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CARCINOMAS DE MAMA INVASORES PRÉ E
PÓS-QUIMIOTERAPIA NEOADJUVANTE E IMPACTO NA SOBREVIVÊNCIA GLOBAL

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Bárbara Jaime Dos Santos

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PÓS-QUIMIOTERAPIA NEOADJUVANTE E IMPACTO NA SOBREVIVÊNCIA GLOBAL**

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Co-orientadora: Prof^º. Dr^ª. Débora Balabram

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“CARCINOMAS DE MAMA INVASORES PRÉ E PÓS-QUIMIOTERAPIA NEOADJUVANTE E IMPACTO NA SOBREVIVÊNCIA GLOBAL”

BÁRBARA JAIME DOS SANTOS

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“Nossas dúvidas são traidoras e nos fazem perder o que, com frequência, poderíamos ganhar, por simples medo de arriscar.”

William Shakespeare

RESUMO

Introdução e objetivo: Fatores preditores clínicos e patológicos bem validados são necessários para guiar decisões terapêuticas, para que os pacientes recebam um tratamento individualizado com maior chance obter benefício da quimioterapia neoadjuvante (Qtneo). A Qtneo pode levar à alterações nos carcinomas de mama invasores (CMI), influenciando as decisões no cenário adjuvante. O objetivo foi identificar fatores que possam prever o nível de resposta do tumor à Qtneo de acordo com o *Residual Cancer Burden* (RCB), além de identificar alterações nos tumores e verificar sua associação com o tempo de sobrevida global (SG). **Materiais e métodos:** Cento e cinco CMI tratados com Qtneo entre 2008 e 2018 foram estudados e comparados parâmetros histológicos e imuno-histoquímicos (IHQ) de amostras de CMI pré e pós-Qtneo e associados à SG. **Resultados:** Após a Qtneo, a maioria dos tumores teve resposta patológica parcial (89; 84,8%) e 16 tumores (15,2%) tiveram resposta patológica completa (pCR), que apresentaram menor risco de morte (HR 0,27; IC: 0,11-0,68; $p = 0,006$). Os seguintes parâmetros foram associados à classe de RCB: tamanho do tumor, com predominância de cT4 na classe III e cT3 nas demais ($p=0,011$); linfonodos regionais, onde apenas o RCB II teve casos N0 ($p<0,001$); células inflamatórias infiltradas no tumor, com predominância de alto índice apenas no RCB 0/1 ($p=0,018$); e expressão de IHQ para linfócitos TCD8+, onde apenas no RCB II predominou baixa expressão ($p=0,016$). Os fatores prognósticos independentes que apresentaram maior risco de morte foram a história familiar de câncer de mama (HR 3,16; $p=0,003$), RCB de classe III (HR 8,77; $p<0,001$) e CMI grau II (HR 3,07; $p = 0,015$) ou grau III (HR 3,97; $p=0,005$). Após a Qtneo foram encontradas alterações no escore de pleomorfismo nuclear do tumor ($p=0,025$); contagem mitótica ($p=0,002$); células inflamatórias infiltradas no tumor ($p=0,016$); carcinoma in situ ($p=0,001$); invasão linfovascular (LVI; $p=0,002$); e expressão de receptores de estrogênio (RE) ($p=0,003$) e progesterona (RP; $p=0,019$); e Ki67 ($p=0,003$). Vinte e seis tumores (30,2%) alteraram o perfil IHQ ($p=0,050$). As características associadas a um maior risco de morte foram: grau histológico III pré-Qt-neo (hazard ratio, HR, 2,94; $p=0,007$), alto pleomorfismo nuclear (HR: 2,53; $p=0,036$) e alto índice Ki67 (HR: 2,47; $p<0,001$); LVI pós-Qtneo (HR 1,90; $p=0,016$) e perfil Luminal B-like (HR: 2,58; $p=0,031$). Além disso, pré- (HR 2,26; $p=0,011$) e pós-Qtneo (HR 2,12; $p=0,038$) contagem mitótica intermediária, pré- (HR: 4,45; $p<0,001$) e pós-Qtneo (HR: 4,52; $p<0,001$) perfil IHQ triplo negativo. As associações com menor risco foram: pré- (HR:

0,35; $p < 0,001$) e pós-Qtneo (HR: 0,39; $p < 0,001$) RE positivo e pré- (HR: 0,37; $p < 0,001$) e pós-Qtneo (HR: 0,57; $p = 0,044$) RP positivo. Mudanças no perfil IHQ foram associadas a SG mais longa ($p = 0,050$). Na análise multivariada, pré-Qtneo grau III, perfil IHQ pré- e pós-Qtneo triplo negativo provaram ser fatores independentes para menor SG. **Conclusão:** História familiar de câncer de mama, classes de RCB e grau histológico são fatores prognósticos independentes. O estadiamento clínico, a presença de infiltrado inflamatório e linfócitos TCD8 intratumorais foram preditores das classes de RCB. As características do tumor e os biomarcadores podem mudar após a QTNEO com impacto na SG, portanto, devem ser reavaliados em amostras residuais para individualizar as decisões terapêuticas.

Palavras-chave: Neoplasias da Mama; Receptores de Estrógeno; Receptores de Progesterona; Receptor, HER-2; Ki-67; Quimioterapia Neoadjuvante.

ABSTRACT

Purpose: Well-validated clinical and pathological predictors are needed to support therapeutic decisions so that patients receive individualized treatment with the greatest chance of benefit from neoadjuvant chemotherapy (NACT). NACT can change invasive breast carcinomas (IBC) and influence decisions on adjuvant treatments. We aimed to identify factors that might predict the level of tumor response by residual cancer burden class (RCB) to NACT, identify tumor changes and to verify their association with patient's overall survival time (OS). **Materials and methods:** One hundred and five invasive breast carcinomas treated with NACT between 2008 and 2018 were studied and compared histological and immunohistochemical (IHC) parameters of pre- and post-NACT IBC samples and associated them with OS. **Results:** After NACT, most tumors had pathological partial response (89; 84.8%) and 16 tumors (15.2%) had pathological complete response (pCR), which had a lower risk of death (HR 0.27; CI: 0.11-0.68; $p = 0.006$). The following parameters were associated with the RCB class: tumor size, with a predominance of cT4 in the RCB III and cT3 in the others ($p=0.011$); regional lymph nodes, where only RCB II had N0 patients ($p<0.001$); tumor infiltrated inflammatory cells, with a predominance of high index only in RCB 0/1 ($p=0.018$); and IHC expression for TCD8+ lymphocytes, where only in RCB II low expression predominated ($p=0.016$). The independent prognostic factors showing higher risk of death were family history of breast cancer (HR 3.16; $p=0.003$), RCB class III (HR 8.77; $p<0.001$) and grade II (HR 3.07; $p = 0.015$) or grade III (HR 3.97; $p=0.005$). Changes were found in tumor nuclear pleomorphism ($p=0.025$); mitotic count ($p=0.002$); tumor-infiltrating inflammatory cells ($p=0.016$); *in situ* carcinoma ($p=0.001$); lymphovascular invasion (LVI; $p=0.002$); and expression of estrogen ($p=0.003$) and progesterone receptors ($p=0.019$); and Ki67 ($p=0.003$). Twenty-six tumors (30.2%) changed the IHC profile ($p=0.050$). Characteristics associated with a higher risk of death were: pre-NACT histological grade III (hazard ratio, HR, 2.94; $p=0.007$), high nuclear pleomorphism (HR: 2.53; $p=0.036$) and high Ki67 index (HR: 2.47; $p<0.001$); post-NACT LVI (HR 1.90; $p=0.016$) and Luminal B-like profile (HR: 2.58; $p=0.031$). Also, pre- (HR 2.26; $p=0.011$) and post-NACT (HR 2.12; $p=0.038$) intermediate mitotic count, pre- (HR: 4.45; $p<0.001$) and post-NACT (HR: 4.52; $p<0.001$) triple-negative IHC profile. Associations with lower risk were: pre- (HR: 0.35; $p<0.001$) and post-NACT (HR: 0.39; $p<0.001$) ER positive, and pre- (HR: 0.37;

p<0.001) and post-NACT (HR: 0.57; p=0.044) PR positive. Changes in IHQ profile were associated with longer OS (p=0.050). In multivariate analysis, pre-NACT grade III and pre-NACT and post-NACT triple negative IHC profile proved to be independent factors for shorter OS. **Conclusion:** Family history of breast cancer, RCB classes and histological grade are independent prognostic factors. Clinical staging, presence of inflammatory infiltrate and intratumoral TCD8 lymphocytes were predictors of RCB classes. Tumor characteristics and biomarkers can change after NACT with an impact on OS, therefore they should be reassessed on residual samples to improve therapeutic decisions.

Keywords: Breast Neoplasms; Receptors, Estrogen; Receptors, Progesterone; Receptor, ErbB-2; Ki-67 Antigen; Lymphocytes, Tumor-Infiltrating; Neoadjuvant Therapy.

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LISTA DE ABREVIATURAS E SIGLAS

AJCC	<i>American Joint Committee on Cancer</i>
ASCO	<i>American Society of Clinical Oncology</i>
CAP	<i>College of American Pathologists</i>
CBN	Cristiana Buzelin Nunes
CMI	Carcinomas mamários invasores
COEP	Comitê de ética em pesquisa
H.E.	Hematoxilina e eosina
HC	Hospital das Clínicas
HER2	Receptor Epidérmico Humano 2
IHQ	Imuno-histoquímica
INCA	Instituto Nacional do Câncer
OMS	Organização Mundial da Saúde
RE	Receptor de Estrógeno
RP	Receptor de Progesterona
RPC	Resposta Patológica Completa
RPP	Resposta Patológica Parcial
TILs	Linfócitos intratumorais
Qtno	Quimioterapia neoadjuvante
TNM	<i>American Joint Committee on Cancer tumor, node and metastasis</i>
UFMG	Universidade Federal de Minas Gerais

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1. Introdução

O câncer de mama é a neoplasia maligna mais frequente em mulheres, apresentando um risco estimado de 66,54 casos a cada 100 mil mulheres e 73.610 novos casos para o ano de 2023 (INCA, 2022). É a principal causa de morte por câncer em mulheres em todo o mundo. Houve uma diminuição da incidência nos países desenvolvidos no início dos anos 2000, como Estados Unidos, Canadá, França e Austrália, parcialmente atribuída a redução do uso de terapia hormonal, porém com o envelhecimento da população, houve um aumento global de novos casos nas últimas décadas (RAKHA, EA; ALLISON, 2020).

Estima-se que até 2040, o número de novos casos de carcinoma mamário deverá crescer em mais de 40%, chegando em torno de 3 milhões de casos por ano. Da mesma forma, a mortalidade deve aumentar em 50%, de 685.000 em 2020 para 1 milhão em 2040 (ARNOLD *et al.*, 2022). Nos Estados Unidos, o número de novos casos estimados em 2022 foi de 287.850 (15% de todos os novos casos de câncer), com 43.250 mortes por câncer de mama (SEER, 2023). No Brasil, o número esperado de novos casos é de 73.610 em 2023 (INCA, 2022). Em países desenvolvidos, existem programas de conscientização e rastreamento eficazes, que permitem a detecção de lesões menores, o que diminui o índice de mortalidade. Já em países subdesenvolvidos, muitas vezes, os tumores são diagnosticados quando se tornam palpáveis, em estágios avançados da doença (BRAY *et al.*, 2018; JATOI; MILLER, 2003).

1.1 Desenvolvimento e progressão

Os principais fatores de risco para o desenvolvimento do câncer de mama podem ser hormonais e/ou genéticos, sendo divididos em esporádicos ou hereditários. Os carcinomas hereditários se devem a mutações genéticas herdadas que são responsáveis por cerca de 12% de todos os tumores, sendo as mutações mais conhecidas, as dos genes BRCA1 e BRCA2 cujas portadoras desenvolvem o tumor em 30 a 90% dos casos dependendo da mutação existente (BRAY *et al.*, 2018; MENEN; TESHOME, 2019). Os

carcinomas esporádicos estão ligados à exposição hormonal, como idade da menarca, menopausa tardia, primeira gravidez acima de 30 anos, nuliparidade, uso de anticoncepcionais por mais de 10 anos e terapia de reposição hormonal. Alguns fatores de risco ambientais podem aumentar a chance de desenvolvimento do câncer, como consumo de álcool, sobrepeso, tabagismo e radioterapia para tratamento de tumores prévios (BRAY *et al.*, 2018; MENEN; TESHOME, 2019).

Tanto os fatores genéticos, quanto os epigenéticos, levam a alterações no ciclo e na divisão celular, não ativando o processo de apoptose, reparo do DNA e supressão tumoral, levando ao surgimento de células que adquirem capacidade de proliferação autônoma, invasão de tecidos adjacentes e que podem desenvolver a capacidade de se destacar do tumor inicial, migrar para outros locais (metástase) e proliferar em outros órgãos (BRASILEIRO FILHO, 2021). A migração destas células pode ocorrer através da corrente sanguínea, chegando a órgãos mais distantes, ou do sistema linfático, aonde podem desenvolver metástases nos linfonodos mais próximos ao sítio primário (BRASILEIRO FILHO, 2021).

Além da capacidade de metastatizarem, as células tumorais adquirem outras propriedades; uma delas é a evasão do sistema imune, que é ocasionada pela interação das células tumorais com outras células do microambiente tumoral, local onde há troca de sinais celulares entre as células tumorais e do estroma, resultando em inibição ou ativação de resposta inflamatória, de cujo balanço depende o desenvolvimento, progressão e a regressão dos carcinomas mamários invasores (CMI)(WÜRFEL *et al.*, 2018).

1.2 Microambiente tumoral

O estroma das neoplasias é composto por células endoteliais de vasos sanguíneos e linfáticos, fibroblastos, mastócitos e vários tipos de células imunitárias de defesa inata e adaptativa (células dendríticas, linfócitos, macrófagos, neutrófilos e eosinófilos). Inicialmente acreditava-se que a presença destas células significava presença de resposta imune antitumoral, mas alguns estudos conseguiram mostrar que diferentes tipos de células e de

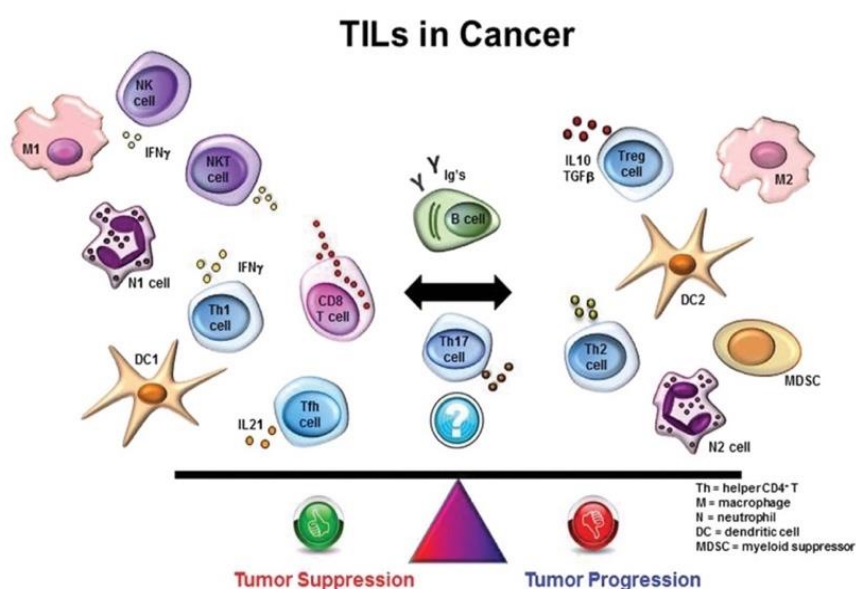
resposta inflamatória poderiam levar a efeitos diferentes nos tumores (HAN *et al.*, 2022; LI, YING *et al.*, 2021; LIANG *et al.*, 2021).

Os linfócitos TCD4⁺ podem se diferenciar exercendo dois tipos de resposta, TH1 Th2 ou Th17. Se a interleucina (IL) 12 predomina no microambiente, produzida por células apresentadoras de antígenos, ocorrerá a ativação deste linfócito com diferenciação em Th1. Esse linfócito produz a IL-2 e interferón-gama, que além de serem indutores de proliferação de outros linfócitos TCD4⁺ e TCD8⁺, conseguem atrair e ativar macrófagos iniciando resposta imunitária celular. Quando a IL-4 predomina no microambiente, a diferenciação será em Th2, produtor de várias interleucinas que são capazes de recrutar e ativar linfócitos B, além de recrutar mastócitos, basófilos e eosinófilos, induzindo resposta humoral e celular. Se houver produção de IL-23 e no microambiente houver IL-6 e TGF- β , esses linfócitos se diferenciam em Th17, produtores de IL-17, que induz ativação endotelial e de leucócitos, potencializando resposta inflamatória local (BEYER; SCHULTZE, 2009; HAN *et al.*, 2022).

Os linfócitos citotóxicos TCD8⁺ são considerados células eficazes na destruição das células tumorais, reconhecendo o alvo quando este se associa a antígenos do MHC de classe I, levando a lise celular. Algumas células tumorais não expressam o MHC de classe I, impedindo a atuação citotóxica (BEYER; SCHULTZE, 2009; HAN *et al.*, 2022).

Os macrófagos são ativados também de acordo com o tipo de quimiocinas e citocinas presentes no microambiente, podendo se diferenciar em M1 e M2 (MURRAY *et al.*, 2014). Os macrófagos M1 ou classicamente ativados, são ativados por lipopolissacarídeos de substratos microbianos, ligantes para receptores do tipo Toll e citocinas, como o interferón-gama. Esses macrófagos produzem fator de necrose tumoral alfa, as interleucinas 1 β e 12 (pró-inflamatórias), espécies reativas de oxigênio e nitrogênio, que serão essenciais para resposta imune anti-tumoral. Já os macrófagos M2 ou alternativamente ativados, são ativados pelas interleucinas 4 e 13. Sua principal função será de estimular a resposta Th2, com produção de IL-10, TGF- β e quimiocinas, acabando por suprimir a resposta Th1 (MURRAY *et al.*, 2014; YUNNA *et al.*, 2020).

Então, os linfócitos TCD4+ produtores de IFN- γ (Th1), macrófagos ativados do tipo M1 e linfócitos citotóxicos TCD8+ agem levando à supressão tumoral e, quando eles são a maioria das células inflamatórias presentes no tumor, estes apresentam melhor prognóstico; já em contraste, tumores com predomínio de linfócitos Th2, macrófagos alternativamente ativados M2 e células mieloides supressoras, teriam pior prognóstico devido à progressão tumoral (Figura 1) (BRASILEIRO FILHO, 2021; LI, YING *et al.*, 2021; LIANG *et al.*, 2021).



From: The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014

Figura 1: Influência do tipo de células inflamatórias nos tumores

A contagem e identificação das células inflamatórias nos tumores é realizada através de avaliação visual ao microscópio óptico, em lâmina com coloração de rotina pela hematoxilina e eosina (HE) (PELEKANOU *et al.*, 2017). As células inflamatórias podem ainda, ser identificadas e separadas em células que conferem bom prognóstico com ação supressora, e células que conferem mau prognóstico, levando à progressão tumoral, através de utilização de

marcadores fenotípicos de células inflamatórias. Essa identificação ocorre através da análise da existência dos tipos celulares, quimiocinas e citocinas que permitem conhecer o tipo de resposta imune que está ocorrendo no microambiente tumoral (BRASILEIRO FILHO, 2021; LIANG *et al.*, 2021).

As células imunitárias vêm sendo cada vez mais valorizadas e estudadas, principalmente os linfócitos intratumorais (TILs), mostrando que sua presença confere melhor prognóstico para as pacientes, além de serem reconhecidos como um fator preditivo para a resposta à quimioterapia (CASTANEDA *et al.*, 2016; DENARDO; COUSSENS, 2007; DENKERT *et al.*, 2010; DIECI, MARIA VITTORIA *et al.*, 2018; LIANG *et al.*, 2021; TAN *et al.*, 2018).

O tratamento por quimioterapia pode levar à formação de novos epitopos imunogênicos, secreção de citocinas, apresentação cruzada de antígenos, ativação de células dendríticas e indução de células T citotóxicas específicas de tumores, levando à indução da resposta imune antitumoral (PELEKANOU *et al.*, 2017). Alguns estudos com pacientes que receberam agentes citotóxicos, como antraciclinas e taxanos, verificaram ainda, o aumento do número de linfócitos intratumorais, além da indução de resposta imune antitumoral (DEMARIA *et al.*, 2001; DIECI, M. V. *et al.*, 2014; LADOIRE *et al.*, 2008). Apesar desta característica positiva, a quimioterapia também pode, por outro lado, exercer efeito citotóxico nas células do sistema imune, impedindo sua atuação contra as células tumorais (GARCÍA-MARTÍNEZ *et al.*, 2014; GONZALEZ-ANGULO *et al.*, 2012).

1.3 Tratamento

O tratamento do câncer de mama é baseado no controle local (cirúrgico) associado ou não à terapia sistêmica e radioterapia (WILLIAMS *et al.*, 2006). A terapia sistêmica pode ser realizada através de quimioterapia e de terapias alvo-específicas. A quimioterapia utiliza medicamentos que destroem as células tumorais durante seu processo de divisão celular através de toxicidade e as terapias alvo-específicas visam alvos moleculares, impedindo a proliferação tumoral, podendo ser realizada em dois momentos, antes da ressecção

cirúrgica (neoadjuvante) ou após a ressecção cirúrgica do tumor (adjuvante) (ALLRED, 2010; GOWN, 2008).

A quimioterapia adjuvante é administrada após a ressecção cirúrgica da lesão, a fim de atingir as células tumorais residuais ou que se desprenderam do tumor primário, que poderiam gerar metástases (KUMAR; ABBAS; FAUSTO, 2010).

A quimioterapia neoadjuvante é um método que visa reduzir o volume do tumor antes da ressecção cirúrgica. Inicialmente, quando começou a ser utilizada na rotina clínica dos anos 70, era indicada para pacientes com tumores localmente avançados e inoperáveis para que estes se tornassem operáveis (AL-SALEH *et al.*, 2021; REDDY; APPLE, 2017). Atualmente, a quimioterapia neoadjuvante também é indicada para pacientes com tumores operáveis; porém, muito grandes, que inicialmente seriam submetidas à mastectomia, diminuindo o volume tumoral e tornando possível a realização de cirurgia conservadora, e para pacientes com tumores em estágios iniciais, especialmente nos tumores HER2 positivos ou triplo-negativos, por apresentarem melhor resposta a quimioterapia neoadjuvante (CANDÁS *et al.*, 2021; REDDY; APPLE, 2017).

