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ESTUDO DE BIOMARCADORES E DA ENZIMA SUPERÓXIDO DISMUTASE 2 NA RESISTÊNCIA AO TAMOXIFENO ADJUVANTE EM PACIENTES COM CÂNCER DE MAMA LUMINAL

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Estudo de biomarcadores e da enzima superóxido dismutase 2 na resistência ao tamoxifeno adjuvante em pacientes com câncer de mama luminal

Tese submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

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Resumo

Introdução: O carcinoma mamário é uma doença complexa e heterogênea, com subtipos biológicos distintos, e devido à incidência e mortalidade é considerada um grave problema de saúde. Novas metodologias estão permitindo estratificar e classificar o câncer de mama de acordo com a expressão proteica e genômica, estabelecendo assim assinaturas genéticas em relação à possibilidade de resposta a terapia. Essa medicina personalizada é baseada na compreensão da carcinogênese molecular, farmacocinética, farmacogenômica, e nas diferenças genéticas de cada indivíduo. Aproximadamente 70% dos diagnósticos são do subtipo Luminal, devido a expressão de receptores de estrogênio ou/e receptores de progesterona. Pacientes com esse perfil recebem terapia anti-estrogênio, sendo o tamoxifeno (TMX) o fármaco de primeira linha mais utilizado para o tratamento. No entanto, aproximadamente 30% dos pacientes com câncer de mama desenvolvem resistência a terapia com o TMX, levando à recidiva da doença e consequentemente ao aumento da mortalidade. **Objetivos:** Elucidar processos biológicos envolvidos na resistência ao TMX e buscar possíveis biomarcadores preditores de recorrência nas pacientes com câncer de mama em uso do TMX adjuvante. **Material e Métodos:** No estudo 1 foi realizado uma revisão de escopo limitada aos trabalhos publicados nos últimos 5 anos para encontrar estudos clínicos que investigassem biomarcadores envolvidos na resposta ao TMX. Uma análise *in silico* foi realizada em conjunto, para avaliar a inter-relação entre marcadores selecionados e o envolvimento com processos biológicos. O estudo 2 foi um estudo de coorte retrospectivo de pacientes com câncer de mama luminal que usaram TMX adjuvante e tiveram um acompanhamento de no mínimo cinco anos. Os genótipos do polimorfismo Val16Ala da enzima superóxido dismutase 2 (SOD2) foram avaliados por PCR em tempo real e os marcadores de apoptose e proliferação foram realizados por imuno-histoquímica. O estudo dos diferentes genótipos foi realizado através de uma análise multivariada de regressão de Cox (backward wald) ajustada para grau, estadiamento clínico e marcadores BCL-2 e KI67. **Resultados:** No estudo 1, quarenta e cinco estudos foram selecionados. Após análise por agrupamento e ontologia gênica, 23 marcadores moleculares foram significativamente associados, formando três grupos de forte correlação com processos biológicos do ciclo celular, transdução de sinal de estímulos proliferativos e resposta hormonal. No estudo 2, tivemos uma amostra de 72 pacientes, 36% apresentaram recidiva, com idade média de diagnóstico de 46 ± 12,68 anos, 35% apresentaram grau histológico 3 e 29.6 % apresentaram estágio clínico III. As frequências genotípicas de SOD2 foram Ala/Ala = 33.3%, Val/Val = 36.1% e Ala/Val = 30.6%. Na análise multivariada, a presença do alelo Val mostrou uma tendência a ser um fator de risco para recorrência (RR = 2,14 (IC 95% 0,84-5,47). Dos 36% pacientes com recidiva, 73,1% apresentaram expressão positiva para o marcador Bcl2 (p = 0,015), indicando um número reduzido de células apoptóticas no tumor primário. **Conclusão:** Com isso, nossos dados encontrados são geradores de hipótese, tanto do artigo I quanto do artigo II, que sugerem mecanismos e biomarcadores promissores para predição da resistência ao Tamoxifeno®. E assim, contribuindo para estudos futuros e uma medicina personalizada.

Palavras-chave: Biomarcadores, estresse oxidativo, luminal, polimorfismo Val-16Ala-SOD2, processos biológicos, resistência, tamoxifeno.

