.8% ORR (CI 12.7–24.0%), including 7 complete responses, with a manageable safety profile [83]. Durvalumab was approved in February 2018 for patients with stage III unresectable NSCLC whose disease has not progressed following platinum-based chemoradiotherapy. In the phase III PACIFIC trial, consolidation with durvalumab dramatically increased PFS in more than 10 months over placebo ($p<0.001$) [84].

IDO Inhibitors

Indoleamine 2,3 dioxygenase (IDO) is an enzyme that catabolizes the first and rate-limiting step in the degradation of the essential amino acid tryptophan to kynurenine. The tryptophan depletion and production of kynurenine and other catabolites limit antigen-dependent T cell activation, resulting in immune tolerance to antigens in tissue microenvironments. In the normal physiologic state, IDO is important to create an environment that limits damage to tissues due to an overactive immune system. But on the other hand, it can facilitate the survival and growth of tumor cells expressing unique antigens that would be recognized by immune system. IDO expression has been correlated with decreased OS and PFS in several clinical studies [85].

An increasing number of trials including IDO inhibitors are still ongoing, but some data already published present controversial results. The combination of pembrolizumab plus epacadostat, an inhibitor of IDO1, has been evaluated in phase I/II study ECHO-202/KEYNOTE-037 for advanced tumors. The data of the melanoma cohort, in treatment-naive patients, have shown attractive results, in which ORR was 56% (25/45; 6 CR, 19 PR), regardless of PD-L1 and *BRAF* mutation status, with just 17.2% of patients experiencing grade 3 or greater immune-related adverse events [86]. Nonetheless, the data from a phase III ECHO-301/KEYNOTE-252 showed that epacadostat plus pembrolizumab in this setting did not result in significantly longer PFS versus placebo plus pembrolizumab (median PFS 4.7 versus 4.9 months, HR 1.00; 95% CI 0.83–1.21; $p=0.517$). And findings were consistent across PD-L1 and *BRAF* subgroups [87]. Likewise, the initial data from phase II ECHO-204 study with the combination epacadostat plus nivolumab for advanced melanoma showed promising antitumor activity, with 62% of ORR (9 CRs, 22 PRs). Unfortunately, the regimen was very toxic, with 48% of the patients presented grade 3 or more toxicity [88].

As for other non-melanoma cancers, ECHO-202/KEYNOTE-037 evaluated some cohorts of solid tumors from other sites. In the platinum-refractory urothelial carcinoma cohort, the combination of pembrolizumab and epacadostat showed ORR of 35% (13/37; all PR). In the renal cell carcinoma cohort, 33 patients were evaluated, 64% were MSKCC intermediate risk, reaching an impressive 47% of ORR (9/19; 1 CR, 8 PR) and, at the date cutoff, 100% of responders were still ongoing. Grade 3 or 4 adverse events occurred in only 15% of the patients [89].

Indoximob, another IDO inhibitor, was evaluated in a phase II trial in association with investigator choice checkpoint inhibitor for patients with heavy-treated advanced

melanoma. The results showed great benefit among patients treated with pembrolizumab and indoximob; ORR was 55.7% (39/70) and CR was 18.6% (13/70), having been well tolerated with easily manageable side effects [90]. As a promising drug in the field of immunotherapy, with interesting preliminary results, many studies are ongoing assessing its efficacy and safety in different settings of solid tumors as monotherapy or in combined regimens.

Conclusion

Systemic therapy for a long time has been mastered by cytotoxic chemotherapy. But recently, the concept of immunotherapy as a modulator of the patient's immune system to battle neoplastic cells became an important weapon against cancer. Immune checkpoint blockade has certainly been one of the most imposing developments made in cancer in recent decades. Immuno-oncology has become a field under rapid and exuberant evolution, with many agents being developed and studied for their potential to improve long-term survival of several tumor types in different settings with considerable success. Throughout this review, many important data regarding the new treatment proposals have been presented.

Predicting clinical efficacy to immune checkpoint blockade remains as a major challenge. The lower response rate to the agents in some settings and the higher cost of drugs with a significant economic impact on the health care systems demand a careful personalized approach. Searching for reliable predictive biomarkers, several strands have focused on tumor immune phenotype, somatic genomic features, or the gut microbiome.

Several studies in different tumor types have shown that patients whose tumors express PD-L1, detected by immunohistochemical assays, have higher response rates to PD-1/PD-L1 blockade than patients who do not express PD-L1. Despite this, specific groups of patients who do not express PD-L1 can still have some degree of response to PD-1/PD-L1 blockade, demonstrating that other biomarkers are still needed to provide better prediction of patients who benefit from the immunotherapeutic treatment [91].

Solid tumors with high number of non-synonymous genomic mutations (MSI-H) have increased T cell infiltration and higher responses to immune checkpoint blockade. TCGA data have demonstrated that mutations in *JAK1* were associated with high mutation burden and microsatellite instability occurring in multiple tumor types including endometrial, colorectal, stomach and prostate carcinomas and may play a role in immune evasion and evasion to checkpoint inhibitors [92].