O tipo de resposta à terapia neoadjuvante é considerado importante fator prognóstico, podendo ser de dois tipos, resposta patológica completa (RPC) e resposta patológica parcial (RPP). Podem ser classificados como RPC, casos em que ocorreu o desaparecimento total do tumor na mama e nas metástases, mesmo em presença de carcinoma *in situ* residual; e como RPP, casos que ainda apresentam células tumorais invasivas ou metastáticas residuais após o tratamento (AMOROSO *et al.*, 2015; KRUG *et al.*, 2018; KUMAR; ABBAS; FAUSTO, 2010). Uma importante ferramenta desenvolvida pelo MD Anderson Cancer Center é a calculadora da massa tumoral residual, conhecida como *Residual Cancer Burden* (RCB), que utiliza alguns parâmetros identificados na avaliação da peça cirúrgica após o tratamento com quimioterapia neoadjuvante, como tamanho e porcentagem de células neoplásicas *in situ* e invasivas do tumor residual, presença e tamanho das metástases em linfonodos axilares, e o resultado do cálculo está diretamente relacionado a progressão do tumor e sobrevida global dos pacientes (MITTENDORF *et al.*, 2011). A maior importância da quimioterapia neoadjuvante, que difere da

quimioterapia adjuvante, é a possibilidade de se observar os efeitos da quimioterapia diretamente no tumor, verificando se este responde ou não àquele tipo de terapia, possibilitando a revisão terapêutica em tumores com baixa resposta (AMOROSO *et al.*, 2015; KRUG *et al.*, 2018; KUMAR; ABBAS; FAUSTO, 2010).

A quimioterapia apresenta toxicidade significativa e vários efeitos colaterais que interferem na qualidade de vida da paciente, além dos custos elevados. A grande variedade de terapias existentes atualmente, tem impacto na clínica de rotina, e se baseia, cada vez mais, na avaliação de fatores prognósticos e preditivos, clínicos e patológicos bem validados para dar suporte às decisões terapêuticas (MCSHANE *et al.*, 2005). Para garantir maior chance de obter melhor benefício com menor efeito colateral à paciente, existe uma necessidade crescente na correta identificação e validação de biomarcadores como fatores prognósticos e preditivos no câncer de mama (RAKHA, EMAD A.; ELLIS, 2011).

1.4 Fatores Prognósticos e Preditivos

Vários fatores são considerados como prognósticos e/ou preditivos. A presença de metástases linfonodais, por exemplo, é considerada um dos principais fatores prognósticos para pacientes com câncer de mama, juntamente com a avaliação imuno-histoquímica (IHQ) de biomarcadores presentes nas células tumorais, a diferenciação da lesão (tipo histológico e grau do tumor) e o índice proliferativo (KUMAR; ABBAS; FAUSTO, 2010; WALKER *et al.*, 2012).

As características clínicas das pacientes e os aspectos patológicos e biológicos dos tumores permitem prever a evolução clínica da doença ou sobrevida das pacientes no momento do diagnóstico inicial, auxiliando na decisão terapêutica, sendo considerados marcadores prognósticos (MCSHANE *et al.*, 2005). Estes marcadores são determinados através de exame clínico e exame histopatológico do tumor, incluindo variáveis tempo-dependentes, biológicas e fatores como a idade e status menopausal das pacientes. As variáveis tempo-dependentes são as mesmas que são utilizadas para composição do estadiamento da *American Joint Committee on Cancer tumor, node and*

metastasis (TNM), que leva em conta o tamanho do tumor, a presença de metástases em linfonodos e à distância, cujo resultado nos dá uma importante informação prognóstica (HORTOBAGYI *et al.*, 2018). As variáveis biológicas são aquelas identificadas através da análise histopatológica do tumor, como definição do tipo e grau histológicos, índice proliferativo, índice de crescimento e parâmetros moleculares (expressão de biomarcadores e perfil molecular ao nível de DNA, RNA ou proteico) (ELSTON; ELLIS, 1991).

Já os marcadores preditivos, são características clínicas, patológicas e biológicas que permitem avaliar a resposta individualizada que uma paciente pode obter em tratamentos específicos, onde o alvo é uma molécula expressa pelo tumor envolvida no crescimento e na proliferação celular. Assim espera-se melhor resposta ao tratamento quando as células tumorais apresentam alta expressão de moléculas alvo, ou podendo-se evitar a utilização de terapia em tumores sem expressão dessas moléculas, predizendo resposta negativa a certos tratamentos (HAMBURG; COLLINS, 2010; LEONG; COOPER; LEONG, 2010).

Apesar de vários marcadores terem sido estudados, atualmente somente são utilizados como fatores preditivos de resposta terapêutica em câncer de mama, os receptores hormonais (RH - receptores de estrógeno e progesterona) e o Receptor do fator de crescimento epidérmico humano-2 (HER2) (ALLISON *et al.*, 2020; ELSTON; ELLIS, 1991; GOBBI, 2012; UNTCH *et al.*, 2013; WOLFF *et al.*, 2018). Estes marcadores também fornecem informação prognóstica isoladamente, ou em conjunto com a avaliação da proteína Ki67, marcador de proliferação celular, que nos permite categorizar os tumores de acordo com o subtipo molecular. Os tumores podem ser categorizados como Luminal A-like (RH positivos, HER2 negativo e Ki67 baixo); Luminal B-like (RE positivo com qualquer expressão de PR e HER2 positivo ou alto Ki67); HER2-like (RH negativos, HER2 positivo e qualquer expressão de Ki67); ou Triplo negativo (RH e HER2 negativos com qualquer expressão de Ki67) (GOLDHIRSCH *et al.*, 2013). O Ki-67 tem sido indicado para definir quimioterapia em pacientes com tumores receptores hormonais positivos (NIELSEN *et al.*, 2021).

1.5.1 Receptores hormonais

Em 1896, Beatson observou em uma jovem mulher com carcinoma inflamatório que a ooforectomia provocava respostas em tumores localmente avançados. Estudos posteriores comprovaram o valor da terapia endócrina para a evolução das pacientes, mudando seu prognóstico (BEATSON, 1896).

Vários tipos de terapia endócrina são utilizados em combinação ou isoladamente: ablação cirúrgica de órgãos produtores de estrógeno ou medicamentos que inibem a hipófise; medicamentos que atuam nos receptores de estrógeno e inibidores da aromatase (ALLRED, 2010). A terapia endócrina é a mais utilizada atualmente em tumores sensíveis a hormônios. O tamoxifeno, medicação mais utilizada nas terapias anti-hormonais em tumores RE e RP positivos, se liga aos receptores de estrógeno, impedindo a ligação do hormônio a seu receptor, tendo como consequência positiva a inibição do crescimento induzido pelo estrógeno. Estudos mostram que o Tamoxifeno aumenta a sobrevida e reduz as chances de recorrência em pacientes com tumores de mama RE positivos (ALLRED, 2010).

Os receptores de estrógeno (RE) são membros da superfamília de receptores nucleares de fatores de transcrição induzidos por ligante, são ativados pelo hormônio estrógeno e regulam o crescimento e a diferenciação de células epiteliais normais na mama. Em tumores, esta via de sinalização celular pode estar ativa em diferentes níveis, sendo prejudicial, pois leva ao crescimento das células tumorais induzido por estrógeno (ALLRED, 2010).

Os receptores de progesterona (RP) são membros dos fatores de transcrição ativados por ligante da família de receptores nucleares esteroides, e são ativados pelo hormônio progesterona. Estes receptores auxiliam a regulação de funções celulares normais primordiais, como a proliferação celular, portanto sua ativação é prejudicial em tumores por levar a proliferação. A presença de RP positiva indica que a via de sinalização celular do crescimento induzida por estrógeno está funcionando perfeitamente, pois a expressão do RP é regulada pelo RE. Em pacientes tratados com terapia hormonal, a presença de RP nos tumores está associada com baixa taxa de recorrência (ALLRED, 2010).

A avaliação dos receptores hormonais nos tumores tem como principal função avaliar a resposta à terapia endócrina (ARIAS; MAZZA; FUNKE, 2003; OYAMA *et al.*, 2007). Devido à importância clínica destes marcadores, um grupo de pesquisadores da *American Society of Clinical Oncology (ASCO)* e do *College of American Pathologists (CAP)* definiram recomendações e diretrizes para os testes de IHQ e as análises destes marcadores (ALLISON *et al.*, 2020; HAMMOND *et al.*, 2011).

1.4.2 Fator de Crescimento Epidérmico Humano 2

Pertencente ao grupo de receptores do fator de crescimento epidérmico, composto pelo EGFR (HER1), HER2, HER3 e HER4, o fator de crescimento epidérmico humano 2 ou HER2 (c-erbB-2 ou HER2-neu) é uma proteína transmembrana de 185 kd codificada pelo gene localizado no cromossomo 17 com atividade de tirosina-quinase (ALLRED, 2010; GOWN, 2008; LEONG; COOPER; LEONG, 2010; ROSS *et al.*, 2009). Expressa em baixos níveis em tecidos normais, a proteína receptora HER2 regula várias funções celulares normais como a proliferação, sobrevivência e a apoptose. Em 10 a 20% dos tumores, está superexpressa e associada a proliferação, diferenciação, adesão e motilidade celular, progressão tumoral, metastatização, angiogênese e redução de apoptose (ALLRED, 2010; GOWN, 2008; LEONG; COOPER; LEONG, 2010; ROSS *et al.*, 2009).

A superexpressão de HER2 está associada a pior prognóstico e má resposta ao tamoxifeno e quimioterapia convencional, e a melhor resposta terapêutica à terapia-alvo anti-HER2 (GOWN, 2008; HAMBURG; COLLINS, 2010; WOLFF *et al.*, 2013). Os tumores com superexpressão do HER2 são mais comumente de grau histológico intermediário ou alto, mais frequentemente são negativos para receptores de estrógeno e progesterona, e apresentam metástase linfonodal ao diagnóstico inicial (GOWN, 2008; HAMBURG; COLLINS, 2010; NUNES, C. B. *et al.*, 2013; WOLFF *et al.*, 2013).

A avaliação da superexpressão do HER2 inicialmente é realizada por IHQ e, havendo resultados equívocos (2+), é necessária confirmação da amplificação do gene através de hibridização *in situ* (ISH). A ASCO/CAP desenvolveu

algoritmos de análise para HER2, tanto por IHQ, quanto por FISH, além de indicar diretrizes para a fase pré-analítica das reações IHQ (WOLFF *et al.*, 2013, 2018).

Um marco na trajetória do tratamento das pacientes foi a liberação de terapias alvo-específicos pela *Food and Drug Administration (FDA)*. A medicação mais utilizada em tumores com superexpressão de HER2 é o Trastuzumab (Herceptin™), anticorpo monoclonal humanizado anti-HER2, que se utilizado em tumores HER2 positivos, melhora a taxa de sobrevida e reduz o risco de recorrência e mortalidade, mas além dele existem outros, como lapatinib (Tykerb™), pertuzumab (Perjeta™), e trastuzumab-emtansine (Kadcyla™), também conhecida como T-DM1 (GOWN, 2008; SATTI, 2011; WOLFF *et al.*, 2013).

Recentemente, ensaios clínicos e novas evidências demonstraram benefícios clínicos significativos para o uso de novos conjugados droga-anticorpo direcionados ao HER2 em carcinomas mamários avançados com expressão baixa da proteína: "HER2 low" (pontuação HER2 IHC de 1+ ou 2+ sem amplificação gênica). Estas evidências fortalecem a necessidade de uma avaliação correta da expressão de HER2, definindo e favorecendo pacientes com indicação para este novo tratamento. Ainda, novos estudo clínicos estão sendo validados para uso destes medicamentos em tumores iniciais com expressão "HER2 low" (MARCHIÒ *et al.*, 2021; ZHANG *et al.*, 2022).

1.4.3 Ki67

A proteína Ki67 foi denominada de Ki em homenagem a cidade e Universidade de Kiel na Alemanha e 67 devido ao número do anticorpo capaz de identificá-lo. A proteína codificada pelo gene MK167, localizado no cromossomo 10q25-ter está envolvida nas etapas iniciais de síntese de RNA ribossomal, e é expressa em todas as fases do ciclo celular, exceto em G0 (ALLISON, 2018).

O Ki67 é considerado um dos principais marcadores de proliferação celular e sua superexpressão é associada a piores taxas de sobrevida (MATSUBARA *et al.*, 2013; YAMAZAKI *et al.*, 2015). Sua avaliação é utilizada tanto para distinguir os tipos do câncer de mama, como para auxiliar na decisão

terapêutica sendo informação prognóstica (MATSUBARA *et al.*, 2013; YAMAZAKI *et al.*, 2015). A marcação do índice de proliferação celular e o número de mitoses estão diretamente relacionados com o crescimento de tumores, assim sendo, quanto maior o índice proliferativo, mais rápida a evolução tumoral (YERUSHALMI *et al.*, 2010).

Existem muitas diferenças de opiniões em relação à porcentagem de células marcadas que define como alto ou baixo índice de proliferação, conferências como a de Saint Gallen na Suíça recomendam pontos de corte (*cut-off*) para a análise deste marcador que variaram ao longo dos anos e também do Grupo internacional de estudo em Ki67 no câncer de mama, sendo que o ponto de corte mais utilizado é acima de 20% de núcleos marcados sendo considerado como alto índice (GNANT; HARBECK; THOMSEN, 2017; NIELSEN *et al.*, 2021; UNTCH *et al.*, 2013). Em 2019 houve uma reunião de consenso do Grupo Internacional de Pesquisa do Ki67 em Câncer de Mama (International Ki67 in Breast Cancer Working Group, IKWG), com as mesmas recomendações de consenso para avaliação dos receptores hormonais e HER2, recomendando avaliação visual das marcações imuno-histoquímicas e reconhecendo a importância do Ki67 como marcador prognóstico em tumores receptores hormonais positivos e HER2 negativos, identificando pacientes que não necessitam realizar quimioterapia adjuvante. Neste grupo de pacientes (T1-2, N0-1), o consenso IKWG é de Ki67 igual ou inferior a 5%, ou igual ou superior a 30%, podem ser usados para estimar prognóstico (NIELSEN *et al.*, 2021).

1.4.4 Avaliação dos biomarcadores

Inicialmente, a avaliação dos biomarcadores é feita em amostras de biópsia incisional ou biópsia por agulha grossa (*core biopsy*) em todos os tumores recém diagnosticados para avaliação do tipo e grau histológicos, além de avaliar a possibilidade de indicação de terapia neoadjuvante e, posteriormente, também podem ser avaliados nas amostras de ressecção cirúrgica (VON MINCKWITZ *et al.*, 2011).

A avaliação dos receptores hormonais e do Ki67 é realizada através da imuno-histoquímica (IHQ), método que, normalmente, utiliza tecidos previamente fixados em formalina, incluídos em parafina, submetidos à microtomia e à utilização de anticorpos específicos para a identificação de produtos celulares ou marcadores de superfície através de imunomarcação (HAMMOND *et al.*, 2011; KUMAR; ABBAS; FAUSTO, 2010; ROCHA *et al.*, 2009).

A avaliação do HER2 pode ser realizada tanto por imuno-histoquímica, avaliando a superexpressão da proteína, quanto por métodos de hibridização *in situ* fluorescente (FISH) ou cromogênica (CISH), avaliando a amplificação gênica (ROCHA *et al.*, 2009). Rotineiramente, é feita pela IHQ por ser um método de custo mais baixo e de mais fácil execução (DOWSETT *et al.*, 2007; NUNES, C. B. *et al.*, 2008; WOLFF *et al.*, 2013, 2018).

1.4.5 Expressão de biomarcadores em diferentes amostras do tumor

Alguns pacientes apresentam resistência à terapia-alvo, sendo uma explicação para isto, a possibilidade de discordância entre o perfil molecular do tumor primário em diferentes amostras (AITKEN *et al.*, 2010; BA; LIU; JIN, 2014; RAICA *et al.*, 2014). Há várias teorias que suportam a ideia de diferenças entre o perfil imuno-histoquímico da amostra obtida inicialmente por biópsia de agulha e a amostra proveniente da ressecção tumoral, como as diferenças na execução da técnica e na análise das reações de IHQ, a evolução biológica, a heterogeneidade do tumor de mama e o recebimento de terapia neoadjuvante (CANDÁS *et al.*, 2021; KATAYAMA *et al.*, 2021; LI, PEIFENG *et al.*, 2013; NIIKURA *et al.*, 2016). Essas alterações podem levar a mudanças no estado dos receptores, levando à resistência à terapia (BA; LIU; JIN, 2014).

Vários autores relatam divergência na expressão dos biomarcadores entre as amostras, mas a falta de recomendações específicas para casos de pacientes que receberam quimioterapia neoadjuvante faz com que a escolha terapêutica no cenário adjuvante seja divergente entre os médicos, onde alguns alteram o terapia se houve mudança e outros preferem considerar o status dos biomarcadores pré-terapia (AL-SALEH *et al.*, 2021; CANDÁS *et al.*, 2021; KATAYAMA *et al.*, 2021; NIIKURA *et al.*, 2016; REDDY; APPLE, 2017). Se

não há avaliação do perfil biológico e imuno-histoquímico de todas as amostras do tumor, pacientes podem estar recebendo terapias desnecessárias em tumores em que o status do receptor era positivo na amostra anterior, mas negativo em outra amostra, ou ainda, estar deixando de receber uma terapia que poderia aumentar a sobrevida em tumores que os receptores eram negativos, mas se tornaram positivos em outra amostra (ADAMCZYK *et al.*, 2012; YAO *et al.*, 2014).

2. Justificativa

É importante conhecer os efeitos da quimioterapia neoadjuvante nos carcinomas mamários invasores, como o tipo de resposta patológica, completa ou incompleta, e quais fatores poderiam influenciar no tipo de resposta. O tipo de resposta orienta a conduta clínica e terapêutica, pois informa a eficácia da terapia *in vivo*. Ainda, as características histopatológicas e expressão dos biomarcadores tumorais podem sofrer mudanças no tumor residual, podendo levar a possíveis alterações na condução da terapia, evitando que pacientes deixem de receber terapias que poderiam aumentar a sobrevida e que terapias desnecessárias, potencialmente tóxicas e de alto custo, poderiam ser evitadas naquelas pacientes que não responderiam a elas.

Conhecer o impacto da mudança destas características histológicas e dos biomarcadores na sobrevida global das pacientes, é importante para definir a real necessidade de reavaliação histológica e IHQ nos tumores residuais, podendo auxiliar na decisão de melhor conduta clínica após a terapia neoadjuvante. Atualmente, ainda não há consenso na literatura se os biomarcadores devem ser reavaliados nas amostras de tumores residuais, ficando a critério clínico do oncologista.

3. Objetivos

3.1 Objetivos gerais:

Estudar os padrões de resposta patológica e os efeitos da quimioterapia neoadjuvante nos carcinomas invasores de mama buscando identificar os fatores patológicos relacionados com melhor resposta de acordo com a classe de RCB à quimioterapia neoadjuvante e maior sobrevida das pacientes.

3.2 Objetivos específicos:

- Avaliar os tipos de resposta patológica à quimioterapia neoadjuvante nos tumores de pacientes tratadas no Hospital das Clínicas da UFMG e correlacionar cada tipo de resposta patológica com a sobrevida global das pacientes;
- Correlacionar os parâmetros clínicos das pacientes, histológicos e imuno-histoquímicos das amostras tumorais pré-terapia com o tipo de resposta patológica;
- Correlacionar os parâmetros histológicos e imuno-histoquímicos das amostras de tumores residuais (resposta patológica parcial) pós-terapia com a sobrevida das pacientes;
- Comparar as características histológicas e imuno-histoquímicas dos tumores em amostras pré-terapia neoadjuvante com as amostras da doença residual e avaliar se a mudança nestes parâmetros impactou na sobrevida das pacientes com tumores residuais pós-terapia;

4. Materiais e métodos

4.1 Seleção dos casos

Trata-se de avaliação retrospectiva de carcinomas mamários invasores de pacientes que realizaram terapia neoadjuvante no período de 2008 a 2018. Foram selecionados 105 casos diagnosticados, tratados clínica e cirurgicamente no Hospital das Clínicas da Universidade Federal de Minas Gerais (HC-UFMG), e que tinham material arquivado no Laboratório de Patologia Mamária da Faculdade de Medicina da UFMG. As pacientes foram separadas de acordo com o tipo de resposta patológica dos tumores à terapia: parcial ou completa.

4.2 Critérios de inclusão e exclusão

Foram incluídos os casos que preencheram os seguintes critérios:

- Ter realizado diagnóstico inicial (biópsia), terapia e cirurgia no HC-UFMG;
- Ter as lâminas coradas em H.E. e blocos de parafina dos tumores disponíveis no arquivo;
- Ter material suficiente dos tumores nas amostras pré e pós-terapia para realizar as análises.

Foram excluídos os casos que preencheram os seguintes critérios:

- Ter realizado diagnóstico inicial em outro serviço;
- Casos em que as lâminas ou blocos não foram encontrados no arquivo;
- Amostras insuficientes no bloco de parafina para novos cortes.

4.3 Avaliação Histológica

Foi realizada revisão de todas as lâminas originais (coradas em H. E.) dos casos selecionados, pré e pós terapia neoadjuvante, quanto ao diagnóstico,

classificação e graduação histológica dos tumores, de acordo com os critérios diagnósticos propostos por Elston e Ellis e pela Organização Mundial da Saúde (ELSTON; ELLIS, 1991; LAKHANI SR, ELLIS IO, SCHNITT SJ, TAN PH, 2012). O grau histológico final foi baseado na soma de três parâmetros, sendo eles: formação tubular, pleomorfismo nuclear e índice mitótico, que foram graduados de I a III (ELSTON; ELLIS, 1991; LAKHANI SR, ELLIS IO, SCHNITT SJ, TAN PH, 2012) . A avaliação do infiltrado inflamatório foi realizada de acordo com os critérios propostos pelo *International TILs Working Group*, agrupando-se os níveis de infiltrado em ausente, baixo (1 a 20%) e alto (acima de 21%) (DIECI, MARIA VITTORIA *et al.*, 2018) (Figura 2).