Abstract

Introduction: The mammary carcinoma is a complex and heterogeneous disease, with different biological subtypes, and due to its incidence and mortality, it is considered a serious health issue. New methodologies are making it possible to stratify and classify breast cancer according to protein and genomic expression, thus establishing genetic signatures, both predictive and in relation to the possibility of response to therapy. This personalized medicine is based on an understanding of molecular carcinogenesis, pharmacokinetics, pharmacogenomics, and individual genetic differences, and these biomarkers provide important information to determine the response to certain drugs. Approximately 70% of diagnoses are of the Luminal subtype, due to the expression of estrogen receptors or / and progesterone receptors. Patients with this profile receive anti-estrogen therapy with TMX, the first line drug most used for the treatment. However, approximately 30% of breast cancer patients develop resistance to TMX therapy, leading to disease recurrence and consequently to increased mortality. Drug resistance remains a major obstacle to more effective adjuvant therapy. **Objectives:** To elucidate biological processes involved in resistance to TMX and to search for possible biomarkers that predict recurrence in patients with breast cancer using adjuvant TMX. **Material and Methods:** In study 1, a review of scope limited to the works published in the last 5 years was carried out to find clinical studies that investigated biomarkers involved in the response to tamoxifen. An *in silico* analysis was performed together, to assess the interrelationship between the selected markers and the involvement with biological processes. Further, study 2 was a retrospective cohort study of patients with luminal breast cancer who used adjuvant TMX and had a follow-up of at least five years. The genotypes of the SOD2 enzyme Val16Ala polymorphism were evaluated by real-time PCR and the apoptosis and proliferation markers were performed by immunohistochemistry. The study of the different genotypes performed using a multivariate analysis of Cox regression (backward wald) adjusted for grade, clinical staging and BCL-2 and KI67 markers. **Results:** In study 1, forty-five studies were selected. After cluster analysis and gene ontology, 23 molecular markers were significantly associated, forming three groups of strong correlation with biological processes of the cell cycle, signal transduction of proliferative stimuli and hormonal response. In study 2, we had a sample of 72 patients, 36% had recurrence, with a mean age at diagnosis of 46 ± 12.68 years, 35% had histological grade 3 and 29.6% had clinical stage III. The genotypic frequencies of SOD2 were Ala / Ala = 33.3%, Val / Val = 36.1% and Ala / Val = 30.6%. In the multivariate analysis, the presence of the Val allele showed a tendency to be a risk factor for recurrence (RR = 2.14 (95% CI 0.84-5.47). Of the 36% patients with recurrence, 73.1 % showed positive expression for the Bcl2 marker (p = 0.015), indicating a reduced number of apoptotic cells in the primary tumor. **Conclusion:** Thus, our data found are hypothesis generators, both in Article I and in Article II, which suggest promising mechanisms and biomarkers for predicting resistance to Tamoxifen®. Therefore, contributing to future studies and personalized medicine.

Keywords: Biomarkers, oxidative stress, luminal, Val-16Ala-SOD2 polymorphism, biological processes, resistance, tamoxifen.