Immunological genetic signatures were evaluated in a large study that enrolled 1535 patients with advanced solid tumors treated with immune checkpoint inhibitors. In this trial, the leukocyte antigen class I (HLA-I) genes were factors that significantly influenced survival in patients with melanoma and NSCLC. For instance, the HLA-B44 profile was associated with prolonged OS, whereas the HLA-I homozygosity and loss of heterozygosity (LOH) represented a genetic barrier to effective immunotherapy response [93].

As a future biomarker, the intestinal microbiota is likely to play a role in the development of cancer as a prior toxin secretion and DNA damage, dysbiosis and inflammation with increased pro-inflammatory signals, inducing immunosuppression and tumor evasion. With the development of immunotherapy, it has been proven that the composition of the gut microbiota has also an impact on the response to the anti-PD-1/PD-L1 agents in patients with epithelial tumors or melanoma [94]. These results were suggested in a prospective study, in which tumor biopsies, oral and gut microbiome and blood samples were collected from 112 patients with metastatic melanoma treated with anti-PD1 agents at specific times throughout the treatment to explore genomic alterations, as well as density of tumor-infiltrating lymphocytes. Metagenomic analysis revealed a functional difference in gut bacteria between responders and non-responders. Analysis of patient fecal microbiome samples showed significantly higher alpha diversity ($p < 0.01$) and relative abundance of the Ruminococcaceae family ($p < 0.01$) among responders [95].

Through simultaneous anti-cancer activities on different fronts, involving different mechanisms of action with synergic effect, these new strategies offer the opportunity to defeat the many barriers that protect tumor cells from the innate and adaptive immune system. Therefore, combining immunotherapy with different therapies may improve survival in a greater number of patients when compared with monotherapy. Establishing strategies in how to make progress in this field and how to apply these new therapies most effectively to achieve the best outcomes is crucial. So far, the great challenge is to select the best combined treatment for each setting and overcome the higher limiting dose toxicities that occur in some cases. Finally, balancing risk and cost-saving schemes are completely imperative.

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Anexo C

Emenda de solicitação de isenção do TCLE aprovada pelo cep

ANEXO V - Solicitação de Isenção do Termo de Consentimento Livre e Esclarecido

Referência: Protocolo de pesquisa "**Câncer de mama triplo-negativo: Uma nova perspectiva sobre biomarcadores" aprovado pelo CEP**, versão 3, CAAE nº 61675516.9.0000.5274.
Pesquisador Responsável: Jessé Lopes da Silva

Ao Comitê de Ética em Pesquisa do Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA):

Vimos por meio deste documento solicitar a dispensa de obtenção de um Termo de Consentimento Livre e Esclarecido (TCLE) do projeto intitulado "**Câncer de mama triplo-negativo: Uma nova perspectiva sobre biomarcadores"** proposto por Jessé Lopes da Silva para os participantes falecidos e os sem possibilidades de contato (sem acompanhamento regular na instituição, sem endereço residencial válido e/ou sem contato telefônico após pelo menos três tentativas frustradas), com base na Res. CNS 466/12 item IV.8 que contempla a dispensa do TCLE em situações de impossibilidade de obtenção do mesmo.

Ainda, a dispensa do uso de TCLE se fundamenta por ser um estudo observacional, analítico ou descritivo retrospectivo que contempla o uso de materiais biológicos coletados e armazenados como parte das rotinas institucionais, sem adição de riscos aos participantes de pesquisas ou prejuízos ao bem-estar dos mesmos.

O investigador principal e demais colaboradores envolvidos no projeto acima se comprometem, individual e coletivamente, a utilizar os dados provenientes deste, apenas para os fins descritos e a cumprir todas as diretrizes e normas regulamentadoras descritas na Res. CNS Nº 466/12, e suas complementares, no que diz respeito ao sigilo e confidencialidade dos dados coletados.

Rio de Janeiro, 13 de Março de 2018

Dr. Jessé Lopes da Silva
Oncologia Clínica CRM 94173-5
jessejeu@yahoo.com.br
+ 55 21 982727044
INCA - Instituto Nacional de Câncer José Alencar Gomes da Silva
MINISTÉRIO DA SAÚDE - BRASIL
Hospital de Câncer III – INCA + 55 21 3207-3700

16/03/18

Dr. Jessé Lopes da Silva
Terapia Intensiva
Oncologia Clínica
CRM 52.94173-5

ANEXO D

PARECER DE APROVAÇÃO DO PROJETO E DE EMENDAS PELO CEP

INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA - INCA

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Câncer de mama triplo-negativo: Uma nova perspectiva sobre biomarcadores
Pesquisador: Jessé Lopes da Silva
Área Temática:
Versão: 3
CAAE: 61675516.9.0000.5274
Instituição Proponente: Hospital do Câncer III
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.568.193

Apresentação do Projeto:
- Projeto de Pesquisa Aprovado pelo Parecer Consubstanciado do CEP-INCA de número 1.858.592, datado de 09 de Dezembro de 2016.