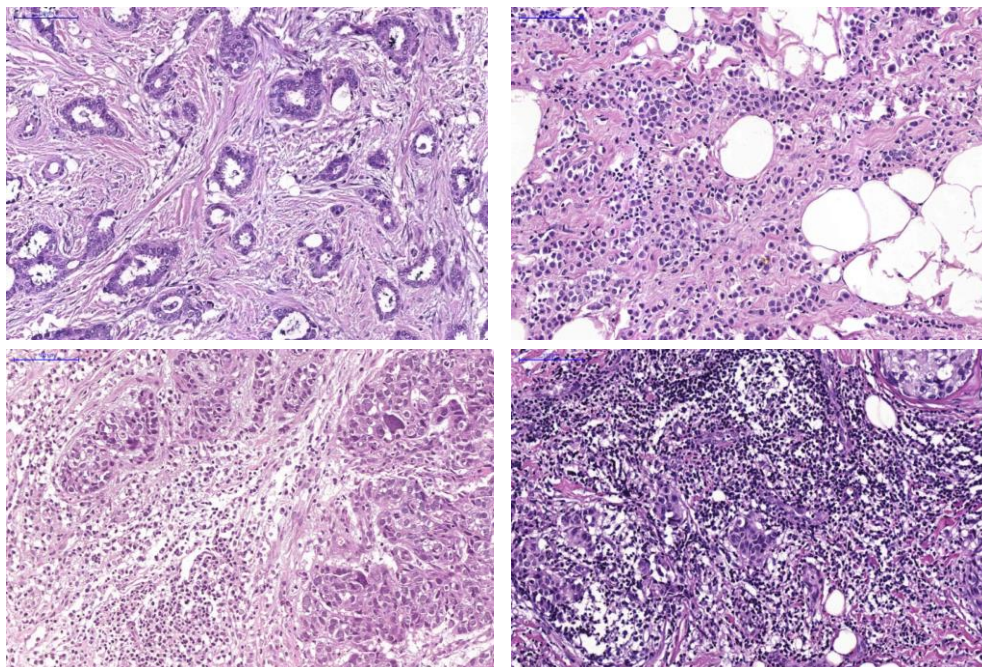


Figura 2: Avaliação dos níveis de infiltrado inflamatório intratumoral - A – Carcinoma Tubular – Ausente/Discreto, B- Carcinoma Lobular Pleomórfico – Discreto, C- CDI – Moderado, D- CDI – Acentuado.

4.4 Imuno-histoquímica

Foram realizadas reações IHQ para RE, RP, HER2 e Ki67 nas amostras pré e pós quimioterapia. Para os receptores hormonais utilizou-se o Kit PharmaDX

(Dako Cytomation); para o Ki67 foi utilizado anticorpo primário Dako (Dako Cytomation) e o kit Novolink (Leica); e para a avaliação do HER2 foi utilizado o Kit HercepTest, (Dako Cytomation). As reações foram realizadas no Laboratório de Patologia Mamária da Faculdade de Medicina da UFMG, utilizando protocolos padronizados, seguindo as recomendações dos fabricantes.

Os blocos de parafina foram submetidos à microtomia, obtendo-se cortes histológicos com espessura de 4µm colocados em lâminas com carga elétrica sendo então, submetidas à técnica de imuno-histoquímica (IHQ).

Os cortes foram desparafinizados, desidratados em álcool e corados manualmente para os marcadores com utilização de protocolos validados previamente em nosso laboratório. Informações sobre os clones dos anticorpos, sistemas de detecção, métodos de reativação antigênica e diluições estão na Tabela 1. As lâminas foram contra-coradas com hematoxilina e montadas com lamínula e verniz.

TABELA 1: Anticorpos, clones, fabricantes, sistemas de detecção, diluições dos anticorpos e recuperação antigênica

Anticorpo	Clone	Fabricante	Sistema de detecção	Diluição	Recuperação antigênica
HER2	A0485 (HercepTest™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Water bath – Epitope retrieval solution, pH 6, Agilent Technologies , 35 minutes
RE	1D5 (PharmaDX™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies , 30 minutes
RP	PGR1294 (PharmaDX™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies , 30 minutes
Ki67	MIB1	Agilent Technologies, United States of America	Detection and amplification system, Novolink, Leica Biosystems, Newcastle upon Tyne, UK	Ready to use	Steamer – Target Retrieval Solution high pH, Agilent Technologies , 30 minutes

CD4	4B12	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Water bath – Epitope retrieval solution, pH 6, Agilent Technologies, 35 minutes
CD8	C8/144B	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies, 30 minutes
CD68	KP1	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies, 30 minutes

As reações imuno-histoquímicas foram realizadas com controles positivos e negativos conhecidos. As reações para RE, RP e HER2 foram realizadas com controles fornecidos pelos fabricantes.

As lâminas foram analisadas por uma patologista experiente (CBN). Os critérios empregados para a análise imuno-histoquímica dos receptores hormonais foram baseados nos guias de recomendação da ASCO/CAP, onde a marcação maior que 1% das células neoplásicas com intensidade moderada ou forte é considerada como resultado positivo (ALLISON *et al.*, 2020; HAMMOND *et al.*, 2011). Os tumores foram também semi-quantitativa e qualitativamente avaliados para RE e RP segundo ALLRED, 2010 (Tabela 2).

TABELA 2: Escore para interpretação de imunocoloração para os receptores hormonais, segundo Allred (2010)

Intensidade da Imunorreatividade	Escore	Proporção de reatividade	Escore
Ausência de reatividade	0	Ausência de reatividade	0
Reatividade fraca	1	<1% núcleos reativos	1
Reatividade moderada	2	1–10% núcleos reativos	2
Reatividade forte	3	11–33% núcleos reativos	3
	-	34–66% núcleos reativos	4
	-	67–100% núcleos reativos	5
Soma dos escores de proporção e intensidade			
Escore total		Interpretação	
0 -2		Negativo	
3 -8		Positivo	

A análise da expressão da proteína HER2 foi baseada no guia de recomendação da ASCO/CAP para HER2 de 2018 (WOLFF *et al.*, 2013, 2018), Tabela 3.

TABELA 3: Sistema de escore recomendado para avaliação do HER2 pelo guia ASCO/CAP (WOLFF *et al.*, 2018)

Escore de interpretação	Achado microscópico
0 NEGATIVO	Não identificada nenhuma coloração na membrana citoplasmática ou Coloração fraca envolvendo parcialmente a circunferência da membrana citoplasmática em $\leq 10\%$ das células neoplásicas.
1+ NEGATIVO	Coloração fraca envolvendo parcialmente a circunferência da membrana citoplasmática em $> 10\%$ das células neoplásicas.
2+ EQUÍVOCO	Coloração fraca/moderada envolvendo toda a circunferência da membrana citoplasmática $> 10\%$ das células neoplásicas.
3+ POSITIVO	Coloração completa, forte, envolvendo toda a circunferência da membrana citoplasmática em $>10\%$ das células neoplásicas.

A interpretação da marcação pelo Ki67 foi semi-quantitativa, verificando-se a porcentagem de núcleos marcados, observados nas áreas de maior marcação (*hot spot*) e em áreas menos marcadas, obtendo-se uma marcação média, avaliando-se pelo menos 300 células consecutivas, quando disponíveis (CURIGLIANO *et al.*, 2013; NIELSEN *et al.*, 2021; UNTCH *et al.*, 2013; YERUSHALMI *et al.*, 2010).

As lâminas coradas para TCD4, TCD8 e CD68 foram digitalizadas usando o 3D Histech Scanner e visualizadas pelo software Case Viewer versão 2.4 (versão de 64 bits) para Windows. As imagens dos slides dos três marcadores foram então alinhadas na tela do computador com ampliação de 20x e fotos de cada marcador foram tiradas de cinco áreas tumorais diferentes. As cinco áreas fotografadas foram iguais nos três marcadores (Figura 3). As fotos foram analisadas pela contagem de todas as células inflamatórias intratumorais marcadas no campo da imagem. Foi realizada a marcação média das 5 fotos e calculada a mediana para definir a marcação baixa e alta (KURODA *et al.*, 2021).

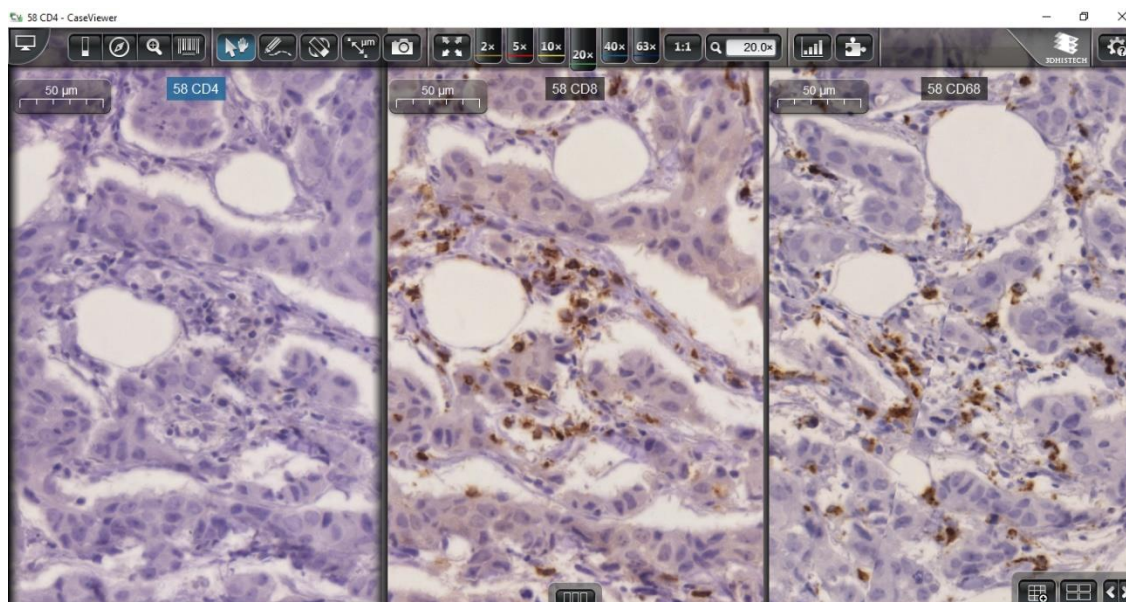


Figura 3 – Método usado para contagem de células marcadas em carcinomas invasivos de mama: três lâminas digitalizadas coradas para TCD4, TCD8 e CD68 alinhadas no mesmo campo na tela do computador.

Os resultados das avaliações dos aspectos histológicos e IHQ foram inseridos em uma planilha do Excel, contendo todos os parâmetros avaliados. Posteriormente, foram comparados os parâmetros dos tumores nas amostras pré e pós-terapia para avaliar os efeitos da terapia nos tumores residuais e a

sobrevida das pacientes de acordo com o perfil histológico e imuno-histoquímico.

4.5 Aspectos éticos

Este trabalho foi desenvolvido no Laboratório de Patologia Mamária da Universidade Federal de Minas Gerais e foi aprovado pelo comitê de ética em pesquisa da UFMG (COEP) sob o parecer da Plataforma Brasil de número 2.109.565 (ANEXO 7.3).

4.6 Análise estatística

Os cálculos estatísticos foram realizados utilizando o programa estatístico SPSS (SPSS, Inc, Chicago, III). Para analisar as diferenças entre as variáveis categóricas de cada grupo (como tipo, grau histológico e positividade de marcadores) foi usado o teste exato de Fisher. Para comparar as variáveis pré e pós-terapia utilizamos o teste de McNemar para variáveis dicotômicas e McNemar-Bowker para variáveis com mais categorias. Para analisar as variáveis contínuas pré e pós-terapia foi utilizado o teste de Wilcoxon. As análises de sobrevida e tempo livre de doença foram realizadas com os métodos de Kaplan-Meier e regressão de Cox. As análises foram consideradas estatisticamente significativas quando $p < 0,05$.

5. Resultados

Esse trabalho é a continuação de nosso estudo no Laboratório de Patologia Mamária sobre resposta à terapia neoadjuvante. No mestrado, realizamos algumas comparações de parâmetros histológicos e IHQ entre a biópsia inicial e a ressecção cirúrgica pós-terapia, além de identificar fatores preditivos de resposta à terapia neoadjuvante. Resultados significativos foram encontrados nas análises dos padrões de resposta patológica à terapia, onde a intensidade do infiltrado inflamatório intratumoral foi considerado fator preditor de melhor resposta à terapia. Realizamos, ainda, algumas comparações entre as amostras dos tumores pré e pós-terapia. Neste trabalho, continuamos a avaliação iniciada no mestrado, repetindo as análises com o acréscimo de pacientes, aprofundando as comparações entre as amostras tumorais pré e pós-terapia e avaliando o impacto na sobrevida global das pacientes em relação aos diversos parâmetros estudados.

Os resultados e discussão serão apresentados nos artigos nos anexos I e II, onde os dois primeiros objetivos serão respondidos pelo artigo no anexo I e os outros dois pelo artigo no anexo II.

6. Conclusões

O estadiamento clínico, Presença de infiltrado inflamatório e marcação para CD8 foram considerados como fatores preditores das classes de RCB.

A história familiar de câncer de mama, as classes de RCB, o grau histológico pré-Qtneo e o subtipo pré e pós-Qtneo foram considerados fatores prognósticos independentes.

Mudanças ocorrem após a Qtneo, então as características das amostras pós-Qtneo também são importantes para definição prognóstica.

Recomendamos a reavaliação do tumor pós-Qtneo para que seja tomada a melhor decisão terapêutica de forma individualizada.

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8. Anexos

8.1 Artigo 1

Prognostic and predictive factors of response to neoadjuvant chemotherapy in breast cancer and association with survival time: a single Brazilian institution study

Original article

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Running title: Prognostic and predictive factors of response to neoadjuvant chemotherapy in breast cancer

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Abstract

Purpose: Well-validated clinical and pathological predictors are needed to support therapeutic decisions so that patients receive individualized treatment with the greatest chance of benefit. We aimed to identify factors that might predict the level of tumor response by residual cancer burden class (RCB) to neoadjuvant chemotherapy (NACT) and to verify their association with patient's overall survival time. **Materials and Methods:** One hundred and five invasive breast carcinomas treated with NACT between 2008 and 2018 were studied. **Results:** After NACT, most tumors had pathological partial response (89; 84.8%) and 16 tumors (15.2%) had pathological complete response (pCR), which had a lower risk of death (HR 0.27; CI: 0.11-0.68; $p = 0.006$). The following parameters were associated with the RCB class: tumor size, with a predominance of cT4 in the RCB III and cT3 in the others ($p=0.011$); regional lymph nodes, where only RCB II had N0 patients ($p<0.001$); tumor infiltrated inflammatory cells, with a predominance of high index only in RCB 0/1 ($p=0.018$); and IHC expression for TCD8+ lymphocytes, where only in RCB II low expression predominated ($p=0.016$). The independent prognostic factors showing higher risk of death were family history of breast cancer (HR 3.16; $p=0.003$), RCB class III (HR 8.77; $p<0.001$) and grade II (HR 3.07; $p = 0.015$) or grade III (HR 3.97; $p=0.005$). **Conclusion:** Family history of breast cancer, RCB classes and histological tumor grade are independent prognostic factors. Clinical staging, presence of inflammatory infiltrate and intratumoral TCD8 lymphocytes were predictors of RCB classes.

Keywords: Breast Neoplasms; Receptors, Estrogen; Receptors, Progesterone; Receptor, ErbB-2; Ki-67 Antigen; Lymphocytes, Tumor-Infiltrating; Neoadjuvant Therapy.

Introduction

Breast cancer is the most common malignancy disease in women, being one of the main causes of morbidity, disability and mortality from cancer (1,2). In Brazil, it has an estimated risk of 66.54 per 100,000 women and 73,610 new cases will be diagnosed in 2023 (3).

There is a relative disparity in the diagnostic tumor stage around the world. In developed countries, there are effective awareness and early tumor diagnostic programs, which lead to the detection of initial lesions and decreases the mortality rate. In underdeveloped and developing countries, as Brazil, tumors are often clinically diagnosed when they become palpable, in advanced stages of the disease, and have higher mortality rates (2,4–6).

In all newly diagnosed cases, histological characteristics of the tumor and immunohistochemical tests for hormone receptors, HER2 and Ki67 should be assessed (7,8). These markers are prognostic, providing information on clinical outcome and predictive of response to specific targeted therapies, such as endocrine therapy or HER2-targeted treatment (9,10). Ki67, a proliferative marker, is considered to predict response to chemotherapy. (11,12).

Histological analysis can also evaluate the tumor microenvironment, a complex stroma composed of blood and lymphatic endothelial cells, fibroblasts, mast cells and various types of immune cells of innate and adaptive defense (dendritic cells, lymphocytes, macrophages, neutrophils and eosinophils) (13). These immune cells have been extensively studied, as depending on the type of cell and inflammatory response present in the microenvironment, they can lead to tumor progression or suppression of the tumor. The presence of these inflammatory cells within the tumor stroma confers better

prognosis for patients when compared to the absence of these cells, in addition to being considered a predictive factor of response to chemotherapy (14–19).

The clinician will decide about the best therapy for the patient based on the patients' clinical data and the histological and immunohistochemical characteristics of the tumor, with tumor clinical staging. Neoadjuvant chemotherapy (NACT) is becoming a more frequent therapeutic decision, aiming to reduce the size of large tumors for later conservative excisions or for triple-negative and HER2 positive tumors, which are known to have better responses to chemotherapy. Pathologic complete response to therapy is the absence of residual invasive disease in the breast and lymph nodes. Therapy can be continued as adjuvant if the pathologic response of the tumor is not complete, in HER2 positive and triple-negative disease (20–24). The residual cancer burden (RCB) should be calculated by the pathologist, as studies have proved the prognostic effect of RCB in NACT (25).

Chemotherapy is toxic and has side effects that interfere with the patient's quality of life, in addition to high costs. This is a reason why well-validated predictive, clinical and pathological factors are increasingly needed to support therapeutic decisions, with a more individualized treatment and a greater chance to obtain benefit with less collateral effects to the patient (9,26).

In this study we aimed to identify the clinicopathological factors that might predict the level of tumor response to neoadjuvant chemotherapy (residual cancer burden class) in a Brazilian public hospital and to verify their association with patient's overall survival time.

MATERIALS AND METHODS

This is a retrospective cohort study. One hundred and five invasive breast carcinomas diagnosed and treated with neoadjuvant chemotherapy between 2008 and 2018 at the Hospital of Clinics of the Federal University of Minas Gerais were retrospectively selected. Only cases with available formalin-fixed paraffin-embedded (FFPE) samples for analysis were included. Patients received NACT according to standard protocols of Brazilian's unified public health system (SUS) (27).

Patients' clinical data were collected for survival analysis. Tumors were staged according to the American Joint Committee on Cancer 8th edition (anatomic stage) (28). All archived hematoxylin and eosin-stained slides of the cases were reviewed by a breast pathologist to determine tumor histological type, grade, presence of associated *in situ* carcinoma, presence of lymphovascular invasion, percentage of tumor infiltrating inflammatory cells, and the immunohistochemical tests for RH, HER2 and Ki67 index were repeated in our laboratory.

Tumor histological type and grade were based on the World Health Organization criteria and the Nottingham grading system (Scarff-Bloom-Richardson modified) (29,30). For statistical analysis, the histological tumor types were divided in three groups: invasive carcinoma of no special type (Ductal NST), invasive lobular carcinoma, and invasive breast carcinomas of other special types.

Tumor infiltrating inflammatory cells were divided in three levels, based on their percentage compared to the tumor cells: absent (less than 1%), low (1 to 20%) and high (over 21%).

The cases were divided into three groups according to the residual cancer burden (RCB) class of pathological response to neoadjuvant chemotherapy, group RCB classes 0/I with better response, group RCB class II and group RCB class III, with worse response. The RCB was calculated following the criteria proposed by the MD Anderson cancer center which are: primary tumor bed area, percentage of overall residual cancer cellularity, percentage of in situ carcinoma, number of positive lymph nodes and diameter of largest metastasis. The RCB calculator is free and available at <http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3> (25)

Immunohistochemical assays

Using standardized protocols according to manufacturers' recommendations with known positive and negative controls, FFPE blocks from the 105 cases were submitted to IHC tests for ER, PR, HER2, Ki67, TCD4, TCD8 and CD68 at the Breast Pathology Laboratory of the School of Medicine of UFMG. Antibody clones and dilutions, detection systems and antigen retrieval are described in supplementary table 1.

The last American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines were followed to evaluate the IHC scores. RH was considered positive when one percent of cells were stained, and the HER2 3+ score was considered positive, 2+ equivocal, and 1+ or 0 were considered negative (7,8). Ki67 expression analysis was semi-quantitative, with the average percentage of tumor-stained nuclei, in at least 300 tumor cells. The median was calculated using the percentage of Ki67 expression of all cases, and the cut-off point was defined for low (< 28%) or high (> 28%) Ki67 index (31–33).

The tumors were divided into 4 groups, according to the biomarker's expression: Luminal A-like: positive hormone receptors, negative HER2 and low Ki67; Luminal B-

like: positive ER, any expression of PR, positive or negative HER2 and high Ki67; HER2-positive: ER and PR negative, HER2 positive and any expression of Ki67; and Triple-negative: ER, PR and HER2 negative with any expression of Ki67 (34).

All the slides stained for TCD4, TCD8 and CD68 were scanned using the 3D Histech Scanner and visualized by the Case Viewer software version 2.4 (64-bit version) for Windows. The slide images of the three markers were then aligned at the computer screen at 20x magnification and pictures of each marker were taken of five different tumor areas. The same areas were photographed for each of the markers (Figure 1). The digital images were analyzed by counting all intratumoral inflammatory cells labeled in the picture field. The average staining of the 5 images was performed and the median was calculated to define low and high staining (35).