Lista de abreviaturas

4HT: 4-Hidroxitamoxifeno

Ala-SOD2: Alanina- Superóxido Dismutase Dois

Bcl-2: Linfoma de Células B2

BRCA1: Breast Cancer 1

BRCA2: Breast Cancer 2

CAT: Catalase

DNA: Ácido Desoxirribonucleico

DSREs: Degradadores Seletivos de Receptores de Estrogênio

ERA: Elemento de Resposta Antioxidante

ERNs: Espécies Reativas de Nitrogênio

EROs: Espécies Reativas de Oxigênio

GH: Grau Histológico

GPx: Glutationa peroxidase

GSH: Glutationa Reduzida

H_2O_2: Peroxido de Hidrogênio

HER2: do inglês: *Human Epidermal Growth Factor Receptor-type 2*

IA: Inibidores de Aromatase

INCA: Instituto Nacional do Câncer

Ki-67: Antígeno nuclear utilizado como marcador de proliferação celular

$LO^{\bullet}$: Radicais Alcoxila

$LOO^{\bullet}$: Peroxila

MSREs: Moduladoras Seletivas de Receptores de Estrogênio

MTS: do inglês: *Mitocondrial Target Sequence*

NF- κ: Fator Nuclear Ќb

$NO^{\bullet}$: Óxido Nítrico

NRF2: Fator Nuclear Eritróide 2

O_2: Oxigênio

$O_2^{\bullet-}$: Superóxido

$OH^{\bullet}$: Radical Hidroxila

$ONOO^-$: Peroxinitrito

RE+: Receptor de Estrógeno Positive

RH+: Receptores Hormonais Positivos

RL: Radicais livres

RNAm: Ácido Ribonucleico mensageiro

SNP: Polimorfismo de Nucleotídeo Simples

SOD: Superóxido Dismutase

S-PH: Superóxido-Peróxido de Hidrogênio

TMX: Tamoxifeno

Val-SOD2: Valina-Superóxido Dismutase 2

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Sumário

1. REFERENCIAL TEÓRICO

1.1. Morfologia da mama

A mama feminina normal (**figura 1**) é composta principalmente por lóbulos (glândulas que produzem leite), ductos (tubos que transportam o leite dos lóbulos ao mamilo) e estroma (tecido conjuntivo e adiposo, vasos sanguíneos e linfáticos). O câncer de mama é uma doença causada pela multiplicação descontrolada de células da mama. Esse processo gera células anormais que se multiplicam, formando uma neoplasia. Frequentemente ele ocorre nas células que revestem os ductos (câncer ductal) e em células que revestem os lóbulos (câncer lobular), enquanto que, uma pequena parcela inicia-se em outros tecidos (1, 2).

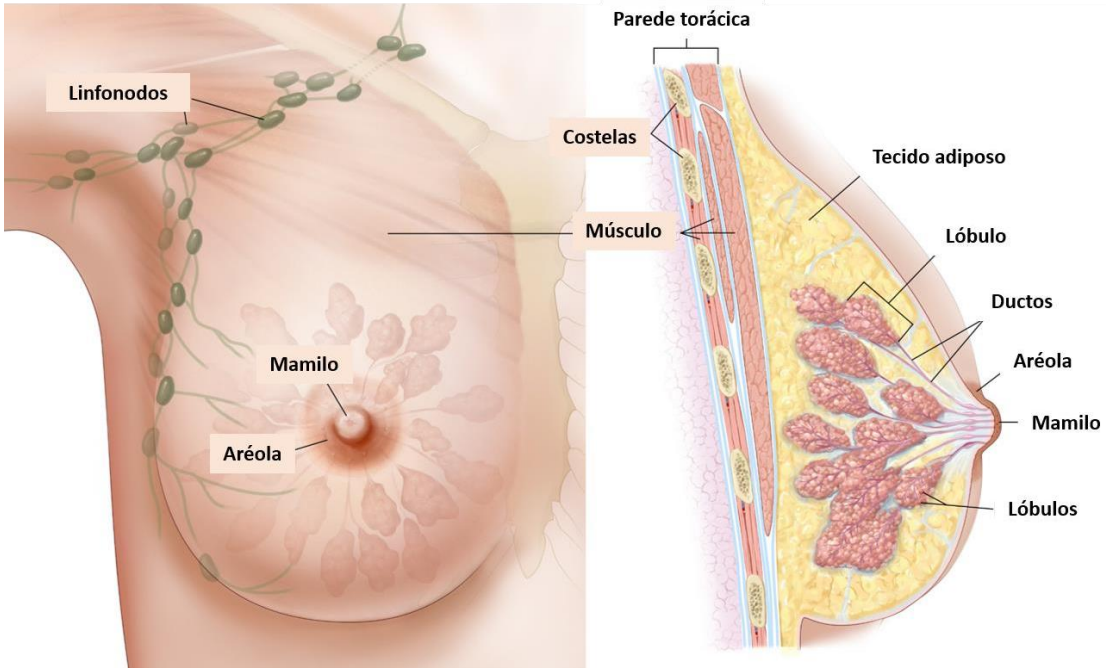

Fig 1. Anatomia da mama normal (Fonte: Adaptada de Breast Cancer Treatment and Pregnancy, 2015).

Os tipos de anormalidades proliferativas nos lóbulos e ductos incluem diversas doenças. Dentre os tumores malignos, o carcinoma ductal infiltrante é o tipo histológico mais

comum e compreende cerca de 80 e 90% do total de casos. Sendo que, as células cancerígenas podem se disseminar da mama para diferentes partes do corpo através do sistema linfático ou da circulação sanguínea (3, 4).