- A presente Emenda trata da apresentação das seguintes solicitações / modificações (arquivos "Anexo1_Emenda1_detalhada.pdf"; "AnexoIV_Orcamento_atualizado.pdf"; "FormularioCEP_assinaturas.pdf"; "AnexoIII_Declaracao_financiamento.pdf"; "AnexoV_Solicitacao_isencao_TCLE.pdf"; "AnexoVI_Cronograma_atualizado.pdf"):
- Inclusão de co-investigadores
- Inclusão de novos biomarcadores
- Atualização de detalhes sobre fontes de financiamento do estudo
- Atualização do orçamento do estudo
- Solicitação de dispensa da obtenção do Termo de Consentimento Livre e Esclarecido (TCLE) para pacientes não encontrados pelos meios de comunicação (telefone, telegrama via correios)
- Atualização do cronograma

Objetivo da Pesquisa:
- A presente Emenda trata da apresentação das seguintes solicitações / modificações (arquivos "Anexo1_Emenda1_detalhada.pdf"; "AnexoIV_Orcamento_atualizado.pdf"; "FormularioCEP_assinaturas.pdf"; "AnexoIII_Declaracao_financiamento.pdf";

Endereço: RUA DO RESENDE, 128 - SALA 203
Bairro: CENTRO CEP: 20.231-092
UF: RJ Município: RIO DE JANEIRO
Telefone: (21)3207-4550 Fax: (21)3207-4556 E-mail: cep@inca.gov.br

"AnexoV_Solicitacao_isencao_TCLE.pdf"; "AnexoVI_Cronograma_atualizado.pdf"):
- Inclusão de co-investigadores
- Inclusão de novos biomarcadores
- Atualização de detalhes sobre fontes de financiamento do estudo
- Atualização do orçamento do estudo
- Solicitação de dispensa da obtenção do Termo de Consentimento Livre e Esclarecido (TCLE) para pacientes não encontrados pelos meios de comunicação (telefone, telegrama via correios)
- Atualização do cronograma

Avaliação dos Riscos e Benefícios:

- A presente Emenda trata da apresentação das seguintes solicitações / modificações (arquivos "AnexoI_Emenda1_detalhada.pdf"; "AnexoIV_Orcamento_atualizado.pdf"; "FormularioCEP_assinaturas.pdf"; "AnexoIII_Declaracao_financiamento.pdf"; "AnexoV_Solicitacao_isencao_TCLE.pdf"; "AnexoVI_Cronograma_atualizado.pdf"):
- Inclusão de co-investigadores
- Inclusão de novos biomarcadores
- Atualização de detalhes sobre fontes de financiamento do estudo
- Atualização do orçamento do estudo
- Solicitação de dispensa da obtenção do Termo de Consentimento Livre e Esclarecido (TCLE) para pacientes não encontrados pelos meios de comunicação (telefone, telegrama via correios)
- Atualização do cronograma

Comentários e Considerações sobre a Pesquisa:

- A presente Emenda trata da apresentação das seguintes solicitações / modificações (arquivos "AnexoI_Emenda1_detalhada.pdf"; "AnexoIV_Orcamento_atualizado.pdf"; "FormularioCEP_assinaturas.pdf"; "AnexoIII_Declaracao_financiamento.pdf"; "AnexoV_Solicitacao_isencao_TCLE.pdf"; "AnexoVI_Cronograma_atualizado.pdf"):
- Inclusão de co-investigadores
- Inclusão de novos biomarcadores
- Atualização de detalhes sobre fontes de financiamento do estudo
- Atualização do orçamento do estudo
- Solicitação de dispensa da obtenção do Termo de Consentimento Livre e Esclarecido (TCLE) para pacientes não encontrados pelos meios de comunicação (telefone, telegrama via correios)
- Atualização do cronograma

Considerações sobre os Termos de apresentação obrigatória:

- A presente Emenda trata da apresentação das seguintes solicitações / modificações (arquivos "AnexoI_Emenda1_detalhada.pdf"; "AnexoIV_Orcamento_atualizado.pdf"; "FormularioCEP_assinaturas.pdf"; "AnexoIII_Declaracao_financiamento.pdf"; "AnexoV_Solicitacao_isencao_TCLE.pdf"; "AnexoVI_Cronograma_atualizado.pdf"):
- Inclusão de co-investigadores
- Inclusão de novos biomarcadores
- Atualização de detalhes sobre fontes de financiamento do estudo
- Atualização do orçamento do estudo
- Solicitação de dispensa da obtenção do Termo de Consentimento Livre e Esclarecido (TCLE) para pacientes não encontrados pelos meios de comunicação (telefone, telegrama via correios)
- Atualização do cronograma

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

Não se aplica.

Considerações Finais a critério do CEP:

Diante do exposto, o Comitê de Ética em Pesquisa do Instituto Nacional de Câncer (CEP-INCA), de acordo com as atribuições definidas na Resolução CNS Nº 466/2012 e na Norma Operacional CNS Nº 001/2013 manifesta-se pela aprovação da Emenda ao projeto de pesquisa proposto.