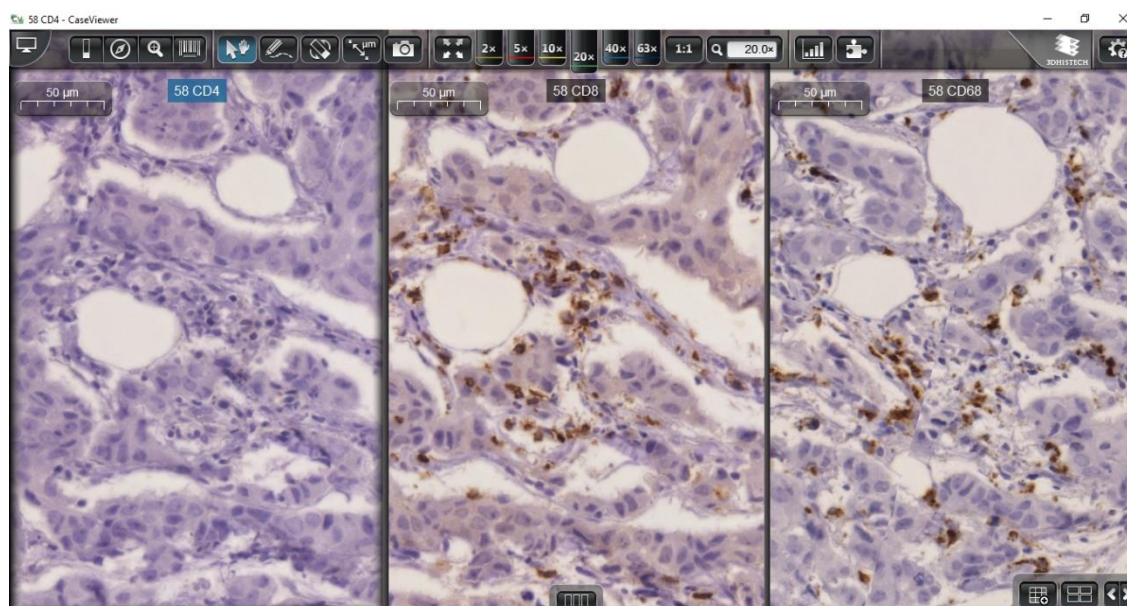


Figure 1 –Method used to count stained cells in invasive breast carcinomas: three scanned slides stained for TCD4, TCD8 and CD68 aligned at the same field on the computer screen.

Statistical analysis

Statistical analysis was performed using the SPSS statistical program, version 29 (SPSS, IBM Inc, Chicago, IL). Categorical variables associated with RCB classes were analyzed using Fisher's exact test and the Kruskal Wallis test was used for continuous variables. Factors associated with overall survival time of patients were analyzed using Kaplan-Meier curves and compared with the log-rank and Cox tests. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated. The tests were considered statistically significant when p value was < 0.05 .

Ethical statement

This study was approved by the Research Ethics Committee of the Federal University of Minas Gerais (COEP-UFMG) under protocol # 2.109.565. Since is a retrospective study, no informed consent was required from patients.

RESULTS

Clinicopathological Characteristics and overall survival

Clinicopathological characteristics are shown in Table 1. Patients mean age was 49.19 years, ranging from 24 to 75 years. Survival data was available from 101 patients, with a mean follow-up time of 46 months (range, 8 to 161 months). Sixty-six patients (62.9%) died during follow-up. Overall survival time of the patients was analyzed, and cases with and without pathological tumor response after NACT were compared.

A family history of breast cancer was found in 19 cases (18.1%) and was associated with a higher risk of death (HR 2.48; CI:1.30 – 4.73; $p = 0.006$) when comparing with no family history of cancer. Most tumors were in the right breast (58/105; 55.2%). Forty-four were multiquadrant (41.9%). After tumor staging based on TNM AJCC system there were no clinical stage I cases. Tumor stage III was the most prevalent (78/105; 74.3%), followed by stage II (15 cases, 14.3%) and stage IV (12 cases, 11.4%). Tumor size category 4 was the most common (48; 45.7%) followed by T3 (40; 38.1%); regional lymph nodes (cN category) were positive in most of the cases, N2 (51; 48.6%) and N1 (42; 40.0%). Distant metastases were not evident on clinical and radiographic studies (M0) in 93 (88.6%) of the cases. The presence of clinical regional movable ipsilateral Level I, II axillary lymph node metastasis was associated with longer overall survival time.

After NACT, most tumors had pathological partial response (89; 84.8%; figures 1A to 1D) and 16 tumors (15.2%) showed no residual tumor (pCR, figure 2E e 2F). Patients with tumor pathological complete response had a lower risk of death when comparing with patients with tumor partial response (HR 0.27; CI: 0.11-0.68; $p = 0.006$).

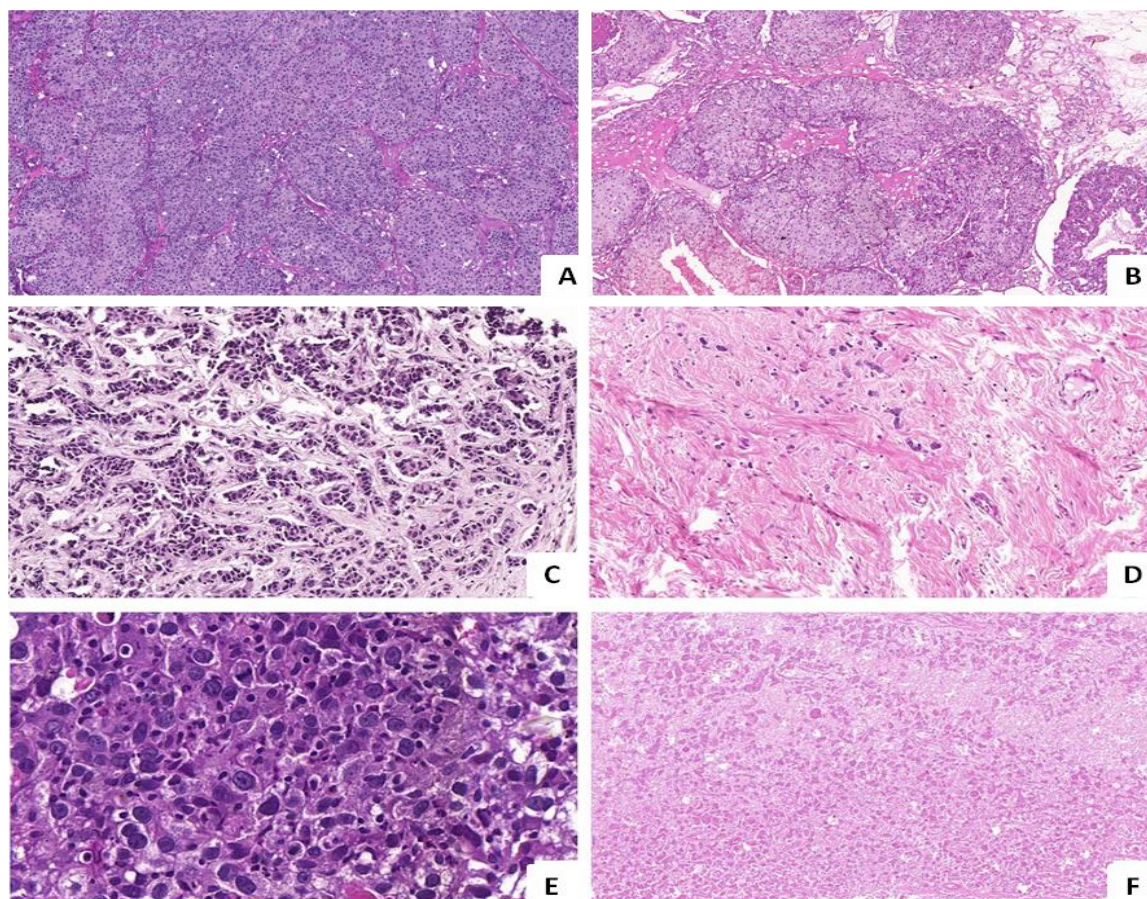


Figure 2 - Grades of response to neoadjuvant chemotherapy (NACT) in invasive breast carcinomas (Hematoxylin/Eosin; 200x magnification). Case 31, A before and B after NACT with 90% of residual disease; Case 18, C before and D after NACT with 5 to 10% of residual disease; and Case 19, E before and F after NACT with complete pathologic response.

We calculated the residual cancer burden (RCB) class of each case using the MD Anderson on-line calculator. 55 cases were classified as RCB III (52.4%), 33 as RCB class II (31.4%), one as RCB class I (1.0%) and 16 as RCB class 0 (15.2%, those with pCR). Since only one patient had an RCB of one, this case was grouped with cases with pCR. Patients with RCB class III tumors had a higher risk of death when compared with patients with RCB 0 (HR 4.14, CI 1.64 – 10.47; $p = 0.003$; figure 3), and those patients with RCB III almost doubled the risk of death when compared with patients with RCB II (HR 2.44, CI 0.89 – 6.65, $p = 0.081$).

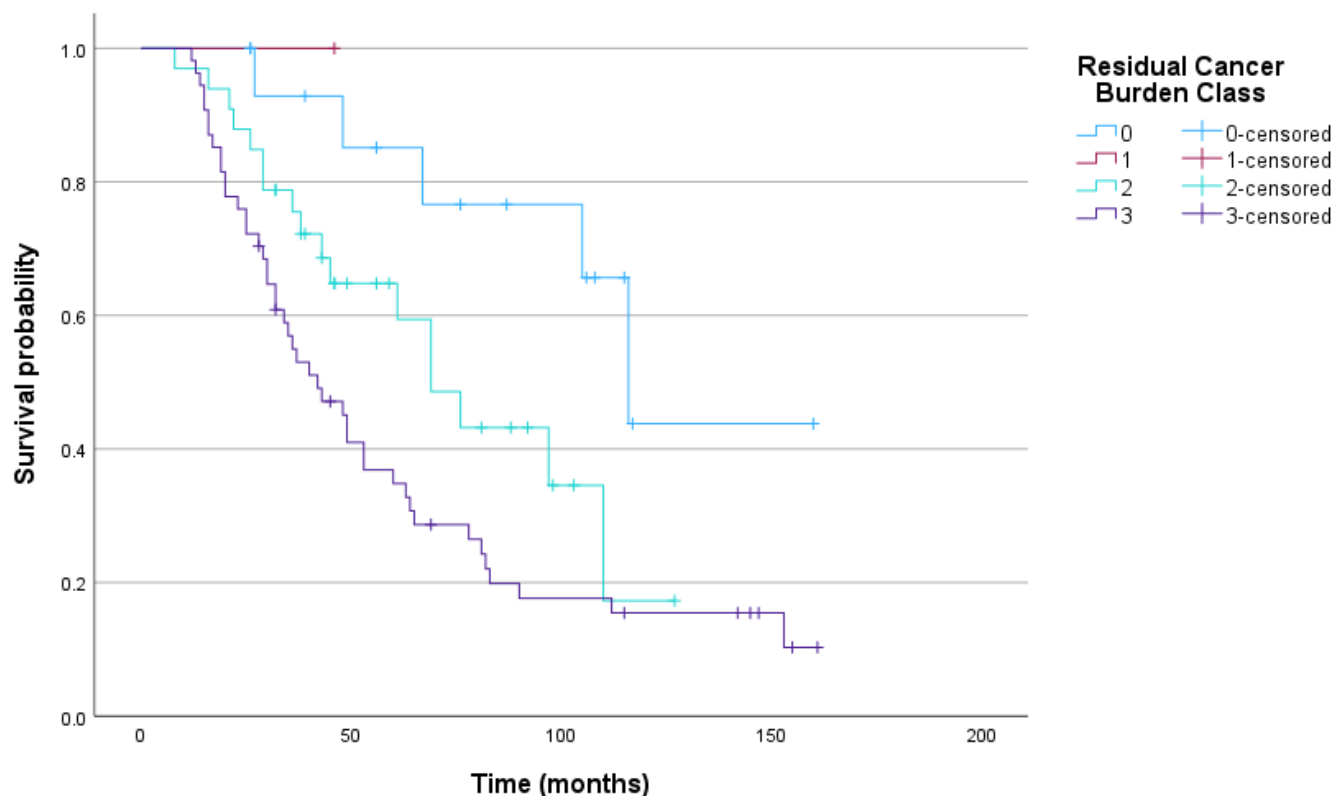


Figure 3: Survival probability of patients with invasive breast carcinoma with different residual cancer burden class ($p = 0.005$).

Invasive carcinoma of no-special type was the most frequent histological subtype (78/105; 74.3%), followed by other subtypes (18.1%) and invasive lobular carcinoma (7.6%). The most common histological grade was III (48.6%), followed by II (37.1%) and I (14.3%). Tumor histological type did not influence overall survival of patients. When compared to grade I, patients with grade III tumors had the highest risk of death (HR: 2.34, CI 1.08 – 5.09; $p = 0.031$).

When we evaluated each parameter of the histological grade, most tumors had low tubular formation (86.7%) and high nuclear pleomorphism (63.8%). Having a mitotic count of 2 versus 1 was associated with shorter overall survival (HR 2.41, CI 1.31 – 4.44, $p = 0.005$).

Most lesions had a low percentage of tumor inflammatory cells (63 cases; 60.0%), followed by over 21% (29 cases; 27.6%) and absence (13 cases; 12.4%). There was no correlation with the percentage of inflammatory cells and overall survival.

Most cases did not have associated *in situ* carcinoma (89 cases, 84.8%). Having *in situ* carcinoma was associated to longer overall survival, with a hazard ratio of 0.47 (CI 0.20 – 0.98) and $p = 0.045$.

Most tumors were estrogen receptor positive (56 cases; 53.3%) and progesterone receptor negative (58 cases; 55.2%). Both markers influenced overall survival with a hazard ratio of 0.47 (CI 0.29 – 0.77; $p = 0.003$) and 0.51 (CI 0.31 – 0.83; $p = 0.007$) respectively. Most tumors were negative for HER2 protein overexpression (85.7%), and HER-2 positivity was not associated with survival time.

Having a high Ki67 index was associated with a higher risk of death (HR 1.98, CI 1.21 – 3.24; $p = 0.007$).

The most common immunohistochemical profile was triple-negative (39 cases; 37.1%) and Luminal B-like (36 cases; 34.3%). Having a triple-negative profile was associated with a hazard ratio for death of 3.11 (CI 1.54 – 6.28; $p = 0.002$) when compared to luminals A-like breast cancer.

The mean score for TCD4 was 1.10, ranging from 0 to 9; for TCD8 it was 20.9, ranging from 0 to 129.8; and for CD68 it was 24.6, ranging from 2 to 85.8. TCD4 and TCD8 counts were not associated with survival time, with a hazard ratio of 0.83 (CI: 0.68-1.01) and $p=0.063$ and of 1.00 (CI: 0.99-1.01) and $p=0.545$. On the other hand, TCD68 count influence the patients' overall survival, with a risk ratio of 1.02 (CI: 1.00-1.03) and $p=0.035$.

A multivariate analysis between overall survival and the parameters that were significant is shown in table 2. The independent prognostic factors showing higher risk of death were the patient having a family history of breast cancer (HR 3.16; CI 1.47 – 6.78; $p = 0.003$), having a tumor RCB class III in relation to classes 0 and I (HR 8.77; CI 2.93 – 26.22; $p < 0.001$) and having a grade II versus grade I (HR 3.07; CI 1.24 – 7.61; $p = 0.015$) or grade III versus grade I tumor (HR 3.97; CI 1.51 – 10.46; $p = 0.005$).

Table 1: Clinicopathological characteristics of invasive breast carcinomas treated with NACT and overall survival by univariate analysis

Parameters		Total n = 105	N of events	Hazard Ratio (95%CI)	p Value
Age (mean 49.19, ranging from 24 to 75 years)		-	-	0.97 (0.95 – 1.00)	0.029
Family history	Absent	48 (45.7)	22 (45.8)	1	-
	Breast cancer	19 (18.1)	17 (89.5)	2.48 (1.30 – 4.73)	0.006
	Other types of cancer	24 (22.9)	16 (66.7)	1.40 (0.73 – 2.67)	0.312
	Both breast and other types	14 (13.3)	7 (50)	0.97 (0.41 – 2.28)	0.944
Clinical Stage	II	15 (14.3)	11 (73.3)	1	-
	III	78 (74.3)	43 (55.1)	0.84 (0.43 – 1.64)	0.618
	IV	12 (11.4)	9 (75)	1.49 (0.61 – 3.62)	0.376
Tumor size category	T1	2 (1.9)	2 (100)	1	-
	T2	15 (14.3)	11 (73.3)	0.53 (0.12 – 2.40)	0.408
	T3	40 (38.1)	18 (45)	0.41 (0.09 – 1.78)	0.235
	T4	48 (45.7)	32 (66.7)	0.58 (0.14 – 2.44)	0.458
Regional lymph nodes category	N0	9 (8.6)	7 (77.8)	1	-
	N1	42 (40.0)	22 (52.4)	0.41 (0.17 – 0.96)	0.041
	N2	51 (48.6)	32 (62.7)	0.53 (0.23 – 1.23)	0.129
	N3	3 (2.8)	2 (66.7)	0.90 (0.19 – 4.37)	0.898
Distant metastasis category	M0	93 (88.6)	54 (58.1)	1.71 (0.84 – 3.49)	0.138
	M1	12 (11.4)	9 (75)		
Pathological response	Partial	89 (84.8)	61 (68.5)	0.27 (0.11 – 0.68)	0.006
	Complete	16 (15.2)	5 (31.3)		
RCB class	0	16 (15.2)	5 (31.3)	1	-
	I	1 (1.0)	0 (0)	0.00 (0.00 – 5.12)	0.978
	II	33 (31.4)	17 (51.5)	2.44 (0.89 – 6.65)	0.081
	III	55 (52.4)	44 (80)	4.14 (1.64 – 10.47)	0.003
Histological subtype	Non-special type carcinoma	78 (74.3)	50 (64.1)	1	-
	Invasive lobular carcinoma	8 (7.6)	3 (37.5)	0.39 (0.12 – 1.16)	0.117
	others	19 (18.1)	13 (68.4)	1.16 (0.63 – 2.14)	0.634

Histological grade	I	15 (14.3)	8 (53.3)	1	-
	II	39 (37.1)	24 (61.5)	1.46 (0.65 – 3.26)	0.355
	III	51 (48.6)	34 (66.7)	2.34 (1.08 – 5.09)	0.031
Tubule formation score	1	4 (3.8)	2 (50)	1	-
	2	10 (9.5)	6 (60)	1.29 (0.26 – 6.42)	0.754
	3	91 (86.7)	58 (63.7)	1.66 (0.40 – 6.81)	0.482
Nuclear Pleomorphism score	1	10 (9.5)	6 (60)	1	-
	2	28 (26.7)	18 (64.3)	1.52 (0.60 – 3.85)	0.373
	3	67 (63.8)	42 (62.7)	1.87 (0.79 – 4.41)	0.154
Mitotic count score	1	44 (41.9)	26 (59.1)	1	-
	2	28 (26.7)	19 (67.9)	2.41 (1.31 – 4.44)	0.005
	3	33 (31.4)	21 (63.6)	1.54 (0.86 – 2.75)	0.144
Intratumoral inflammatory cells	0%	13 (12.4)	10 (76.9)	1	-
	1 to 20%	63 (60.0)	42 (66.7)	0.75 (0.37 – 1.50)	0.417
	above 21%	29 (27.6)	14 (48.3)	0.54 (0.24 – 1.21)	0.135
In situ carcinoma	Absent	89 (84.8)	59 (66.3)	0.45 (0.20 – 0.98)	0.045
	Present	16 (15.2)	7 (43.8)		
Estrogen receptor status	Negative	49 (46.7)	34 (69.4)	0.47 (0.29 – 0.77)	0.003
	Positive	56 (53.3)	32 (57.1)		
Progesterone receptor status	Negative	58 (55.2)	40 (69)	0.51 (0.31 – 0.83)	0.007
	Positive	47 (44.8)	26 (55.3)		
HER2 overexpression	Negative	90 (85.7)	59 (65.6)	0.79 (0.36- 1.74)	0.563
	Positive	15 (14.3)	7 (46.7)		
Ki67 index	Low	53 (50.5)	29 (54.7)	1.98 (1.21 – 3.24)	0.007
	High	52 (49.5)	37 (71.2)		
IHC profile	Luminal A-like	20 (19.0)	11 (55)	1	-
	Luminal B-like	36 (34.3)	21 (58.3)	1.15 (0.55 – 2.39)	0.710
	HER2	10 (9.5)	4 (40)	0.82 (0.26 – 2.59)	0.737
	Triple negative	39 (37.1)	30 (76.9)	3.11 (1.54 – 6.28)	0.002
TCD4 count	Low	57 (54.3)	39 (68.4)	0.78 (0.48 – 1.29)	0.342
	High	48 (45.7)	27 (56.3)		
TCD8 count	Low	55 (52.4)	34 (61.8)	1.09 (0.67 – 1.78)	0.714
	High	50 (47.6)	32 (64)		
CD68 count	Low	53 (50.5)	32 (60.4)	1.43 (0.88 – 2.34)	0.147
	High	52 (49.5)	34 (65.4)		
TCD4 count (mean 1.10, ranging from 0 to 9)		-	-	0.83 (CI: 0.68-1.01)	0.063
TCD8 count (mean 20.9, ranging from 0 to 129.8)		-	-	1.00 (CI: 0.99-1.01)	0.545
CD68 count (mean 24.6, ranging from 2 to 85.8)		-	-	1.02 (CI: 1.00-1.03)	0.035

NACT – neoadjuvant chemotherapy; N: number of cases; HER2 - human epidermal growth factor receptor 2; IHC – immunohistochemical profile; CD4 – cluster of differentiation 4; CD8 – cluster of differentiation 8; CD68 – cluster of differentiation 68; ^a p-value calculated by cox regression test. Statistically significant results are highlighted in bold.