Ressalto o(a) pesquisador(a) responsável deverá apresentar relatórios semestrais a respeito do seu estudo.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_925827_E1.pdf	27/03/2018 13:55:18		Aceito
Outros	FormularioCEP_assinaturas.pdf	26/03/2018 11:19:11	Jessé Lopes da Silva	Aceito
Outros	AnexoI_Emenda1_detalhada.docx	22/03/2018 10:06:50	Jessé Lopes da Silva	Aceito
Declaração de Pesquisadores	AnexoIII_Declaracao_financiamento.doc	22/03/2018 10:05:44	Jessé Lopes da Silva	Aceito
Orçamento	AnexoIV_Orcamento_atualizado.docx	22/03/2018	Jessé Lopes da Silva	Aceito

Orçamento	AnexoIV_Orcamento_atualizado.docx	10:05:07	Jessé Lopes da Silva	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	AnexoV_Solicitacao_isencao_TCLE.doc	22/03/2018 10:04:43	Jessé Lopes da Silva	Aceito
Cronograma	AnexoVI_Cronograma_atualizado.docx	22/03/2018 10:04:29	Jessé Lopes da Silva	Aceito
Outros	Carta_Encaminhamento_Emenda1.doc	20/03/2018 13:03:27	Jessé Lopes da Silva	Aceito
Outros	AnexoI_Emenda1_detalhada.pdf	20/03/2018 13:02:53	Jessé Lopes da Silva	Aceito
Orçamento	AnexoIV_Orcamento_atualizado.pdf	20/03/2018 13:01:54	Jessé Lopes da Silva	Aceito
Outros	Carta_Encaminhamento_Emenda1.pdf	19/03/2018 16:03:25	Jessé Lopes da Silva	Aceito
Declaração de Pesquisadores	AnexoIII_Declaracao_financiamento.pdf	19/03/2018 15:59:11	Jessé Lopes da Silva	Aceito
Projeto Detalhado / Brochura Investigador	AnexoII_Projeto_atualizado.pdf	19/03/2018 15:58:54	Jessé Lopes da Silva	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	AnexoV_Solicitacao_isencao_TCLE.pdf	19/03/2018 15:58:41	Jessé Lopes da Silva	Aceito
Cronograma	AnexoVI_Cronograma_atualizado.pdf	19/03/2018 15:58:28	Jessé Lopes da Silva	Aceito
Folha de Rosto	folhaderostofinal.pdf	03/11/2016 23:28:20	Jessé Lopes da Silva	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIO DE JANEIRO, 28 de Março de 2018

Assinado por:
Carlos Henrique Debenedito Silva
(Coordenador)

APÊNDICE

APÊNDICE A - METODOLOGIA

11 MATERIAIS E MÉTODOS

11.1 Aspectos gerais do desenho do estudo

Trata-se de um estudo retrospectivo, observacional e translacional do tipo coorte. O CMTN foi caracterizado como ausência de expressão (<1%) do RE e RP, assim como HER-2 negativo pela IHQ (0 ou 1+) ou duvidoso pela IHQ (2+) com teste de amplificação por FISH negativos.

11.2 População do estudo

O estudo incluiu toda a população de mulheres elegíveis consecutivamente matriculadas no INCA de janeiro de 2010 a dezembro de 2014. Este período foi escolhido por já se ter consolidado as rotinas de indicação de estratégia padrão de quimioterapia neoadjuvante para pacientes com CMTN localmente avançado na instituição, assim como melhor padronização da análise patológica do material da core biópsia e da peça cirúrgica pelo Departamento de patologia (DIPAT/INCA).

11.2.1 Critérios de inclusão

I. Mulheres com mais de 18 anos;
II. CMTN invasivo com histologia epitelial;
III. Estadiamento localmente avançado (T3-4NqqM0; TqqN1-3M0). Para o propósito deste estudo, a definição de CMLA abrange tumores de estadio II e III.
IV. Ausência de metástase a distância ao diagnóstico;
V. Pacientes matriculadas no INCA entre janeiro de 2010 e dezembro de 2014;
VI. Pacientes obrigatoriamente submetidas a pelo menos um ciclo de quimioterapia neoadjuvante padrão no INCA;
VII. Obrigatoriamente submetidas a abordagem cirúrgica curativa no INCA;
VIII. Material patológico obrigatoriamente analisado no DIPAT/INCA.

11.2.2 Critérios de exclusão

I. Outros subtipos de câncer de mama não-CMTN (LUMINAL, HER-2 enriquecido, etc). Tumores com positividade para algum dos receptores RE, RP e/ou HER2, ou no material de biópsia ou na peça cirúrgica com tumor residual;
II. Tumores exclusivamente não-invasores (CDIS) na core biópsia;
III. Outros tumores primários sincrônicos ou anacrônicos no mesmo sítio de mama e/ou em outros sítios diferentes;
IV. Pacientes tratadas previamente com antineoplásicos para outros tumores (imunoterápicos, anti-angiogênicos, quimioterápicos, etc);
V. Pacientes que tiveram evento de progressão durante a quimioterapia padrão e não conseguiram resposta satisfatória com tratamento de resgate complementar sistêmico (com capecitabina ou cisplatina) e/ou radioterápico, não tendo se submetido a abordagem cirúrgica;
VI. Pacientes participantes do estudo institucional Neosamba[1] somente serão excluídas para análises dos *subitens II e III do item 2.2.*

11.3 Definição das variáveis analisadas no estudo

11.3.1 Variáveis clínicas e sociodemográficas

I. Raça/cor da pele pelo Instituto Brasileiro de Geografia e Estatística (IBGE): apesar da conhecida miscigenação brasileira, essa variável foi definida pela autodeclaração da paciente na primeira consulta no INCA.
II. Data do diagnóstico: Data do laudo patológico do DIPAT para a core biópsia.
III. Idade ao diagnóstico: calculada a partir da data de nascimento na data do diagnóstico.
IV. Escolaridade: essa variável foi categorizada de acordo com o sistema antigo de educação no Brasil constado nos registros de primeira avaliação no INCA (nenhuma alfabetização, ensino fundamental incompleto, ensino fundamental completo, ensino médio incompleto, ensino médio completo, ensino superior incompleto, ensino superior completo, pós-graduação incompleta e pós-graduação completa).

[1] Estudo com o objetivo de avaliar a sequência neoadjuvante usual e reversa de uma antraciclina seguida de taxano em câncer de mama localmente avançado.

V. Tabagismo: os dados de exposição ao tabagismo ativo (dicotomizado em “exposta” ou “não-exposta”) foram colhidos em prontuário, incluindo carga tabágica em maços-ano e exposição ao tabagismo na adolescência.

VI. Consumo de bebida alcoólica: os dados de exposição a bebida alcoólica (dicotomizado em “expostas” ou “não-expostas”) foram colhidos em prontuário, incluindo exposição ao etilismo na adolescência.

VII. Distância do domicílio para o centro de tratamento: calculada por meio do aplicativo Google Maps (https://www.google.com.br/maps) a distância do domicílio da paciente até o endereço do HC3/INCA. Foi arbitrariamente escolhida uma distância de corte de 28 Km para comparação.

VIII. Quimioterapia neoajuvante padrão: para o tratamento neoadjuvante de CMLA no HC3/INCA, já foi muito usado anteriormente o regime FAC (fluorouracil 500mg/m² + doxorrubicina 50mg/m² + ciclofosfamida 500mg/m², administrado intravenoso de 21/21 dias por 6 ciclos). Posteriormente, foi incorporado o taxane com o esquema FAC-T (fluorouracil 500mg/m² + doxorrubicina 50mg/m² + ciclofosfamida 500mg/m², administrado intravenoso de 21/21 dias por 3 ciclos, seguido de docetaxel 100mg/m² de 21/21 dias, por mais 3 ciclos). Nos últimos anos, um outro regime também baseado em antraciclina-taxane, o AC-T (doxorrubicina 60mg/m² + ciclofosfamida 600mg/m², administrado intravenoso de 21/21 dias por 4 ciclos, seguido de docetaxel 100mg/m² intravenoso de 21/21 dias por mais 4 ciclos, ou seguido de paclitaxel 80 mg/m² semanal intravenoso por 12 semanas consecutivas sem intervalo, aqui definido como um total de 4 ciclos de 3 semanas), tem sido adotado até o momento como padrão. Outras opções não-antraciclina para casos selecionados em discussão de mesa redonda do serviço de oncologia por impedimentos pessoais de algumas pacientes para uso de antraciclina podiam ser oferecidas: TC docetaxel 75 mg/m² + ciclofosfamida 600mg/m² administrado intravenoso de 21/21 dias por 4 ciclos).

IX. Quimioterapia padrão completa: administração de 6 ciclos completos de FAC ou 6 ciclos de FAC-T ou 8 ciclos de AC-T.

X. Quimioterapia padrão incompleta: por motivos diversos (toxicidade grave inaceitável, questões sociais, progressão de doença em vigência de tratamento), define-se como administração de quantidade menor de quimioterapia do que a definida como padrão.

XI. Tratamento complementar: pacientes consideradas inoperáveis na primeira avaliação oncológica após quimioterapia padrão, eram discutidos na mesa redonda da oncologia para fazer quimioterapia (cisplatina ou capecitabina) e/ou radioterapia neoadjuvante para conseguir resposta com intuito de depois fazer abordagem cirúrgica curativa.

XII. Tempo do diagnóstico para início do tratamento: esse dado foi calculado como tempo da data do laudo patológico da core biópsia pelo DIPAT até a data da primeira dose de quimioterapia neoadjuvante padrão.

XIII. Tempo do fim da quimioterapia neoadjuvante padrão para a cirurgia: definido como o tempo da data do último ciclo da quimioterapia padrão até a data da abordagem cirúrgica.

11.3.2 Variáveis patológicas

I. **Estadio clínico:** será classificado de acordo com o TNM de acordo com os grupos de estadios anatômicos clínicos do 8° AJCC (*figura 11*).

II. **T clínico:** será classificado de acordo com o TNM de acordo estadio anatômicos do tumor primário clínico do 8° AJCC (*figura 7*).

III. **N clínico:** será classificado de acordo com o TNM de acordo estadio anatômicos do *status* nodal clínico do 8° AJCC (*figura 8*).

IV. **Estadio patológico:** será classificado de acordo com o TNM de acordo com os grupos de estadios anatômicos patológicos do 8° AJCC (*figura 11*).

V. **T patológico:** será classificado de acordo com o TNM de acordo estadio anatômicos do tumor primário patológico do 8° AJCC (*figura 7*).

VI. **N patológico:** será classificado de acordo com o TNM de acordo estadio anatômicos do *status* nodal patológico do 8° AJCC (*figura 9*).

VII. **Grau tumoral:** serão considerados grau 1 (tumores bem diferenciados); grau 2 (tumores moderadamente diferenciados) e grau 3 (tumores pouco diferenciados).