Table 2: Clinicopathological characteristics of 105 breast cancer treated with neoadjuvant therapy and overall survival by multivariate analysis

Parameters	Hazard Ratio (95%CI)	p Value ^a
Age	-	-

Family history	Absent	1	-
	Breast cancer	3.16 (1.47 – 6.78)	0.003
	Others types	1.39 (0.68-2.86)	0.366
	Both	1.25 (0.45-3.49)	0.666
Clinical Stage	II		
	III	-	-
	IV		
RCB class	0	1	-
	I	0.00(0.00-0.00)	0.984
	II	2.22 (0.69-7.10)	0.179
	III	8.77 (2.93-26.22)	< 0.001
Histological grade	I	1	-
	II	3.07 (1.24-7.61)	0.015
	III	3.97 (1.51-10.46)	0.005
In situ carcinoma	Absent	-	-
	Present		
IHC profile	Luminal A-like	1	-
	Luminal B-like	0.64 (0.27-1.52)	0.308
	HER2	0.62 (0.17-2.26)	0.466
	Triple negative	2.02 (0.86-4.75)	0.108

RCB – residual cancer burden; IHC – immunohistochemical; ^{a)}p-values calculated by cox regression

Clinicopathological characteristics correlation with RCB class

We associated the clinicopathological parameters with the tumor RCB class in an attempt to identify factors that might predict the level of response to neoadjuvant chemotherapy. All clinicopathological characteristics in each RCB class cases are shown in table 3.

The majority of the 17 RCB class 0/I cases were clinical prognostic stage III (14 cases, 82.4%), tumor size T3 (10 cases, 58.8%) and none of 17 cases had N0. One tumor had distant metastasis at diagnosis (5.9%). Invasive carcinoma NST (14 cases, 82.4%) and grades III and II (9 cases, 52.9% and 8 cases, 47.1% respectively) predominated. There was no grade I tumor with good response to therapy. All tumors had low tubular formation, most with high nuclear pleomorphism (13 cases, 76.5%), low mitotic count (9 cases, 52.9%), absence of lymphovascular invasion (15 cases, 88.2%) and high intratumor inflammatory cells (10 cases, 58.8%). The immunohistochemical

expressions were hormonal receptor negative (10 ER negative cases, 58.8% and 11 PR negative cases, 64.7%), HER2 negative (12 cases, 70.6%) with high ki67 index (10 cases, 58.8%). Triple negative immunohistochemical profile predominated (7 cases, 41.2%). Low and high TCD4 expression were similar (9 cases low, 52.9% and 8 cases high, 47.1%), high expression of TCD8 predominated (10 cases, 58.8%) and also high CD68 expression (11 cases, 64.7%).

33 cases were identified with RCB class II, 24 were staged III (72.7%), with cT3 (19 cases, 57.6%) axillary positive lymph nodes (25 cases, 75.7%) and absence of distal metastasis (31 cases, 93.9%). No special histologic type of invasive carcinoma (24 cases, 72.7%) and grades III and II (17 cases, 51.5% and 13 cases, 39.4% respectively) predominated. 29 tumors had low tubular formation (87.9%), most with high nuclear pleomorphism (22 cases, 66.7%), low and high mitotic count (10 cases, 30.3%, and 14 cases, 42.4%, respectively), absence of lymphovascular invasion (23 cases, 69.7%) and intermediate intratumoral inflammatory cells (20 cases, 60.6%). The immunohistochemical expressions were estrogen receptor positive and progesterone negative (17 cases, 51.5%), HER2 negative (30 cases, 90.9%) with low ki67 index (21 cases, 63.6%). Triple negative immunohistochemical profile also predominated (14 cases, 42.4%). The mean TCD4 count was 1.19 (CI: 0.57-1.81), the mean TCD8 count was 14.56 (CI: 6.11-23.02) and the mean CD68 count was 23.36 (CI: 18.08- 23.02). TCD4 low and high expression were similar (16 cases low, 48.5% and 17 cases high, 51.5%), but low expression of TCD8 predominated (24 cases, 72.7%) and low CD68 expression (19 cases, 57.6%).

Most of our cases were classified as RCB III (55 cases, 52.4%). Most of them were also on stage III (40 cases, 72.7%), with 33 cases cT4 (60%), positive axillary lymph nodes

in all cases and absence of distant metastasis in 46 cases (83.6%). No special type of invasive carcinoma (40 cases, 72.7%) and grade III (26 cases, 47.3%) also predominated. Forty-five tumors had low tubular formation (81.8%), most with high nuclear pleomorphism (32 cases, 58.2%), low and intermediate mitotic count (25 cases, 45.5%, and 17 cases, 30.9%, respectively), absence of lymphovascular invasion (40 cases, 72.7%) and intermediate intratumor inflammatory cells (36 cases, 65.5%). The immunohistochemical expressions were estrogen receptor positive (32 cases, 58.2%) and progesterone receptor negative (30 cases, 54.5%), HER2 negative (48 cases, 87.3%) with similar high and low ki67 index (25 low ki67 cases, 45.5%, and 30 high ki67 cases, 54.5%). Luminal B-like (21 cases, 38.2%) and triple negative (18 cases, 32.7%) immunohistochemical profiles predominated. The mean TCD4 count was 0.75 (CI: 0.47- 1.02). Mean TCD8 count was 17.77 (CI: 13.69-21.85) and the CD68 mean count was 23.89 (CI: 20.09- 21.85). TCD4 low expression predominated (32 cases, 58.2%), but low and high expression of TCD8 and CD68 were similar (24 low TCD8 cases, 43.6%, 31 high TCD8 cases, 56.4%, 28 low CD68 cases, 50.9%, and 27 high CD68 cases, 49.1%).

Graphic representations of the expression level distribution for CD4, CD8 and CD68 are available in figure 4.

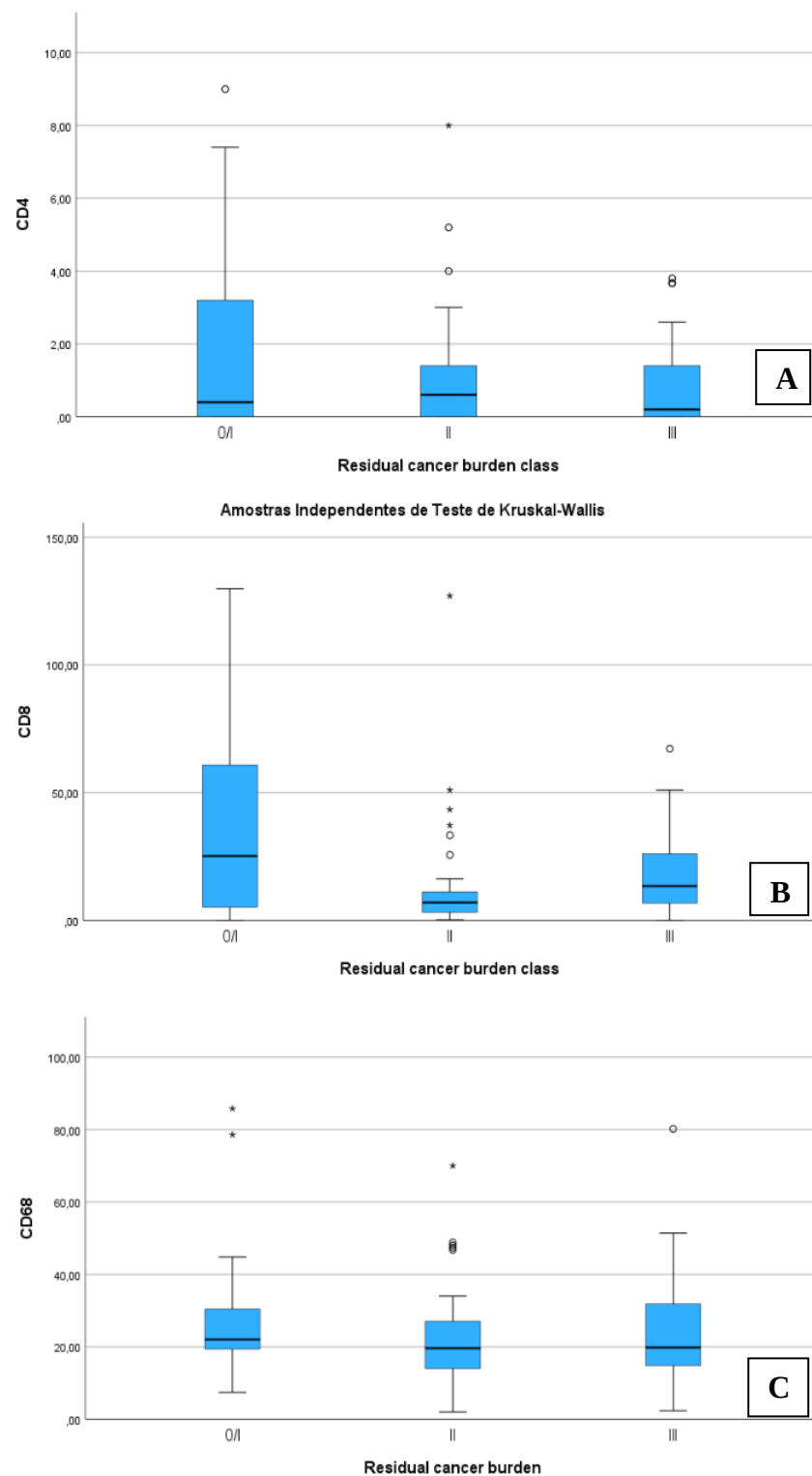


Figure 4: Expression level distribution of (A) CD4; (B) CD8; and (C) CD68 according to RCB class.

The clinicopathological characteristics that might predict the RCB class after neoadjuvant therapy were tumor size (T3 in RCB classes 0/I and II, and T4 in RCB III) ($p = 0.011$), presence of axillary lymph node metastasis in RCB classes 0/I and III tumors ($p < 0.001$), over 21% of intratumoral inflammatory cells in RCB class 0/I, and between 1% and 20% in RCB class II tumors ($p = 0.018$). High expression of TCD8 cells was associated with RCB classes 0 or pCR ($p = 0.031$), classes 0/I and III ($p = 0.016$).

Table 2: Association between clinicopathological characteristics of breast cancer and level of response to neoadjuvant therapy

Parameters		RCB 0/I n = 17 (%)	RCB II n = 33 (%)	RCB III n = 55 (%)	Total n = 105 (%)	P value ^{a)}
Clinical Stage	II	2 (11.8)	7 (21.2)	6 (10.9)	15 (14.3)	0.454
	III	14 (82.4)	24 (72.7)	40 (72.7)	78 (74.3)	
	IV	1 (5.8)	2 (6.1)	9 (16.4)	12 (11.4)	
Tumor size category	T1	0 (0.0)	0 (0.0)	2 (3.6)	2 (1.9)	0.011
	T2	3 (17.6)	3 (9.1)	9 (16.4)	15 (14.3)	
	T3	10 (58.8)	19 (57.6)	11 (20.0)	40 (38.1)	
	T4	4 (23.5)	11 (33.3)	33 (60.0)	48 (45.7)	
Regional lymph nodes category	Negative (N0)	0 (0.0)	8 (24.3)	0 (0.0)	8 (7.6)	< 0.001
	Positive (N1, N2 or N3)	17 (100.0)	25 (75.7)	55 (100.0)	97 (92.4)	
Distant metastasis category	Negative (M0)	16 (94.1)	31 (93.9)	46 (83.6)	93 (88.6)	0.314
	Positive (M1)	1 (5.9)	2 (6.1)	9 (16.4)	12 (11.4)	
Histological subtype	Non-special type carcinoma	14 (82.4)	24 (72.7)	40 (72.7)	78 (74.3)	0.963
	Invasive lobular carcinoma	1 (5.9)	3 (9.1)	4 (7.3)	8 (7.6)	
	Other	2 (11.8)	6 (18.2)	11 (20.0)	19 (18.1)	
Histological grade	I	0 (0.0)	3 (9.1)	12 (21.8)	15 (14.3)	0.139
	II	9 (52.9)	13 (39.4)	17 (30.9)	39 (37.1)	
	III	8 (47.1)	17 (51.5)	26 (47.3)	51 (48.6)	
Tubular formation score	1	0 (0.0)	1 (3.0)	3 (5.5)	4 (3.8)	0.572
	2	0 (0.0)	3 (9.1)	7 (12.7)	10 (9.5)	
	3	17 (100.0)	29 (87.9)	45 (81.8)	91 (86.7)	
Nuclear Pleomorphism score	1	0 (0.0)	2 (6.1)	8 (14.5)	10 (9.5)	0.453
	2	4 (23.5)	9 (27.3)	15 (27.3)	28 (26.7)	
	3	13 (76.5)	22 (66.7)	32 (58.2)	67 (63.8)	
Mitotic count score	1	9 (52.9)	10 (30.3)	25 (45.5)	44 (41.9)	0.199
	2	2 (11.8)	9 (27.3)	17 (30.9)	28 (26.7)	
	3	6 (35.3)	14 (42.4)	13 (23.6)	33 (31.4)	
Lymphovascular invasion	Absent	15 (88.2)	23 (69.7)	40 (72.7)	78 (74.3)	0.367
	Present	2 (11.8)	10 (30.3)	15 (27.3)	27 (25.7)	

Intratumoral inflammatory cells	0% 1 to 20% 21 to 50%	0 (0.0) 7 (41.2) 10 (58.8)	7 (21.2) 20 (60.6) 6 (18.2)	6 (10.9) 36 (65.5) 13 (23.6)	13 (12.4) 63 (60.0) 29 (27.6)	0.018
Estrogen receptor status	Negative Positive	10 (58.8) 7 (41.2)	16 (48.5) 17 (51.5)	23 (41.8) 32 (58.2)	49 (46.7) 56 (53.3)	0.462
Progesterone receptor status	Negative Positive	11 (64.7) 6 (35.3)	17 (51.5) 16 (48.5)	30 (54.5) 25 (45.5)	58 (55.2) 47 (44.8)	0.694
HER2 overexpression	Negative Positive	12 (70.6) 5 (29.4)	30 (90.9) 3 (9.1)	48 (87.3) 7 (12.7)	90 (85.7) 15 (14.3)	0.155
Ki67 index	Low High	7 (41.2) 10 (58.8)	21 (63.6) 12 (36.4)	25 (45.5) 30 (54.5)	53 (50.5) 52 (49.5)	0.193
IHC profile	Luminal A-like Luminal B-like HER2 Triple negative	2 (11.8) 5 (29.4) 3 (17.6) 7 (41.2)	7 (21.2) 10 (30.3) 2 (6.1) 14 (42.4)	11 (20.0) 21 (38.2) 5 (9.1) 18 (32.7)	20 (19.0) 36 (34.3) 10 (9.5) 39 (37.1)	0.794
TCD4 count	Low High	9 (52.9) 8 (47.1)	16 (48.5) 17 (51.5)	32 (58.2) 23 (41.8)	57 (54.3) 48 (45.7)	0.695
TCD8 count	Low High	7 (41.2) 10 (58.8)	24 (72.7) 9 (27.3)	24 (43.6) 31 (56.4)	55 (52.4) 50 (47.6)	0.016
CD68 count	Low High	6 (35.3) 11 (64.7)	19 (57.6) 14 (42.4)	28 (50.9) 27 (49.1)	53 (50.5) 52 (49.5)	0.336

N: number of cases; HER2 - human epidermal growth factor receptor 2; IHC – immunohistochemical profile; CD4 – cluster of differentiation 4; CD8 – cluster of differentiation 8; CD68 – cluster of differentiation 68; ^a p-value calculated by Fisher's exact test. Statistically significant results are highlighted in bold.

DISCUSSION

In our study, having a lower residual cancer burden was the strongest predictor of longer survival time. Patients with pCR had a lower risk of death ($p = 0.006$), when we analyzed survival by RCB, since having RCB III (HR 4.14, CI 1.64 – 10.47; $p = 0.003$) means having almost twice the risk of death of RCB II (HR 2.44, CI 0.89 – 6.65; $p = 0.081$), confirming that RCB is an important factor that should be used in all cases of patients receiving neoadjuvant therapy (36).

Neoadjuvant chemotherapy is given to patients with advanced stages of the disease or with tumors having characteristics known to have worse prognosis. Therapies have also been approved for HER2-positive and triple-negative tumors without a complete response to NACT (20–24). The RCB can better predict a patients' prognosis in relation

to just categorizing tumors as complete or partial response, thus better tailor adjuvant treatments.

Our results have shown that the main characteristic in the tumors that were linked to a better response to NACT was the inflammatory infiltrate. Only the total quantification of inflammatory cells in routine hematoxylin-eosin stained slides can help predict response to treatment, without the need for additional tests. Our findings related to the inflammatory infiltrate point to a possible application of this information to help better predict response to chemotherapy after NACT. Studies have been carried out and much has been discussed regarding the possibility of this information becoming applicable in clinical practice as an isolated marker of response to neoadjuvant chemotherapy (15,37–39).

Our results regarding the levels of intratumoral inflammatory infiltrate are in line with reports from other studies, with statistically significant results when associated with the pathological response, since all patients with pCR had the presence of inflammatory cells. This leads us to believe that the inflammatory infiltrate plays a role by interacting with chemotherapy drugs, increasing the effect on the response to therapy (40).

In the tumor microenvironment, stromal cells including inflammatory cells, adipocytes, endothelial cells, macrophages, fibroblasts and mast cells dictate the progression from normal mammary gland to *in situ*, invasive and metastatic carcinomas. The presence of CD4⁺ and CD8⁺ T lymphocytes exerting a Th1-type inflammatory response represents a better prognosis with the action of the anti-tumor immune system (41,42).

Despite being reported that CD4⁺ T lymphocytes play a central role in the tumor microenvironment through the coordination of adaptive immune responses in epithelial tissues, all cases had low expression of this marker (43).

Cytotoxic CD8⁺ T lymphocytes are the most effective in destroying tumor cells, which can lead to the lysis of these cells. When we compare the expression of this marker between the classes, we verify a positive association, proving this fact. Although this marker has proved to be an independent predictor of response, as only quantification could already predict the response to neoadjuvant chemotherapy, and CD8⁺ T lymphocytes testing is useless and add additional cost (35,36).

Activated M1 macrophages have the power to lyse tumor cells through production of tumor necrosis factor alpha, release of oxygen free radicals, or through antibody-dependent cellular cytotoxicity. The CD68 expression was highest in RCB grades 0/1 and was associated with longer overall survival time (41).

All the results of these markers contribute to the idea that a Th1-type immune response occurs in cases with better response to neoadjuvant chemotherapy.

It is already known that the effect of chemotherapy on tumor cells creates an immunogenic environment necessary for successful therapy (44,45), through the formation of new immunogenic epitopes, secretion of cytokines, cross-presentation of antigens, activation of dendritic cells and induction of tumor-specific cytotoxic T cells, leading to the induction of anti-tumor immune response (13). Some studies with patients who received cytotoxic agents, such as anthracyclines and taxanes, found an increase in intratumoral lymphocytes, in addition to the induction of an antitumor immune response (18,46,47)

In our study, tumor stage was not associated with survival time. We believe this is due to our sample size and to fact that some of the patients already had metastatic disease, which was not diagnosed at first.

By associating the histological tumor type and grade with the class of pathological response, we found results similar to those in the literature, with no association between these parameters and the response (48,49).

In the analysis of the components that form the histological grade, all cases of classes 0/1 had low tubular formation, none had low nuclear pleomorphism, and most had high pleomorphism and most had low mitotic counts. None of these analyzes were significant. High-histologic grade tumors tend to respond better to neoadjuvant chemotherapy, which explains the results regarding tubular formation and the degree of pleomorphism, since the mitotic count is impaired in core biopsy samples, as the mitotic count is performed at the edges of the tumor and the small sample makes this count difficult. High-histological grade tumors have a worse prognosis, and this was demonstrated in our study, with higher grade tumors associated with a greater risk of death (20–22).

Hormone receptors and HER2 protein are biomarkers that should be routinely tested in invasive breast carcinomas, due to their power to predict response to specific treatments, in addition to providing prognostic information. Triple-negative or HER2 positive tumors usually have a worse prognosis but may have a good response to neoadjuvant therapy. In RCB class 0/I, seven cases had a triple-negative profile, five Luminal B-like profile due to high Ki67, 3 were HER2 positive and only 2 had a Luminal A-like profile, thus showing that, although our results were not significant, there is a predominance of triple-negative and Luminal B-like profiles in patients with pathological complete response. Probably our small sample size influenced these results. Our study agrees with the literature in which hormone receptors negative

tumors, triple-negative tumors or with high expression of Ki67 have a higher risk of death (37,40).

It is well established that patients with hormone receptor positive tumors can benefit from treatment with hormone therapy such as tamoxifen, aromatase inhibitors or ovarian suppression, but hormone receptor negative tumors tend to respond better to neoadjuvant chemotherapy. In the cases in our study that obtained a complete post-therapy response, most of the tumors were negative for hormone receptors, corroborating this fact (11).

We did not identify a relationship between HER2 overexpression and better response to neoadjuvant therapy, as described in the literature, since in all classes, most tumors were negative for HER2 expression. Although the neoadjuvant treatment with the addition of trastuzumab has already been approved by the FDA, in our hospital it is not a routine, this does not happen in the neoadjuvant setting, and this may be an explanation for the lack of differences in the response to NACT. A single patient in our study who received trastuzumab, obtained pCR; so we can think that, if all patients with HER2 positive tumors had received the target-specific treatment, our result would have been relevant in this parameter (11).

The Ki-67 biomarker is well established as a prognostic marker in patients with breast cancer. Tumors with a high Ki-67 respond well to chemotherapy because it primarily targets proliferating cells. Most of the patients' tumors had a high Ki67 index in classes 0/I, with a higher percentage than in classes II and III, but there was no statistical difference regarding the response (11,50,51).

In conclusion, family history of breast cancer, RCB classes and histological grade are independent prognostic factors. Clinical staging, presence of inflammatory infiltrate and intratumoral TCD8 lymphocytes were predictors of RCB classes.

CONFLICTS OF INTEREST

The authors declared no conflict of interest.

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S1 TABLE- Antibodies, clones, manufacturers, detection systems, dilutions and antigenic retrieval of antibodies

Antibody	Clone	Manufacturers	Detection systems	Dilution	Antigenic retrieval
HER2	A0485 (HercepTest™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Water bath – Epitope retrieval solution, pH 6, Agilent Technologies , 35 minutes
RE	1D5 (PharmaDX™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies , 30 minutes
RP	PGR1294 (PharmaDX™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies , 30 minutes
Ki67	MIB1	Agilent Technologies, United States of America	Detection and amplification system, Novolink, Leica Biosystems, Newcastle upon Tyne, UK	Ready to use	Steamer – Target Retrieval Solution high pH, Agilent Technologies , 30 minutes
CD4	4B12	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Water bath – Epitope retrieval solution, pH 6, Agilent Technologies , 35 minutes
CD8	C8/144B	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies, 30 minutes
CD68	KP1	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies, 30 minutes

HER2 - human epidermal growth factor receptor 2; RE – Estrogen receptor; PR – Progesterone receptor; CD4 – cluster of differentiation 4; CD8 – cluster of differentiation 8; CD68 – cluster of differentiation 68

8.2 Artigo 2

Este artigo será submetido à revista Cancer Research and Treatment antes da defesa.