11.3.3 Variáveis de imunohistoquímica

As lâminas de hematoxilina & eosina (H&E) das core biopsia/biópsias iniciais foram revistas quanto ao grau histológico (método Scarff-Bloom-Richardson, modificado por Elston e Ellis)(MEYER *et al.*, 2005), tipo histológico de acordo com os critérios da Organização Mundial de Saúde (WHO 2012), invasão angiolinfática e presença de infiltrado inflamatório tumoral.

As lâminas da biópsia inicial, por apresentarem material escasso, foram reanalisadas de forma inteira para anticorpos RE, RP, HER2, KI67 e p53. Nelas também foram realizadas análises dos demais anticorpos: EGFR, AR, CK 14, CK 17, CK5/6, PD-L1, PD-L2, PD-1, FOXP3, CD3, CD4, CD8, CD 56, CD68 e CD 117. Já nas peças cirúrgicas, produto da cirurgia realizada após quimioterapia neoadjuvante, foram estudados os mesmos fatores testados na biópsia inicial citados anteriormente para fins descritivos e para análise comparativa. Nesse caso, as áreas de interesse foram selecionadas pela H&E e marcadas nos blocos de parafina para a preparação de TMA (*tissue microarray*). Considerando a heterogeneidade intratumoral, três pequenas punções cilíndricas teciduais de 0,6 mm foram coletadas de áreas com maior grau e celularidade nas seções correspondentes em cada amostra de peça cirúrgica.

Por fim, foram realizadas seções de desparafinação, reidratação e processamento usando um corante automatizado através de métodos padrão. A técnica de IHQ foi realizada em um ou dois dias consecutivos. As lâminas comerciais, previamente tratadas com cargas (StarFrost- Knittel Glass) contendo cortes de 3 micras foram imersas em 3 banhos de 5 minutos em xilol, seguidos de banhos rápidos em álcool 100%, 90%, 80% e 70%. O excesso de álcool foi retirado em água corrente por 3 minutos.

A recuperação antigênica foi realizada em tampão Trilogy (Cell Marque), à temperatura de 98°C, utilizando-se o processo a vapor (Steamer), por 30 minutos. O bloqueio de peroxidase e o bloqueio de proteína foi feito utilizando-se o kit Novolink Max Polymer Detection (Leica Microsystems), por 5 minutos cada. A incubação com o anticorpo primário foi realizada por uma hora (técnica de 1 dia) ou *overnight* (técnica de 2 dias), à geladeira. A *tabela 6.1* apresenta os anticorpos, clones, tempos de incubação e marcações de cada reagente utilizado.

Após a incubação com o reagente primário, as lâminas foram incubadas com o anticorpo pós-primário (Novolink) e com o polímero (Novolink), ambos por 30 minutos. Para a revelação da reação será utilizado o cromógeno DAB (Diaminobenzidina), por 3 minutos (Novolink). A contra-marcação foi feita com a hematoxilina de Harris, por 30 segundos. Após a retirada do excesso de hematoxilina em água corrente, as lâminas foram imersas em banhos de 70%, 80%, 90%, 100% e xilol. Foi utilizado o bálsamo, para a montagem das lâminas, que serão analisadas ao microscópio óptico, observando-se uma marcação específica de cada anticorpo (vide Tabela 11.1).

Todas as técnicas de IHQ foram realizadas com controle positivo e negativo da reação. O Ki67 foi considerado positivo quando houve marcação nuclear e foi definido como a percentagem de células tumorais imunorreativas em relação ao total de células tumorais no campo de aumento 40x, com “cutoff” em 14%, gerando dois grupos: ≤ 14% (atividade proliferativa baixa) e > 14% (atividade proliferativa alta). O PD-1 foi considerado positivo quando houve marcação nuclear e será definido como a percentagem de células tumorais imunorreativas em relação ao total de células tumorais no campo de aumento 40x, sendo posteriormente categorizado em grupos de 10%. O EGFR foi considerado positivo quando houve marcação de membrana das células tumorais ≥ 10%. O RA foi considerado positivo quando houve marcação nuclear e o “cutoff” usado para distinguir positivo de negativo será células tumorais positivas ≥ 1%.

O PD-L1/PD-L2 foram considerados positivos quando houve marcação de membrana celular linfocitária, sendo definido escore percentual contínuo pela proporção de linfócitos positivos sobre células linfo-mononucleares totais no campo de aumento 40x, e posteriormente categorizado em grupos de 10%. As citoqueratinas CK5/6, CK 14 e CK 17 foram consideradas positivas quando houve marcação de membrana das células tumorais ≥ 1% e avaliado quanto a intensidade (0 = negativo; 1+ = fraco; 2+ = moderado; 3+ = forte). Os FOXP3, PD-1, CD3, CD4, CD8, CD 56, CD68 e CD 117 foram considerados positivos quando houve marcação de membrana das células do infiltrado linfoide, e avaliado quanto a intensidade (0 = negativo; 1+ = fraco; 2+ = moderado; 3+ = forte).

Tabela 11.1 - Informações específicas sobre as reagentes utilizados.

	ANTICORPO	Clone	DILUIÇÃO	Fabricante	Método de recupeação antigenica	TÉCNICA	MARCAÇÃO*
1	**RE**	**EP1**	1+2(P.uso)	Dako	Trilogy	1 dia	Nucleo
2	**RP**	**PgR636**	1/1500	Dako	Trilogy	1 dia	Nucleo
3	**KI67**	**SP6**	1/500	Cell Marque	Trilogy	1 dia	Nucleo
4	**CERB**	**SP3**	1/500	Cell Marque	Trilogy	1 dia	Membrana citoplamatica
5	**P53**	**SP5**	1/500	Cell Marque	Trilogy	1 dia	Nucleo
6	**RA**	**SP107**	1+5 (P.USO)	Cell Marque	Trilogy	2 dia	Nucleo
7	**EGFR**	**HPA018530**	1/300	Sigma	Trilogy	2 dia	Citoplasma/membrana citoplasmática
8	**CK5/6**	**D5&16B4**	1/100	Cell Marque	Trilogy	1 dia	Citoplasma/membrana citoplasmática
9	**CK14**	**SP53**	1/1000	Cell Marque	Trilogy	1 dia	Citoplasma
10	**CK17**	**EP98**	1/500	Cell Marque	Trilogy	2 dia	Citoplasma
11	**CD3**	**MRQ-39**	1/1000	Cell Marque	Trilogy	1 dia	Citoplasma/membrana de linfócitos e CA mama
12	**CD4**	**SP35**	1/400	Cell Marque	Trilogy	1 dia	Citoplasma/membrana de linfócitos e CA mama
13	**CD8**	**SP16**	1/1000	Cell Marque	Trilogy	1 dia	Citoplasma/membrana nuclear de linfócitos
14	**CD14**	**EPR3653**	1/200	Cell Marque	Trilogy	1 dia	Citoplasma/membrana de linfócitos e CA mama
15	**CD56**	**123C3.D5**	1/800	Cell Marque	Trilogy	1 dia	Citoplasma/membrana de linfócitos e CA mama
16	**CD68**	**Kp-1**	1/1500	Cell Marque	Trilogy	1 dia	Citoplasma/membrana nuclear de linfócitos e macrófagos
17	**C-KIT**	**CD117**	1/1000	Dako	Trilogy	1 dia	Citoplasma/membrana de CA mama e mastócito
18	**FOX P-3**	**236/E7**	1/5.000	Abcam	Trilogy	2 dia	Nucleo de linfócitos
19	**PD-1**	**NAT105**	1/100	Cell Marque	Trilogy	2 dia	Citoplasma/membrana citoplasmática de linfócitos
20	**PDL-1**	**SP142**	Pronto p uso	Ventana	SS1(Ventana)	Maquina	Citoplasma/membrana citoplasmática de linfócitos
21	**PDL-2**	**ab200377**	1/200	Abcam	Trilogy	1 dia	Citoplasma/membrana citoplasmática de linfócitos

11.4 Desfechos analisados

I. **Resposta patológica completa:** definida como ausência de tumor viável na peça cirúrgica da mama e da axila (ypT0N0), seguindo diretrizes da *Food and Drug Administration* (FDA) (CORTAZAR, 2014).

II. **Tumor residual:** definido como resíduo tumoral na peça cirúrgica, desde grupos celulares, tumor *in situ* ou tumor invasivo.

III. **Resposta clínica satisfatória**: definida como uma análise subjetiva do médico oncologista examinador após último ciclo do tratamento com quimioterapia neoadjuvante padrão como resposta clínica local pela constatação de redução do tamanho tumoral no sítio primário em mama e/ou axila, possibilitando a abordagem cirúrgica com margens livres naquele momento.

IV. **Resposta clínica insatisfatória**: definida como uma análise subjetiva do examinador médico oncologista após último ciclo do tratamento com quimioterapia neoadjuvante padrão como resposta mínima ao tratamento quimioterápico e/ou identificação de aumento do volume tumoral no sítio primário em mama e/ou axila impossibilitando a cirurgia naquele momento.

V. **Sobrevida livre de eventos**: definido como o tempo da data do diagnóstico até a progressão da doença, morte por qualquer causa, ou descontinuação do tratamento para iniciação de tratamento complementar por pouca resposta a quimioterapia padrão. Trata-se de um dos desfechos sugeridos pela FDA para estudos de tratamento neoadjuvante (CENTER FOR DRUG EVALUATION AND RESEARCH (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES), 2018).

VI. **Sobrevida global:** definido como o tempo do diagnóstico até óbito por qualquer causa (CENTER FOR DRUG EVALUATION AND RESEARCH (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES), 2018).

11.5 Gerenciamento e fonte de dados

Uma ficha clínica eletrônica (eCRF) foi criada através do aplicativo *OpenClinica* Enterprise®, já licenciado para uso no INCA. Este sistema é baseado em internet com o nível de segurança apropriado, garantindo a confidencialidade e a rastreabilidade dos dados conforme preconizado pelas Boas Práticas Clínicas. *Backups* diários são

realizados pela equipe de infraestrutura da Divisão de Informática do INCA, conforme procedimentos operacionais padrões.

A eCRF foi customizada pelos gerentes de dados da Área Representativa de Pesquisa Clínica (ARPC/INCA). Os dados estão sendo coletados através de prontuários médicos e outros documentos-fonte, tais como laudos laboratoriais, patológicos e de imagem. A coleta de dados clínicos e inserção na eCRF está sendo realizada pelo investigador principal ou profissional da nossa. O investigador principal é o responsável pela precisão e veracidade dos dados clínicos coletados, assinando eletronicamente as eCRFs ao final do preenchimento.