Changes in invasive breast carcinomas after neoadjuvant chemotherapy can influence adjuvant therapeutic decisions

Original article

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Running title: Breast carcinomas pre- and post-neoadjuvant therapies

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ABSTRACT

Purpose: Neoadjuvant chemotherapy (NACT) can change invasive breast carcinomas (IBC) and influence decisions on adjuvant treatments. We aimed to identify tumor changes and their influence on overall survival time (OS). **Materials and methods:** We compared histological and immunohistochemical (IHC) parameters of 86 pre- and post-NACT IBC samples and associated them with OS. **Results:** Changes were found in tumor nuclear pleomorphism ($p=0.025$); mitotic count ($p=0.002$); tumor-infiltrating inflammatory cells ($p=0.016$); *in situ* carcinoma ($p=0.001$); lymphovascular invasion (LVI; $p=0.002$); and expression of estrogen ($p=0.003$) and progesterone receptors ($p=0.019$); and Ki67 ($p=0.003$). Twenty-six tumors (30.2%) changed the IHC profile ($p=0.050$). Characteristics associated with a higher risk of death were: pre-NACT histological grade III (hazard ratio, HR, 2.94; $p=0.007$), high nuclear pleomorphism (HR: 2.53; $p=0.036$) and high Ki67 index (HR: 2.47; $p<0.001$); post-NACT LVI (HR 1.90; $p=0.016$) and Luminal B-like profile (HR: 2.58; $p=0.031$). Also, pre- (HR 2.26; $p=0.011$) and post-NACT (HR 2.12; $p=0.038$) intermediate mitotic count, pre- (HR: 4.45; $p<0.001$) and post-NACT (HR: 4.52; $p<0.001$) triple-negative IHC profile. Associations with lower risk were: pre- (HR: 0.35; $p<0.001$) and post-NACT (HR: 0.39; $p<0.001$) ER positive, and pre- (HR: 0.37; $p<0.001$) and post-NACT (HR: 0.57; $p=0.044$) PR positive. Changes in IHC profile were associated with longer OS ($p=0.050$). In multivariate analysis, pre-NACT grade III and pre-NACT and post-NACT triple negative IHC profile proved to be independent factors for shorter OS. **Conclusion:** Tumor characteristics and biomarkers can change after NACT with an

impact on OS, therefore they should be reassessed on residual samples to improve therapeutic decisions.

Keywords: Breast Neoplasms; Receptors, Estrogen; Receptors, Progesterone; Receptor, ErbB-2; Ki-67 Antigen; Lymphocytes, Tumor-Infiltrating; Neoadjuvant Therapy.

INTRODUCTION

Invasive breast carcinomas (IBC) treatment involves local control (surgery and radiotherapy), usually associated with systemic therapies, guided by tumor stage, histological characteristics and tumor biomarkers status, including hormone receptors (estrogen - ER and progesterone - PR), human epidermal growth factor receptor 2 (HER2) and Ki67 status, assessed in all new breast cancer cases by immunohistochemical staining (IHC). These prognostic and predictive factors give information about clinical outcome and predict response to specific therapies, thus influence the best individual treatment decision (1–5).

Systemic therapies can be administered before (neoadjuvant) and/or after surgical resection (adjuvant) (6). Neoadjuvant chemotherapy (NACT) is used as the initial treatment for patients with inoperable tumors, for operable tumors to downstage and allow more conservative surgical excisions (both in the breast and in the axilla), for early-stage tumors (especially triple-negative and HER2-positive), and in highly proliferative tumors, as they show better response to systemic therapy (1,4,5,7,8). Information about response to neoadjuvant therapy response also allows adjuvant therapy decision. NACT can result in pathological complete response (pCR), partial response with residual disease, and tumor progression (5,8,9). An important tool developed by a group in the MD Anderson Cancer Center is the Residual Cancer

Burden (RCB) calculator, which includes pathological parameters of the residual tumor, and is related with tumor progression and patients' overall survival (10).

In cases with residual disease after NACT, tumor morphological characteristics and immunohistochemical status may be discordant with those in the initial biopsy sample, due to several factors, like intratumoral heterogeneity (genetic alterations during the clonal evolution of the tumor cells resulting in subclones with different responses to therapy) and sampling variability (1,11,12). In metastatic IBC, the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) guidelines recommend re-biopsy of breast cancer metastases to re-evaluate ER, PR and HER2 status (13). Although several authors have reported pathological changes in residual tumors, there is still no consensus if biomarkers should be retested in post-NACT samples and if these changes should be considered in the adjuvant setting (1,4,5,7,11,12,14).

This study aims to evaluate the effects of NACT on invasive breast carcinomas and identify their influence on patient's overall survival time, in a single Public Health Institution in Brazil.

MATERIALS AND METHODS

Ethical Statement

This is a retrospective cohort study, approved by the Research Ethics Committee of the Federal University of Minas Gerais (COEP-UFMG) under protocol # 2.109.565. Given the retrospective nature of the study, no informed consent was required from research ethics committee.

Study Group and Histological Review

From the archives of the Mammary Pathology Laboratory of the Faculty of Medicine – Universidade Federal de Minas Gerais (UFMG), 86 patients with invasive breast carcinoma treated at the Hospital of Clinics between 2008-2018, who underwent NACT prior to surgical resection were retrospectively selected. All cases with clinicopathological data and both pre- and post-therapy formalin-fixed paraffin-embedded (FFPE) samples available were included. pCR was defined as the absence of residual invasive disease in the breast and lymph nodes (15).

Clinical data were collected via review of the archives and medical records to evaluate disease stage and overall survival time of patients. Patients received NACT according to standard protocols of Brazilian's unified public health system (Sistema Único de Saúde, SUS) (16).

All pathological data were reviewed by a breast pathologist (CBN), as tumor histologic type and grade, percentage of tumor infiltrating inflammatory cells, presence of lymphovascular invasion, presence of associated in situ carcinoma, and immunohistochemical tests for ER, PR, HER2 and Ki67 were repeated in our lab.

Tumors were classified according to the World Health Organization diagnostic criteria (17). For statistical analysis, the histological tumor types were divided in three groups: invasive carcinoma of no special type (Ductal NST), invasive lobular carcinoma, and invasive breast carcinomas of other specific types. Histological grade was evaluated in accordance with the Nottingham (modified Scarff-Bloom-Richardson) system (18). Tumor infiltrating inflammatory cells were divided in three levels, based on their percentage compared to the tumor cells: absent (less than 1%), low (1 to 20%) and high (over 21%). Tumors were staged according to the American Joint Committee on Cancer 8th edition (anatomic stage) (19) .

The RCB was calculated based on primary tumor bed area, percentage of overall residual cancer cellularity, percentage of *in situ* carcinoma, number of positive lymph nodes and diameter of largest metastasis. The RCB calculator is free and available at <http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3> (10).

Immunohistochemical assays and evaluation

The FFPE of pre- and post-therapy blocks were submitted to microtomy, obtaining 4 µm histological sections placed on electric charged slides and submitted to IHC tests for ER, PR, HER2 and Ki67.

All tests were performed at the Mammary Pathology Laboratory of the Medical Faculty of UFMG, using standardized protocols according to the manufacturers' recommendations. Known positive and negative controls were included. Antibody clones and dilutions, detection systems and antigen retrieval are described in S1 table.

The interpretation of the results followed the latest ASCO/CAP guidelines for ER, PR and HER2 protein expression (20,21). A positive ER and PR tumor showed more or equal of 1% of cells staining and results were semi quantitative with percentage and intensity of positive cells (Allred score). The HER2 3+ cases were considered positive, 2+ cases were considered equivocal and sent to reflex HER2 gene amplification analysis in an external service to determine HER2 status and 0 or 1+ were considered negative (21). Ki67 expression analysis was semi-quantitative, with the average percentage of tumor-stained nuclei, in at least 300 tumor cells. The median was calculated using the percentage of Ki67 expression of all cases, and the cut-off point was defined for low (< 28%) or high (> 28%) Ki67 index (22,23).

According to the biomarker's expression, the tumors were divided into 4 groups: Luminal A-like: positive ER and PR, negative HER2 and low Ki67; Luminal B-like: positive ER, any expression of PR, positive or negative HER2 or high Ki67; HER2-positive: negative ER and PR, positive HER2 and any expression of Ki67; and Triple-negative: negative ER, PR and HER2 with any expression of Ki67 (24).

Statistical analysis

Statistical analysis was performed using the SPSS statistical program, version 29 (SPSS, IBM Inc, Chicago, IL). To compare pre- and post-therapy variables, the McNemar test was used for dichotomous variables and the McNemar-Bowker for variables with more categories. The Wilcoxon's test was used to analyze pre- and post-therapy continuous variables.

Factors associated with overall survival time of patients were analyzed using Kaplan-Meier curves and compared with the log-rank test. The Cox proportional hazards model was also used. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated. Two multivariate models were constructed: one based on pre and the other on post-NACT characteristics. A backward-conditional strategy was used. The tests were considered statistically significant when p value was < 0.05 .

RESULTS

Clinicopathological characteristics

The clinical characteristics and survival univariate analysis are summarized in table 1. Patient's age at diagnosis ranged from 24 to 75 years old (mean 49.65 years, standard deviation ± 10.96). The patient's age at the time of diagnosis influenced overall

survival, with a lower risk of death with each additional age year (HR, 0.98, CI 0.96-1.00, $p = 0.046$).

Pre-NACT tumor samples were from core needle biopsies (51 cases, 59.3%) and incisional biopsies (35 cases; 40.7%); post-NACT tumor samples were from mastectomies (70 cases, 81.4%) and breast conserving surgeries (16 cases, 18.6%).

Survival data was available from 85 patients, with a median follow-up time of 43 months (range, 8 to 161 months). Sixty-one patients (70.9%) died during follow-up.

Most patients had locally advanced or metastatic breast cancer at presentation: 68 (79%) had tumor size of T3 or T4, 74 (86%) had clinically positive lymph nodes and 12 (14%) had metastatic disease. Most cases were on pathological stage III (57 cases; 66.3%). Four cases (4.7%) were not staged due to missing information. There were no cases on stage I.

The RCB of the cases with residual disease was: RCB=III (55 cases, 64%), RCB=II (30 cases, 34.9%) and RCB=I (1 case, 1.2%). When analyzing patients' survival according to RCB, cases classified as RCB II had a non-significant lower risk of death when compared to RCB III (HR 0.62, CI 0.15 – 1.09, $p = 0.098$) in univariate analysis. No patient with metastatic disease had a pCR.

Table 1: Clinical characteristics and survival analysis of 86 invasive breast carcinomas

Parameters (%)			N of events (%)	Hazard ratio (95%CI)	p-value ^{a)}
Age, mean 49.65 (SD ± 11.40)			-	0.98 (0.96 – 1.00)	0.046
Stage	II	13 (15.1)	10 (76.9)	1	-
	III	57 (66.3)	40 (70.2)	0.94 (0.47 – 1.89)	0.864
	IV	12 (14)	9 (75)	1.29 (0.52 – 3.19)	0.586
	Missing	4 (4.7)			

	1	2 (2.3)	2 (100)	1	-
	2	12 (14)	9 (75)	0.59 (0.12 – 2.74)	0.497
Tumor size	3	26 (30.2)	18 (69.2)	0.69 (0.16 – 3.00)	0.622
	4	42 (48.8)	30 (71.4)	0.63 (0.15 – 2.64)	0.525
	Missing	4 (4.7)			
	0	8 (9.3)	7 (87.5)	1	-
Positive	1	31 (36)	20 (64.5)	0.56 (0.23 – 1.33)	0.189
Lymph	2	40 (46.5)	30 (75)	0.64 (0.28 – 1.48)	0.302
nodes	3	3 (3.5)	2 (66.7)	0.90 (0.19 – 4.36)	0.899
	Missing	4 (4.7)			
	0	70 (81.4)	50 (71.4)	1.35 (0.66 – 2.74)	0.408
Distant	1	12 (14)	9 (75)		
Metastasis	Missing	4 (4.7)			
Residual					-
Cancer	I	1 (1.2)	0	0.00 (0.00 – 5.18)	0.972
Burden	II	30 (30.4)	17 (56.7)	0.62 (0.35 – 1.09)	0.098
Class	III	54 (64)	44 (81.5)	1	

SD: Standard deviation. ^ap-values calculated by cox proportional hazards model.

Regarding NACT, drug information was available for 82 patients. The most common regimens were doxorubicin plus cyclophosphamide followed by taxane (60 patients, 69.8%) and doxorubicin plus cyclophosphamide (10 patients, 11.6%). Four (8.3%) of 48 pre-NACT ER-positive patients also received hormone therapy and 3 of 11 (27.3%) HER2-positive patients received trastuzumab. In adjuvant setting, all 37 patients with post-NACT ER-positive tumors and 11 patients who tumors tested negative after NACT received hormonal treatment. Six out of 10 post-NACT HER2-positive tumors (60%) received trastuzumab.

The pathological characteristics and survival univariate analysis of the 86 cases with residual disease after NACT are summarized in table 2, and survival curves are shown in figure 5. Survival multivariate analysis are summarized in table 3. The most prevalent initial histological characteristics were invasive carcinoma of no special type (63 cases;

73.3%), histological grade III (42 cases, 48.8%) and low tumor infiltrating inflammatory cells (55 cases, 64.0%). After NACT, the proportion of these characteristics was: 52 cases (60.5%) had invasive carcinoma of no special type (NST), 41 cases (47.7%) had histological grade III and 49 cases (57%) had low tumor infiltrating inflammatory cells. Eight tumors (9.3%) showed rare residual isolated tumor cells, limiting histological type and grade analysis.

Tumor characteristics discordance with pre- and post-NACT were: 19 tumors (22.1%) changed histological type ($p = 0.392$), 10 tumors (52.7%) changed from Ductal NST to pure special types; 31 tumors (36.1%) showed changes on histological grade, 21 (24.4%) changed to a higher and 10 (11.6%) changed to a lower histological grade ($p = 0.115$). Evaluating the three individual features for assessing histological grade, 13 tumors (16.7%) changed tubule formation score ($p = 0.189$), 25 (32%) tumors changed nuclear pleomorphism score ($p = 0.025$), and 37 (47.4%) tumors changed the mitotic count score ($p = 0.002$, supplementary table 2).

The intratumoral infiltrating inflammatory cells changed in 43 (50%) cases, showing a lower percentage in 31 (36%), and in 12 cases (14%) showing a higher percentage in the residual tumor ($p = 0.016$).

Associated *in situ* carcinoma was identify more frequently in surgical specimens post-NACT, with 29 cases (33.7%) compared to 11 cases (12.8%) pre-NACT samples ($p < 0.001$).

Lymphovascular invasion (LVI) was identify in 24 (27.9%) initial samples, and in post-NACT samples was almost the double, with 44 cases (51.2%; $p = 0.002$).

The following pre-NACT tumor characteristics showed association with higher risk of death in univariate analysis: histological grade 3 versus grade 1 (HR 2.94, CI 1.35 – 6.39; $p = 0.007$); nuclear pleomorphism grade 3 versus grade 1 (HR 2.53, CI 1.06 – 6.00; $p = 0.036$); and mitotic count grade 2 versus grade 1 (HR 2.26, CI 1.21 – 4.24; $p = 0.011$). The following post-NACT tumor characteristics showed association with higher risk of death: mitotic count grade 2 versus grade 1 (HR 2.12, CI 1.04 – 4.30; $p = 0.038$) and presence of lymphovascular invasion (HR 1.90, CI 1.12 – 3.19; $p = 0.016$).

Biomarkers' status

The most frequent pre-NACT biomarkers expression of the initial tumors was ER positive (48 cases, 55.8%), PR positive (46 cases, 53.5%) and HER2 negative (73 cases, 87.2%). Two cases were HER2-equivocal (2+), but one showed gene amplification by fluorescence in situ hybridization (FISH) and was interpreted as HER2 positive.

Triple-negative (31 cases, 36.0%) and Luminal B-like (30 cases, 34.6%) were the most frequent immunohistochemical profiles, followed by Luminal A-like (18 cases, 20.9%) and HER2-positive (7 cases, 8.1%).

The most frequent post-NACT biomarkers expression was ER negative (49 cases, 57.0%), PR negative (57 cases, 66.3%) and HER2 negative (76 cases, 88.4%). Most residual tumors had a low Ki67 rate (62 cases, 72.1%).

Except for HER2 expression, all biomarkers had significant changes after NACT, including the IHC profile.

ER status changed in 13 (15.1%) residual tumors ($p = 0.003$), most commonly becoming negative (12 cases; 14%; figure 1A e 1B, supplementary table 2). Ten tumors (11.6%) showed a higher Allred ER score (% and intensity of stained cells), and 24

cases (27.9%) showed a lower ER score. The patient whose tumor turned ER from negative to positive received adjuvant hormone treatment, and died within 32 months. Changes in ER status did not influence the patients' overall survival, with a hazard ratio of 1.02 (CI: 0.51 - 2.03) and $p = 0.958$.

PR status changed in 19 (22.1%) residual tumors ($p = 0.019$); the majority becoming negative (15 cases; 17.4%; figure 1B e 1C). PR Allred score remained the same in 53 tumors (61.6%), seven (8.1%) changed to a higher and 26 tumors (30.2%), to a lower Allred PR score. Four patients but one, whose tumors PR turned to positive received adjuvant hormonal therapy; 13 out of 15 patients whose tumors' PR turned to negative didn't use hormone therapy in the adjuvant setting, and one patient had no information about adjuvant hormone therapy. Changes in PR status did not influence the patients' overall survival, with a hazard ratio of 0.74 (CI: 0.39 – 1.39, $p = 0.352$).

HER2 expression changed from positive to negative in two tumors ($p = 0.051$); one changed from score 2+, FISH amplified to score 0 and the other changed from score 3+ to score 1+ (figure 1D e 1E). These patients did not receive anti-HER2 neoadjuvant treatment, but one of them received anti-HER2 adjuvant treatment. HER2 scores changed in 13 tumors (15.1%, $p = 1.000$). Curiously, one score 3+ tumor changed to score 2+ but was FISH amplified. Changes in HER2 status did not influence the patients' overall survival, with a hazard ratio of 0.05 (CI: 0.00 – 17.22, $p = 0.309$).

Ki67 index changed in 52 tumors (60.5%), the majority to a lower index (figure 1G e 1H). Median Ki67 expression changed from 28% (95% confidence interval, CI, 25.6 to 32.7%) in pre-NACT to 18% (95% CI 19.2 to 28.2%) in post-NACT samples ($p = 0.004$). The distribution of the data in relation to Ki67 expression was different when comparing pre-therapy with post-therapy tumor samples. Before NACT, the data

showed a normal distribution and after NACT the data were skewed to the right (figure 2).

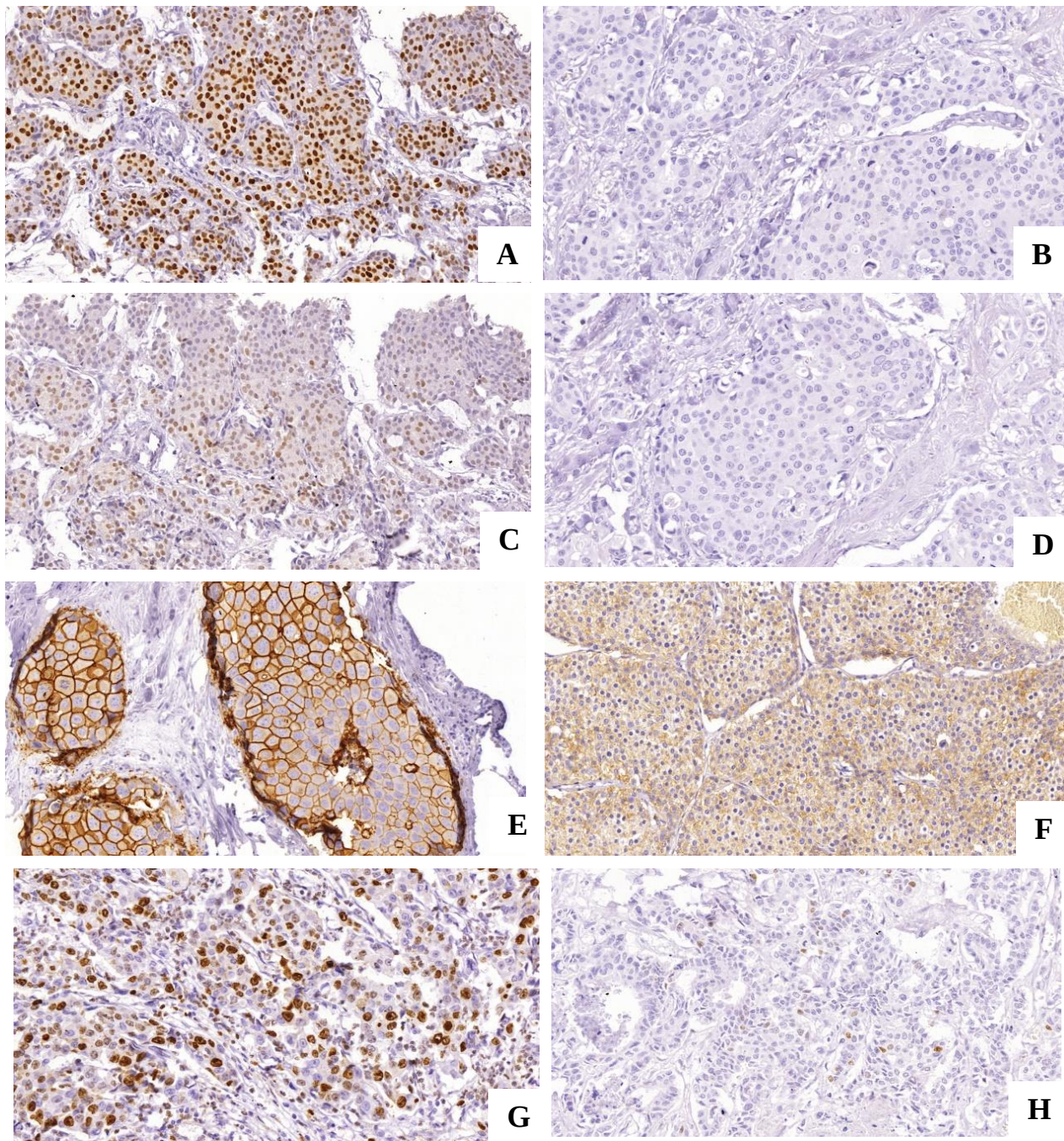
Triple-negative (42 cases, 48.8%) and Luminal A-like (26 cases, 30.2%) were the most frequent immunohistochemical profiles of the residual tumors, followed by Luminal B-like (12 cases, 14.0%) and HER2-like (6 cases, 7.0%). Most tumors maintained the same immunohistochemical profile (60 cases, 69.8%, figure 3). Twenty-six tumors (30.2%) showed changes after NACT ($p < 0.001$): four tumors were initially Luminal-A like, 20 were Luminal-B like and 2 were HER2. Interestingly, none of the initial triple-negative and only one HER2 positive tumor changed subtype in residual tumors. Five metastatic patients changed the subtype: one from luminal B to luminal A-like and four from luminal B-like to triple-negative. No metastatic patients were initially luminal A-like. Changes in subtype were associated with tumor histologic grade: 10 (66.7%) grade 1 tumors changed subtype, versus 6 (20.7%) grade 2 and 11 (26.2%) grade 3 cases ($p = 0.005$).