Dos prontuários médicos foram colhidas as seguintes variáveis: data de nascimento, raça, escolaridade, distância da residência ao centro de tratamento, data de início da quimioterapia neoadjuvante, data de término da quimioterapia neoadjuvante, tipo de quimioterapia neoadjuvante (antraciclinas e/ou taxane) e tratamento neoadjuvante complementar (uso de platina, capecitabina ou radioterapia). Os dados de biomarcadores tumorais a partir das análises de IHQ foram processamento no DIPAT.

11.6 Métodos estatísticos – aspectos gerais

Para descrever as variáveis contínuas, foi utilizada a média (ou mediana, a depender do padrão de distribuição) e do desvio padrão. Já as variáveis categóricas foram apresentadas por frequência absoluta e relativas.

A avaliação de resposta foi realizada para as variáveis numéricas pelo teste T ou, quando aplicável, pelo teste não-paramétrico de Mann-Witney (HART, 2001). Para variáveis categóricas, foi utilizado o teste de Qui-quadrado ou, em situações específicas, o teste exato de Fisher (KIM, H.-Y., 2017).

As análises de sobrevida foram realizadas pelo método de Kaplan-Meier e as curvas foram comparadas pelo teste de log-rank. O HR bruto para cada variável foi calculado pelo modelo de riscos proporcionais de Cox. Para selecionar as variáveis para o modelo múltiplo de Cox, foram utilizadas variáveis com p-valor ≤ 0,1 no HR bruto. O modelo múltiplo final foi escolhido de acordo com critérios de Akaike (VRIEZE, 2012). As estimativas foram consideradas estatisticamente significativas para os

valores de $p < 0,05$. Todas as análises estatísticas estão sendo efetuada usando o Programa R (CHAN, 2018).

11.7 Métodos para minimizar viés

I. Para evitar viés de mensuração, a análise do material patológico foi realizada em amostra inteira na core biópsia e em triplicata na peça cirúrgica, garantindo uma boa cobertura do espécime tumoral.
II. Outra medida para reduzir os erros de mensuração foi a leitura dos escores realizada por dois patologistas experientes do DIPAT cegados para a identificação das amostras. Diante de discordâncias na interpretação dos resultados da IHQ, uma reunião era agendada para estabelecer um consenso.
III. Variáveis sociodemográficas e patológicas que poderiam atuar como confundidores conhecidos foram previamente estabelecidas para ajustes.

11.8 Cálculo amostral e poder do estudo

Como se trata de um estudo que engloba a população inteira consecutiva elegível do INCA num período de cinco anos, originalmente não houve necessidade de um cálculo de tamanho amostral. Entretanto, considerou-se a importância da avaliação do escore do biomarcador linfócito infiltrante tumoral para a conclusão final do estudo.

Baseando-se em dados históricos que sugerem uma taxa de RPC de 45% para pacientes com CMTN com escore moderado a alto de LITs versus 23% para escore baixo, com um erro alfa de 0,05 e um poder de estudo de 80%, calcula-se que seria necessário um total de 140 pacientes para o estudo ter poder analítico estatístico.

11.9 Financiamento

O projeto contou com apoio financeiro da AstraZeneca do Brasil (CNPJ 60.318.797/0001-00) através de um programa de financiamento de projetos de pesquisas de iniciativa do pesquisador, tendo sido investido um total de R$ 106.006,52 para compra de insumos. O estudo está vinculado a Divisão de Pesquisa Clínica e

Desenvolvimento Tecnológico. O sistema utilizado para coleta e gerenciamento de dados (*OpenClinica Enterprise*) é mantido pela Divisão de Pesquisa.

11.10 Aspectos éticos e regulatórios

11.10.1 Riscos aos pacientes do estudo

Por ser estudo de natureza retrospectiva e observacional, foi baseado na utilização de dados de prontuário e amostras teciduais arquivados em parafina no DIPAT, não havendo riscos físicos diretos aos participantes da pesquisa. Já o risco de quebra de confidencialidade foi prevenido utilizando-se uma planilha de codificação dos pacientes onde somente o número do paciente no estudo será utilizado na coleta, no gerenciamento e na análise dos dados coletados para o estudo.

Conforme o *Anexo C*, foi solicitada isenção do Termo de Consentimento Livre e Esclarecido, sendo aprovado pelo CEP.

Casos com revisão da histologia e IHQ para RE, RP, HER2 diferentes do laudo original serão comunicados à chefia do DIPAT e da Oncologia Clínica do HC3/INCA para providências no manejo clínico das pacientes.

11.10.2 Benefícios ao paciente

Pelo caráter retrospectivo e sem nenhum tipo de intervenção, o estudo não trouxe benefícios diretos aos pacientes. Com os resultados alcançados, novas hipóteses poderão ser levantadas para o desenho de futuros ensaios clínicos com terapia-alvo nesse contexto de tratamento sistêmico neoadjuvante.

11.10.3 Aprovação no Comitê de Ética em Pesquisa

Este projeto foi submetido como protocolo de pesquisa à avaliação do Comitê de Ética em Pesquisa da instituição (CEP/ INCA), sendo aprovado sob o número de CAAE 61675516.9.0000.5274 (*Anexo D*).