The following tumor biomarkers were associated with a lower risk of death: being pre- (HR: 0.35, CI 0.21 – 0.58, $p < 0.001$) and post-NACT (HR: 0.39; $p < 0.001$) ER positive, pre- (HR: 0.37, CI 0.20 – 0.63; $p < 0.001$) and post-NACT (HR: 0.57, CI 0.32 – 0.98; $p = 0.044$) PR positive. Having a pre-NACT high Ki67 index (HR: 2.47; $p = 0.001$; figure 4A), post- Luminal B-like profile (HR: 2.58, CI 1.09 – 6.13; $p = 0.031$); and pre- (HR: 4.45, CI 2.12 – 9.32; $p < 0.001$; figure 4B) and post-NACT (HR: 4.52, CI 2.29 – 8.92; $p < 0.001$; figure 4C) triple-negative IHC profile were associated with a higher risk of death. Changes in immunohistochemical profile was associated with overall survival in log-rank test ($p = 0.050$) and in cox-regression showed a tendency to

increase patients' overall survival time, with a hazard ratio of 0.57 (CI: 0.33 – 1.01, $p = 0.054$, figure 4D).

In the multivariate analysis, the pre-NACT histologic grade ($p = 0.021$); and IHC profile in both pre-NACT and post-NACT ($p = 0.003$ and $p < 0.001$, table 3) proved to be an independent factor of overall survival samples.

Figure 1: Biomarkers IHC assays (200x magnification) of invasive breast carcinomas



showing tumors: ER positive in the pre-NACT sample (A) and negative in the post-NACT sample (B); PR positive in the pre-NACT sample (C) and negative in the post-NACT sample (D); Her2 2+ in the pre-NACT sample (E) and 0 in the post-NACT sample (F); high Ki67 index in the pre-NACT sample (G) and a low index in the post-NACT sample (H).

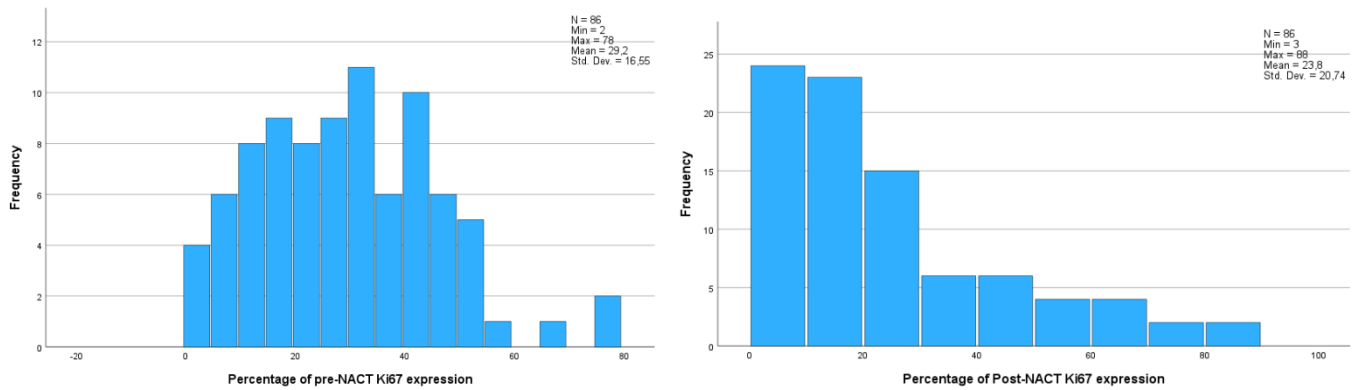


Figure 2: Distribution of pre- and post-NACT Ki67 expression in 86 invasive breast carcinomas ($p = 0.004$)

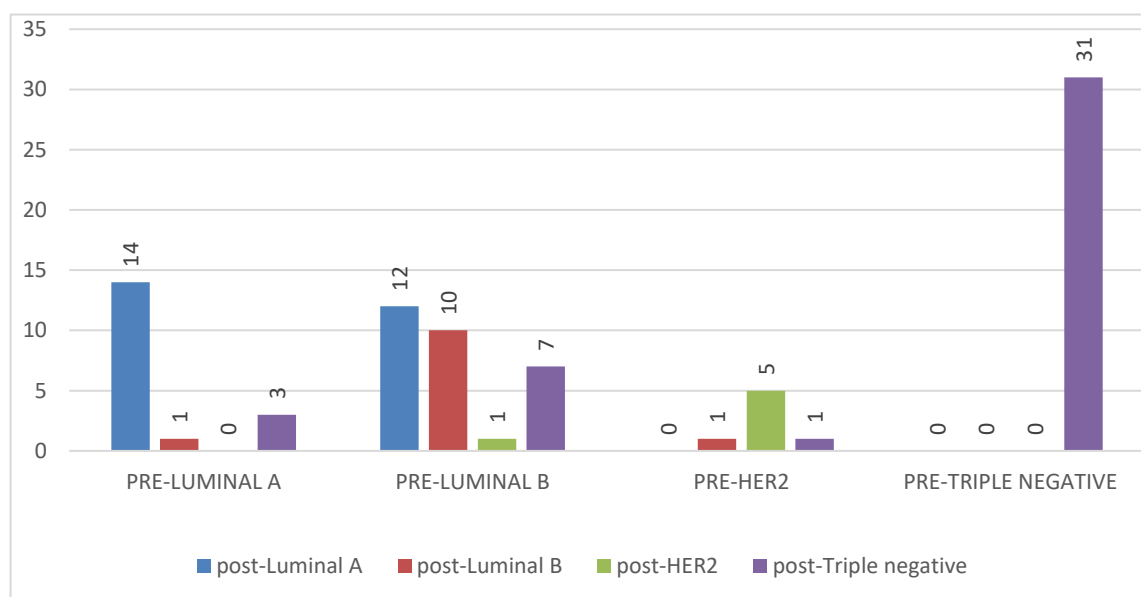


Figure 3: Changes in tumor IHC profiles in pre- and post-NACT samples from 86 invasive breast carcinomas ($p < 0.001$)

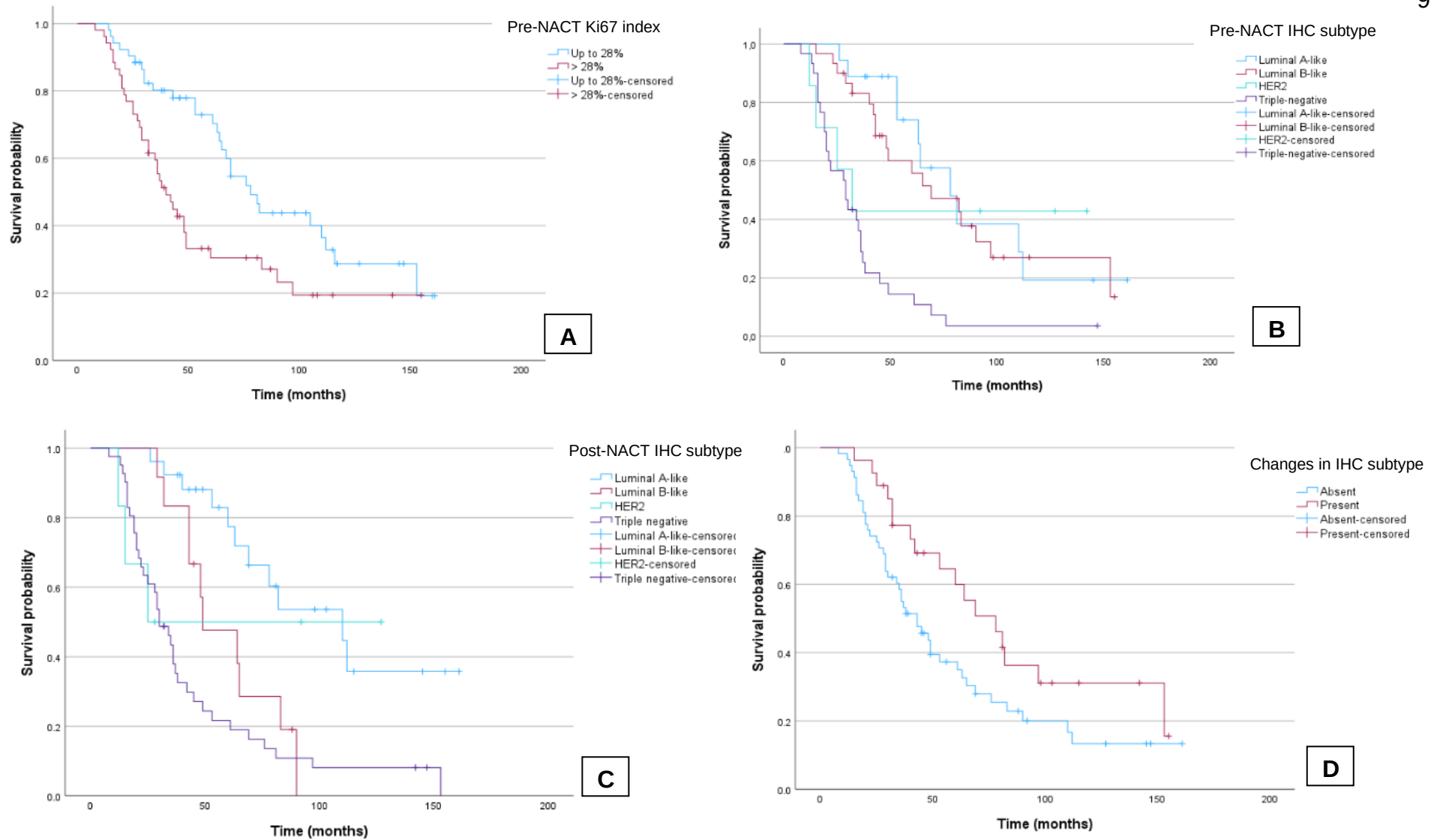


Figure 4 - Survival probability of patients with invasive breast carcinoma according to: (A) Ki67 index in pre-NACT samples ($p = 0.006$); (B) IHC profile in pre-NACT samples ($p < 0.001$); (C) IHC profile in post-NACT samples ($p < 0.001$); and (D) Changes in IHC profiles after NACT ($p = 0.050$).

Parameters	Pre-NACT	Post-NACT	Pre and post-	Pre-NACT survival	Post-NACT survival
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Table 2: Clinicopathological characteristics of invasive breast carcinomas pre- and post-NACT and univariate survival analysis

		samples N (%)	samples N (%)	NACT p-value ^{a)}	N of events (%)	Hazard Ratio (95%CI)	p-value ^{a)}	N of events (%)	Hazard Ratio (95%CI)	p-value ^{a)}
Histological type	Ductal NST	63 (73.3)	52 (60.5)	0.392	47 (75.8)	1	-	36 (70.6)	1	-
	Invasive lobular carcinoma	7 (8.1)	5 (5.8)		3 (42.9)	0.38 (0.12 – 1.24)	0.109	4 (80)	0.93 (0.33 – 2.62)	0.886
	Other special types	16 (18.6)	21 (24.4)		11 (68.7)	0.94 (0.49 – 1.82)	0.856	17 (81)	1.32 (0.74 – 2.35)	0.349
	Isolated cells only	0	8 (9.3)		-	-	-	4 (50)	0.60 (0.21 – 1.69)	0.331
Histological grade	I	15 (17.4)	6 (7.0)	0.115	8 (53.3)	1	-	3 (50)	1	-
	II	29 (33.7)	31 (36.0)		20 (69)	1.91 (0.84 – 4.38)	0.124	21 (72.4)	1.96 (0.58 – 6.60)	0.275
	III	42 (48.8)	41 (47.7)		33 (80.5)	2.94 (1.35 – 6.39)	0.007	33 (82.5)	2.98 (0.91 – 9.75)	0.071
	Not evaluable	0	8 (9.3)		-	-	-	-	-	-
Tubule Formation score	1	4 (4.7)	2 (2.3)	0.189	2 (50)	1	-	1 (50)	1	-
	2	10 (11.6)	17 (19.8)		6 (60)	1.33 (0.27 – 6.58)	0.730	10 (58.8)	1.83 (0.23 – 14.32)	0.566
	3	72 (83.7)	59 (68.6)		53 (74.6)	2.16 (0.52 – 8.89)	0.286	46 (79.3)	3.69 (0.50 – 26.90)	0.198
	Not evaluable	0	8 (9.3)		-	-	-	-	-	-
Nuclear Pleomorphism score	1	10 (11.6)	2 (2.3)	0.025	6 (60)	1	-	0	1	-
	2	23 (26.7)	20 (23.3)		15 (65.2)	1.70 (0.66 – 4.40)	0.373	13 (65)	- (0.00 – ∞)	0.889
	3	53 (61.6)	56 (65.1)		40 (76.9)	2.53 (1.06 – 6.00)	0.036	44 (78.6)	- (0.00 – ∞)	0.881
	Not evaluable	0	8 (9.3)		-	-	-	-	-	-
Mitotic Count score	1	35 (40.7)	32 (38.4)	0.002	22 (62.9)	1	-	21 (65.6)	1	-
	2	25 (29.1)	16 (17.4)		19 (79.2)	2.26 (1.21 – 4.24)	0.011	13 (81.2)	2.12 (1.04 – 4.30)	0.038
	3	26 (30.2)	30 (34.9)		20 (76.9)	1.65 (0.89 – 3.03)	0.109	23 (79.3)	1.48 (0.81 – 2.67)	0.199
	Not evaluable	0	8 (9.3)		-	-	-	-	-	-
Tumor infiltrating Inflammatory cells	0%	13 (15.1)	25 (29.1)	0.016	10 (76.9)	1	-	18 (72)	1	-
	1 to 20%	55 (64.0)	49 (57.0)		39 (70.9)	0.83 (0.42 – 1.69)	0.620	32 (65.3)	0.87 (0.49 – 1.57)	0.656
	over 21%	18 (20.9)	12 (14.0)		12 (70.6)	0.85 (0.37 – 1.98)	0.715	11 (92)	1.32 (0.59 – 2.95)	0.503
Associated <i>in situ</i> carcinoma	Present	11 (12.8)	29 (33.7)	< 0.001	5 (45.5)	0.42 (0.17 – 1.07)	0.068	20 (69)	1.11 (0.66 – 1.88)	0.682
	Absent	75 (87.2)	57 (66.3)		56 (75.7)			41 (71.9)		
Lymphovascular invasion	Present	24 (27.9)	44 (51.2)	0.002	17 (70.8)	1.26 (0.71 – 2.23)	0.422	36 (81.8)	1.90 (1.12 – 3.19)	0.016
	Absent	62 (72.1)	42 (48.8)		44 (72.1)			25 (61.0)		
Estrogen receptor status	Negative	38 (44.2)	49 (57.0)	0.003	32 (86.5)	0.35 (0.21 – 0.58)	< 0.001	40 (83.3)	0.39 (0.23 – 0.67)	< 0.001
	Positive	48 (55.8)	37 (43.0)		29 (60.4)			21 (56.8)		
Progesterone receptor status	Negative	46 (53.5)	57 (66.3)	0.019	38 (84.4)	0.37 (0.2 – 0.63)	< 0.001	43 (76.8)	0.57 (0.32 – 0.98)	0.044
	Positive	40 (46.5)	29 (33.7)		23 (57.5)			18 (62.1)		
HER2 expression	Negative	75(87.2)	76 (88.4)	0.051	54 (73)	1.08 (0.49- 2.38)	0.842	54 (72)	1.21 (0.55 – 2.67)	0.632
	Positive	11(12.8)	10 (11.6)		7 (63.6)			7 (70)		
Ki67 expression	Low	44 (51.2)	62 (72.1)	0.003	26 (60.5)	2.47 (1.47 – 4.15)	0.001	41 (66.1)	1.68 (0.98 – 2.87)	0.060
	High	42 (48.8)	24 (27.9)		35 (83.3)			20 (87.0)		
IHC Profile	Luminal A-like	18 (20.9)	26 (30.2)	< 0.001	10 (55.6)	1	-	11 (42.3)	1	-
	Luminal B-like	30 (34.9)	12 (14.0)		19 (63.3)	1.12 (0.57 – 2.65)	0.595	10 (83.3)	2.58 (1.09 – 6.13)	0.031
	HER-2 positive	7 (8.1)	6 (7.0)		4 (57.1)	1.25 (0.39 – 4.01)	0.712	3 (50)	2.03 (0.56 – 7.30)	0.279
	Triple-negative	31 (36.0)	42 (48.8)		28 (93.3)	4.45 (2.12 – 9.32)	< 0.001	37 (88.1)	4.52 (2.29 – 8.92)	< 0.001

NACT- Neoadjuvant chemotherapy, Ductal NST- Invasive carcinoma of no special type, HER2 - human epidermal growth factor receptor 2, IHC profile- immunohistochemmichal profile.; survival analysis refers to the 85 cases with available survival data; ^ap-value calculated by Mc-Nemar, Mc-Nemar-Bowker, Wilcoxon and cox proportional hazards model.

Table 3: Multivariate survival analysis of patients with invasive breast carcinomas before and after NACT

Included parameters		Final step	
		HR (95%CI)	p-value ^{a)}
Pre-NACT			
Age at diagnosis		-	-
Histological grade	I	1	-
	II	1.87 (0.79 – 4.39)	0.152
	III	2.71 (1.16 – 6.31)	0.021
IHC profile	Luminal A-like	1	-
	Luminal B-like	0.91 (0.41 – 2.04)	0.823
	HER2-like	0.73 (0.21 – 2.50)	0.618
	Triple-negative	3.32 (1.52 – 7.27)	0.003
Pre-NACT			
Age at diagnosis		-	-
Presence of lymphovascular invasion		-	-
Histological grade	I	-	-
	II	-	-
	III	-	-
IHC profile	Luminal A-like	1	-
	Luminal B-like	2.26 (0.95 – 5.35)	0.065
	HER2-like	2.06 (0.45 – 9.35)	0.349
	Triple-negative	4.39 (2.20 – 8.77)	<0.001

NACT- Neoadjuvant chemotherapy; IHC profile – immunohistochemical profile; ^{a)}p-values, calculated by cox proportional hazards model

DISCUSSION

In our study, 26 (30.2%) tumors changed the immunohistochemical subtype. The most frequent changes were from luminal B to luminal A-like subtype (46.2%) and from luminal B-like to triple-negative (27%). The post-NACT IHC profile was still strongly associated with survival in comparison to the pre-NACT sample. Other studies also found post-NACT changes in 16% to 27.2% of cases. Some of them did not consider the Ki67 index in subtype classification (7,25,26).

Positivity of estrogen and progesterone receptors predicts response to endocrine therapy. It also represents a lower risk of recurrence in short term (17). Changes in estrogen and progesterone receptors status from positive to negative were significant. In the literature, similar results have been described, either turning from negative to positive or from positive to negative after neoadjuvant chemotherapy (4,5,7,25,27). Reddy et al described a worse prognosis when PR status changed to negative (1). On the other hand, Chatterjee et al described shorter survival time when hormone receptor negative patients became positive after NACT (28).

HER2 changes can also occur after NACT. Only two cases lost HER2 overexpression, and curiously, one of them was equivocal (score 2+) at the initial biopsy (5,12). Several studies found HER2 expression discordance, mostly losing expression after therapy, ranging from 9.8% to 24.7%, but in some of these studies patients were treated with anti-HER2 target therapy (5,7,25,27). In our study, patients with HER2 positive tumors did not receive anti-HER2 target therapy in the neoadjuvant setting and only one patient out of two whose tumor lost HER2 expression, received adjuvant anti-HER2 therapy. In our study, HER2 expression and changes were not associated with overall survival time. However, only 11 (12.8%) of our

patients were HER2 positive.

The changes in tumor pathological characteristics and biomarkers expression after neoadjuvant therapy are widely debated in the literature. They could directly affect decisions regarding adjuvant therapy. Interestingly, in our study, changes in IHC profile were marginally associated with longer overall survival time (HR 0.57, CI, 0.32-1.01, $p = 0.056$). We believe this is due to the most common changes encountered, as lower Ki67 index, with a change from luminal B to luminal A-like breast cancer. The Ki67 index is a well-established proliferative marker predictive of response to chemotherapy (4,29,30). In our study, nearly half of post-therapy residual tumors (42/86) had lower index of Ki67, representing more favorable clinical outcomes. Many other studies showed lower indexes of Ki67 after NACT (1,31). Those changes may be due to therapy targeting proliferating cells (29). Adjuvant hormonal and anti-HER2 therapies also may have influenced our survival results.

The dynamics of IHC changes is yet to be fully understood. Thus, it is debatable whether targeted treatment should be reviewed according to changes in IHC subtype after NACT. To our knowledge, no prospective randomized trials have been performed addressing this issue. Although there is still no consensus or guideline indicating biomarkers routine retesting in residual tumors (32,33), we are in accordance with previous authors who recommend biomarkers should be retested, especially in cases initially HR or HER2 negative, since these could represent false negative results (1,4,5,7,14,25,27,31).

Basically, two factors can explain changes in tumor characteristics before and after NACT: sampling differences in tumor biopsies versus resection specimens, and changes related to neoadjuvant treatment.

Tumor heterogeneity, small sampling of the initial biopsy, results close to the

negative/positive limit, and false-negative results (pre-analytical and/or analytical factors)(1,27,34,35) have been described. We detected more *in situ* carcinomas and the presence of lymphovascular invasion in residual post-therapy samples than in pre-therapy samples. Core biopsies tends to represent the central area of the tumor, whereas these features predominate in the periphery of the tumor. These changes were significant. (36). Lymphovascular invasion was not an independent predictor of survival time in multivariate analysis (27).

Changes in nuclear pleomorphism and mitotic index may be associated with chemotherapy effects on cells and tumor progression, but have also been reported in surgical samples of patients who didn't undergo neoadjuvant therapy, usually explained by sampling variability with a higher tumor grade in the resection specimen when compared to the initial core needle biopsy (37).

Histological type changes in residual tumors after neoadjuvant chemotherapy have also been described previously (34,35,38). Our study included tumors with more than one histological type (mixed type) in the initial biopsy sample that showed only one special type in post NACT residual tumor, indicating that cell clones could be selected. However, other tumors with one pure special type changed to mixed types after surgical resection. These could represent true changes after NACT in histological tumor type or could have had an under representative tumor sample of a heterogeneous tumor reaching only part of the tumor (1,27). Changes in histological type were not significant in this study.

In In general, there is a good concordance with the percentage of tumor infiltrating inflammatory cells between the initial biopsy and the surgical resection tumors (39). It is already known that a higher percentage of tumor infiltrating inflammatory cells predicts a

good response to chemotherapy. The increase in the percentage of these inflammatory cells after NACT is also associated with longer survival time, especially in triple-negative invasive breast carcinomas (31). In this study, changes in tumor infiltrating inflammatory cells were significant but did not influence the overall survival of our patients.

This study was conducted in a Brazilian public hospital. Our cases were diagnosed and treated at the same hospital, following the same therapy protocols. All histopathological slides were reviewed and analyzed by the same pathologist (CBN). Although a retrospective study, immunohistochemical tests were repeated, using the same protocol and antibodies. All assays were performed by the same person (BJS), seeking to standardize the reactions and eliminate interpretation or technical bias. Unfortunately, all our patients had advanced breast cancer: 86% had positive lymph nodes, 79% had tumors T3 or T4, and 14% had metastatic disease. Most of them died during follow up. Other studies have shown that Brazilian patients are frequently diagnosed with advanced disease. Many don't have access to diagnosis and therapies in due time, which may explain our frequency of high stage disease and short overall survival time (40,41).

In conclusion, we showed changes in tumor characteristics after exposure to NACT. Knowledge of those changes can help predict a patient's risk of death, thus tailor adjuvant treatments. Thus, we suggest that tumors should be reclassified, regraded and IHC biomarkers should be retested on post-neoadjuvant residual tumors.

CONFLICTS OF INTEREST

The authors declared no conflict of interest.

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SUPPLEMENTARY

S1 Table - Antibodies, clones, manufacturers, detection systems, dilutions and antigenic retrieval of antibodies

Antibody	Clone	Manufacturers	Detection systems	Dilution	Antigenic retrieval
HER2	A0485 (HercepTest™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Water bath – Epitope retrieval solution, pH 6, Agilent Technologies, 35 minutes
RE	1D5 (PharmaDX™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies, 30 minutes

RP	PGR1294 (PharmaDX™)	Agilent Technologies, United States of America	Visualization reagent, Agilent Technologies	Ready to use	Steamer – Epitope retrieval solution, high pH, Agilent Technologies, 30 minutes
Ki67	MIB1	Agilent Technologies, United States of America	Detection and amplification system, Novolink, Leica Biosystems, Newcastle upon Tyne, UK	Ready to use	Steamer – Target Retrieval Solution high pH, Agilent Technologies, 30 minutes

HER2 - human epidermal growth factor receptor 2; RE – Estrogen receptor; PR – Progesterone receptor.

S2 table: Changes on histopathological characteristics after NACT in invasive breast carcinomas samples

Parameter	Tumor behavior	N (%)	Type	N of total of cases (%)	N of changes (%)
Histological type	Stable	59 (68.6%)	Ductal NSE to Lobular	1 (1.2)	1 (5.3)
	Changed	19 (22.1%)	Ductal NSE to special types	10 (11.6)	10 (52.6)
	Isolated Cells	8 (9.3%)	Lobular to special types	2 (2.3)	2 (10.5)
			Special types to Ductal NSE	5 (5.8)	5 (26.3)
			Special types to Lobular	1 (1.2)	1 (5.3)
Histological grade	Stable	47 (54.6%)	Grade I to grade II	9 (10.5)	9 (29)
	Changed	31 (36.1%)	Grade I to grade III	1 (1.2)	1 (3.2)
	NE	8 (9.3%)	Grade II to grade I	2 (2.3)	2 (6.5)
			Grade II to grade III	11 (12.8)	11 (35.5)
			Grade III to grade I	0	0
Tubular Formation	Stable	65 (75.6%)	Score 1 to score 2	2 (2.3)	2 (15.4)
	Changed	13 (15.1%)	Score 1 to score 3	1 (1.2)	1 (7.7)
	NE	8 (9.3%)	Score 2 to score 1	0	0
			Score 2 to score 3	2 (2.3)	2 (15.4)
			Score 3 to score 1	1 (1.2)	1 (7.7)
Nuclear Pleomorphism	Stable	53 (61.6%)	Score 3 to score 2	7 (8.1)	7 (53.8)
	Changed	25 (29.1%)	Score 1 to score 2	7 (8.1)	7 (28)
	NE	8 (9.3%)	Score 1 to score 3	2 (2.3)	2 (8)
			Score 2 to score 1	2 (2.3)	2 (8)
			Score 2 to score 3	11 (12.8)	11 (44)
			Score 3 to score 1	0	0
			Score 3 to score 2	3 (3.5)	3 (12)

Mitotic count	Stable	41	Score 1 to score 2	5 (5.8)	5 (13.5)
	Changed	(47.7%)	Score 1 to score 3	5 (5.8)	5 (13.5)
	NE	37 (43%)	Score 2 to score 1	7 (8.1)	7 (18.9)
		8 (9.3%)	Score 2 to score 3	11 (12.8)	11 (29.7)
			Score 3 to score 1	4	4 (10.8)
			Score 3 to score 2	5 (5.8)	5 (13.5)
Inflammatory Infiltrate	Stable	43	0% to 1-20%	3 (3.5)	3 (7)
	Changed	(50.0%)	0% to over 21%	3 (3.5)	3 (7)
		43	1-20% to 0%	16 (18.6)	16 (37.2)
		(50.0%)	1-20% to over 21%	6 (7)	6 (14)
			over 21% to 0%	2 (2.3)	2 (4.6)
			over 21% to 1-20%	13 (15.1)	13 (30.2)
Presence of <i>in situ</i> carcinoma	Stable	62	Absent to present	21 (24.4)	21 (87.5)
	Changed	(72.1%)	Present to Absent	3 (3.5)	3 (12.5)
Lymphovascular invasion		24			
		(27.9%)			
Estrogen receptor status	Stable	48	Absent to present	29 (33.7)	29 (76.3)
	Changed	(55.8%)	Present to Absent	9 (10.5)	9 (23.7)
		38			
		(44.2%)			
Progesterone receptor status	Stable	73	Positive to negative	12 (14)	12 (92.3)
	Changed	(84.9%)	Negative to positive	1 (1.2)	1 (7.7)
		13			
		(15.1%)			
HER2 expression	Stable	67	Positive to negative	15 (17.4)	15 (78.9)
	Changed	(77.9%)	Negative to positive	4 (4.7)	4 (21.1)
		19			
		(22.1%)			
HER2 expression (categories)	Stable	84	Positive to negative	2 (2.3)	2 (100)
	Changed	(94.7%)	Negative to positive	0	0
		2 (5.3%)			
Ki67 expression	Stable	73	0 to 1+	1 (1.2)	1 (7.7)
	Changed	(84.9%)	1+ to 0	8 (9.3)	8 (61.5)
		13	2+ to 0	2 (2.3)	2 (15.4)
		(15.1%)	3+ to 1+	1 (1.2)	1 (7.7)
			3+ to 2+	1 (1.2)	1 (7.7)
IHC Subtype	Stable	52	Low to high	8 (9.3)	8 (23.5)
	Changed	(60.5%)	High to low	26 (30.2)	26 (76.5)
		34			
		(39.5%)			

NACT- Neoadjuvant chemotherapy, HER2 - human epidermal growth factor receptor 2, IHC - immunohistochemical.; NE – not evaluable.

8.3 Aprovação do Comitê de Ética – Plataforma Brasil

UNIVERSIDADE FEDERAL DE
MINAS GERAIS



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeitos da terapia neoadjuvante na expressão de marcadores imuno-histoquímicos e no padrão histológico em carcinomas mamários invasores e nas metástases ganglionares

Pesquisador: Cristiana Buzelin Nunes

Área Temática:

Versão: 1

CAAE: 68441217.3.0000.5149

Instituição Proponente: UNIVERSIDADE FEDERAL DE MINAS GERAIS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.109.565

UNIVERSIDADE FEDERAL DE
MINAS GERAIS



Continuação do Parecer: 2.109.565

Situação do Parecer:

Aprovado

Necessita apreciação da CONEP:

Não

BELO HORIZONTE, 08 de Junho de 2017

8.4 Termo de Consentimento Livre e esclarecido

Consentimento Livre e Esclarecido

Será solicitada dispensa do Termo de Consentimento Livre e Esclarecido ao Comitê de Ética em Pesquisa (COEP), pois conforme disposto na Resolução do Conselho Nacional de Saúde / CNS 466/2012, nos casos em que serão utilizados Dados Secundários como prontuários e arquivos, em que não tragam prejuízos nem benefícios para o paciente ou não interfiram no curso da doença ou no tratamento, este termo poderá ser dispensado após análise, aprovação e parecer favorável do Comitê de Ética em Pesquisa.

Serão preservados o sigilo e a confidencialidade dos dados e do anonimato dos indivíduos pesquisados, garantindo a privacidade dos pacientes.

O foco deste estudo são os efeitos da terapia neoadjuvante em marcadores imuno-histoquímicos e no padrão morfológico, que serão analisados em lâminas e blocos de parafina contendo amostras do tumor de pacientes com câncer de mama, não havendo portanto, contato direto com os pacientes.

Solicitação de Isenção do Termo de Consentimento Livre e Esclarecido

Referência: Efeitos da terapia neoadjuvante na expressão de marcadores imuno-histoquímicos e no padrão histológico em carcinomas mamários invasores e nas metástases ganglionares.

Responsável: Cristiana Buzelin Nunes

Ao Comitê de Ética em Pesquisa Faculdade de Medicina da UFMG

Venho por meio deste documento solicitar a dispensa de obtenção de um Termo de Consentimento Livre e Esclarecido (TCLE) para o estudo intitulado "Efeitos da terapia neoadjuvante na expressão de marcadores imuno-histoquímicos e no padrão histológico em carcinomas mamários invasores e nas metástases ganglionares", proposto por Cristiana Buzelin Nunes.

A dispensa do uso de TCLE se fundamenta:

- a) por ser um estudo observacional, analítico e descritivo retrospectivo, que empregará apenas informações de prontuários médicos, sistemas de informação institucionais e/ou demais fontes de dados e informações clínicas disponíveis na instituição e no laboratório de Anatomia Patológica responsável pela realização dos exames Anatomopatológicos e Imuno-histoquímicos das pacientes com Diagnóstico de Carcinoma Mamário Invasivo tratadas no Hospital das Clínicas da UFMG de Belo Horizonte, sem previsão de coletas de Novos materiais Biológicos nestas pacientes, onde serão objetos de estudos as lâminas e peças já existentes e previamente coletadas durante o diagnóstico e tratamento;
- b) todos os dados serão arquivados e analisados de forma anônima e sigilosa, sem identificação nominal dos participantes de pesquisa;
- c) os resultados decorrentes do estudo serão apresentados de forma agregada, não permitindo a identificação individual dos participantes;
- d) Trata-se de um estudo não intervencionista (sem intervenções clínicas) e sem alterações/influências na rotina/tratamento do participante de pesquisa, e consequentemente sem adição de riscos ou prejuízos ao bem-estar dos mesmos.

O investigador principal e demais colaboradores envolvidos no estudo 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.

Belo Horizonte, 19/04/2017

Cristiana Buzelin Nunes - Pesquisador Responsável

8.5 Protocolo de Imuno-histoquímica – Kit HERCEPTEST™

Padronização adotada pelo Laboratório de Patologia Mamária da Faculdade de Medicina/UFMG

➤ Desparafinizar:

- 1- Imergir as lâminas em 3 banhos de xilol – 2 de 10 minutos + mergulhar 20x
- 2- Imergir as lâminas em álcool absoluto- 2 banhos- mergulhar 20x +1 banho - 3 min.
- 3- Colocar as lâminas em água destilada mergulhar 20x + 1 banho de 5 minutos

➤ □ Reativação antigênica:

- 1- Colocar uma cuba contendo a solução de recuperação do epítipo diluída no banho –maria aquecendo a 95-99/C e fechar a tampa
- 2- Imergir as lâminas na cuba com a solução de recuperação do epítipo aquecida
- 3- Após atingir 95-99/C deixar 35min □
- 4- Deixar as lâminas resfriando em temperatura ambiente por 20 a 30min □
- 5- Depois de frias, enxaguar as lâminas em tampão de lavagem por 5min □

➤ Bloqueio da peroxidase endógena

- 1- Secar ao redor dos cortes e circundar com a caneta DAKOCytomation
- 2- Pingar 3 gotas (100µl) do Reagente de Bloqueio da Peroxidase nos cortes
- 3- Incubar por 5min □
- 4- Lavar as lâminas uma a uma com o Tampão de Lavagem ou água destilada com pisceta (garrafa de esguicho) e imergi-las em 2 banhos com tampão fresco por 5min cada □

➤ Anticorpo primário ou Reagente controle negativo:

- 1- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Anticorpo anti-HER2 ou do Reagente controle negativo nos cortes
- 2- Incubar em câmara úmida em temperatura ambiente por 30min □
- 3- Lavar as lâminas uma a uma com o Tampão de Lavagem ou água destilada com pisceta (garrafa de esguicho) e imergi-las em 2 banhos com tampão fresco por 5min cada □

➤ Reagente de Visualização:

- 1- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Reagente de visualização em cada corte,
- 2- Incubar em câmara úmida por 30min □
- 3- Lavar as lâminas uma a uma com o Tampão de Lavagem ou água destilada com pisceta (garrafa de esguicho) e imergi-las em 2 banhos com tampão fresco por 5min cada □

➤ **Revelação da reação (DAB):**

- 1- Preparar a solução de DAB momentos antes do uso

1 gota do cromógeno para cada ml de substrato

- 2- Secar ao redor dos cortes e pingar 3 gotas +/-100µl da solução de DAB
- 3- Incubar por 10min□
- 4- Colocar as lâminas em um recipiente com água destilada para parar a revelação
- 5- Lavar as lâminas em água corrente por 5min□

➤ **Contracoloração:**

- 1- Mergulhar rapidamente (2seg□) as lâminas na hematoxilina de Harris
- 2- Lavar as lâminas em água corrente por 7min□

➤ **Desidratação, diafanização e montagem:**

- 1- Imergir as lâminas em álcool absoluto- 3'□ em cada uma das 4 cubas
- 2- Imergir as lâminas em xilol: 10X em cada uma das 2 cubas
- 3- Manter as lâminas na 2ª cuba de xilol enquanto realiza a montagem com lamínula e Enthelan®.

8.6 Protocolo de Imuno-histoquímica – Kit PharmaDx™

Padronização adotada pelo Laboratório de Patologia Mamária da Faculdade de Medicina/UFMG

➤ Desparafinizar:

- 1- Imergir as lâminas em 3 banhos de xilol – 2 de 10 minutos + mergulhar 20x
- 2- Imergir as lâminas em álcool absoluto- 2 banhos- mergulhar 20x +1 banho - 3 min.
- 3- Colocar as lâminas em água destilada mergulhar 20x + 1 banho de 5 minutos

➤ □ Reativação antigênica:

- 1- Colocar uma cuba contendo a solução de recuperação do epítipo diluída na panela à vapor, aquecendo a 95-99/C
- 2- Imergir as lâminas na cuba com a solução de recuperação do epítipo aquecida
- 3- Após atingir 95-99/C deixar 30 min □
- 4- Deixar as lâminas resfriando em temperatura ambiente por 20 a 30min
- 5- Depois de frias, enxaguar as lâminas em água destilada

➤ Bloqueio da peroxidase endógena

- 1- Secar ao redor dos cortes e circundar com a caneta DAKOCytomation
- 2- Pingar 3 gotas (100µl) do Reagente de Bloqueio da Peroxidase nos cortes
- 3- Incubar por 5min □
- 4- Lavar as lâminas uma a uma com o Tampão de Lavagem ou água destilada com pisceta (garrafa de esguicho) e imergi-las em 2 banhos com tampão fresco por 5min cada □

➤ Anticorpo primário ou Reagente controle negativo:

- 1- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Anticorpo anti-RE, anti-RP ou do Reagente controle negativo nos cortes
- 2- Incubar em câmara úmida em temperatura ambiente por 1hora
- 3- Lavar as lâminas uma a uma com o PBS da pisceta (garrafa de esguicho) e imergi-las em 2 banhos de tampão fresco por 5min cada

➤ Reagente de Visualização:

- 1- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Reagente de visualização em cada corte,
- 2- Incubar em câmara úmida por 30min
- 3- Lavar as lâminas uma a uma com o PBS da pisceta (garrafa de esguicho) e imergi-las em 2 banhos de tampão fresco por 5min cada

➤ **Revelação da reação (DAB):**

- 1- Preparar a solução de DAB momentos antes do uso

1 gota do cromógeno para cada ml de substrato

- 2- Secar ao redor dos cortes e pingar 3 gotas +/-100µl da solução de DAB
- 3- Incubar por 10min□
- 4- Colocar as lâminas em um recipiente com água destilada para parar a revelação
- 5- Lavar as lâminas em água corrente por 5min□

➤ **Contracoloração:**

- 1- Mergulhar rapidamente (2seg□) as lâminas na hematoxilina de Harris
- 2- Lavar as lâminas em água corrente por 7min□

➤ **Desidratação, diafanização e montagem:**

- 1- Imergir as lâminas em álcool absoluto- 3'□ em cada uma das 4 cubas
- 2- Imergir as lâminas em xilol: 10X em cada uma das 2 cubas
- 3- Manter as lâminas na 2ª cuba de xilol enquanto realiza a montagem com lamínula e Enthelan®.

8.7 Protocolo de Imuno-histoquímica – Kit Novolink™

Padronização adotada pelo Laboratório de Patologia Mamária da Faculdade de Medicina/UFMG

Desparafinizar:

- 1- Imergir as lâminas em 3 banhos de xilol – 2 de 10 minutos + mergulhar 20x
- 2- Imergir as lâminas em álcool absoluto- 2 banhos- mergulhar 20x +1 banho - 3 min.
- 3- Colocar as lâminas em água destilada mergulhar 20x + 1 banho de 5 minutos

Reativação antigênica:

- 1- Colocar uma cuba contendo a solução de recuperação do epítipo diluída na panela à vapor, aquecendo a 95-99/C
- 2- Imergir as lâminas na cuba com a solução de recuperação do epítipo aquecida
- 3- Após atingir 95-99/C deixar 30min□
- 4- Deixar as lâminas resfriando em temperatura ambiente por 20 a 30min□
- 5- Depois de frias, enxaguar as lâminas em água destilada

Bloqueio da peroxidase endógena

- 1- Secar ao redor dos cortes e circundar com a caneta DAKOCytomation
- 2- Pingar 3 gotas (100µl) do Reagente de Bloqueio da Peroxidase nos cortes
- 3- Incubar por 5min □
- 4- Lavar as lâminas uma a uma com o Tampão de Lavagem ou água destilada com pisceta (garrafa de esguicho) e imergi-las em 2 banhos com tampão fresco por 5min cada□

Anticorpo primário ou Reagente controle negativo:

- 1- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Anticorpo anti-RE, anti-RP ou do Reagente controle negativo nos cortes
- 2- Incubar em câmara úmida em temperatura ambiente por 1 hora
- 3- Lavar as lâminas uma a uma com o PBS da pisceta (garrafa de esguicho) e imergi-las em 2 banhos de tampão fresco por 5min cada□

Reagente de Visualização:

- 1- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Reagente de amplificação em cada corte,
- 2- Incubar em câmara úmida por 30min□
- 3- Lavar as lâminas uma a uma com o PBS da pisceta (garrafa de esguicho) e imergi-las em 2 banhos de tampão fresco por 5min cada□
- 4- Secar ao redor dos cortes e pingar 3 gotas +/-100µl do Reagente de visualização em cada corte,
- 5- Incubar em câmara úmida por 30min□
- 6- Lavar as lâminas uma a uma com o PBS da pisceta (garrafa de esguicho) e imergi-las em 2 banhos de tampão fresco por 5min cada□

Revelação da reação (DAB):

- 1- Preparar a solução de DAB momentos antes do uso

1 gota do cromógeno para cada ml de substrato

- 2- Secar ao redor dos cortes e pingar 3 gotas +/-100µl da solução de DAB
- 3- Incubar por 10min□
- 4- Colocar as lâminas em um recipiente com água destilada para parar a revelação
- 5- Lavar as lâminas em água corrente por 5min□

Contracoloração:

- 1- Mergulhar rapidamente (2seg□) as lâminas na hematoxilina de Harris
- 2- Lavar as lâminas em água corrente por 7min□

Desidratação, diafanização e montagem:

- 1- Imergir as lâminas em álcool absoluto- 3'□ em cada uma das 4 cubas
- 2- Imergir as lâminas em xilol: 10X em cada uma das 2 cubas
- 3- Manter as lâminas na 2ª cuba de xilol enquanto realiza a montagem com lamínula e Enthelan®.