Os linfonodos intramamários podem ser um local de disseminação regional dadoença. O comprometimento axilar é considerado um fator prognóstico importante, influenciando nas decisões de tratamento de acordo com a sua presença ou ausência de metástase nos linfonodos (5).

O câncer de mama é uma doença conhecida por ser heterogênea, a qual, compreende vários elementos tumorais associados a diferentes padrões histológicos e características biológicas, com abordagens e comportamentos distintos. Estas características podem ser facilmente observadas pela variedade de manifestações morfológicas e clínicas e por diferentes assinaturas genéticas, que levam a respostas distintas ao tratamento (4, 6, 7).

1.2. Epidemiologia do câncer de mama

A incidência do câncer de mama se configura entre as primeiras posições mundiais de neoplasias malignas. Foram estimados aproximadamente 2.1 milhões de novos casos de câncer de mama em todo o mundo em 2018, equivalendo a 24.2% dos casos em relação aos outros tipos de câncer em mulheres (**figura 2**). A taxa de mortalidade mundial foi de 627.000 (8).

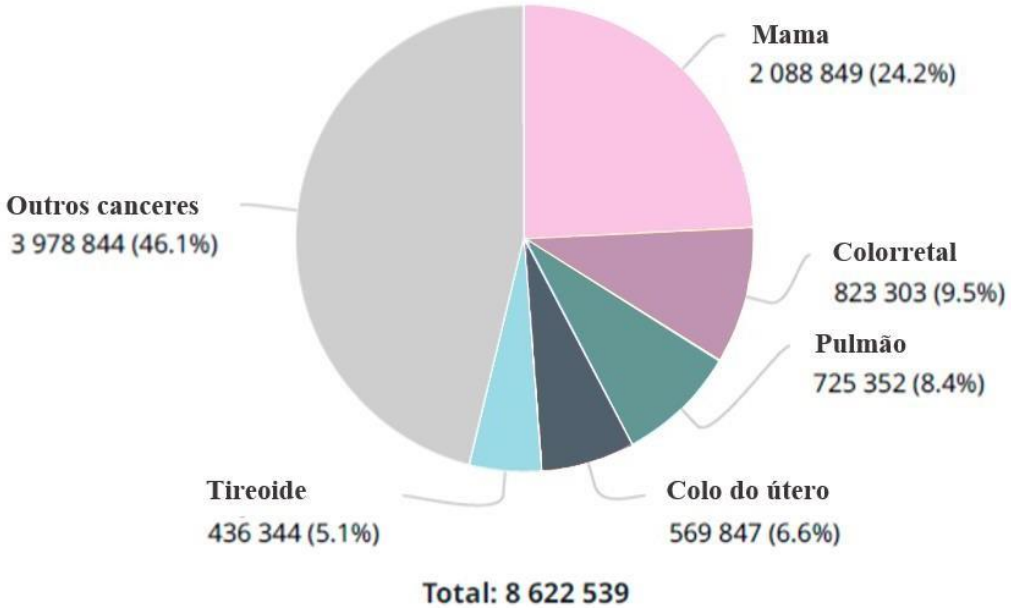

Fig. 2 Número de novos casos em 2018 no mundo, somente em mulheres, de todas as idades (Fonte: Adaptada IARC, 2018).

Segundo o Instituto Nacional do Câncer (INCA) são estimados para o Brasil, 66.280 mil casos novos de câncer de mama, para cada ano do triênio 2020-2022. Esse valor corresponde a um risco estimado de 61,61 casos novos a cada 100 mil mulheres. Desconsiderando os tumores de pele não melanoma, o câncer de mama ocupa a primeira posição em todas as regiões brasileiras, com um risco estimado de 81,06/100 mil na Região Sudeste; de 71,16/100 mil na Região Sul; de 45,24/100 mil na Região Centro-Oeste; de 44,29/100 mil na Região Nordeste e de 21,34/100 mil na Região Norte (3).

Avaliando exclusivamente o estado do Rio Grande do Sul, estimam-se 4.050 mil novos casos, representando um risco de 69,50 casos para cada 100 mil habitantes, e para a capital, Porto Alegre, as taxas são maiores ainda, representando 81,82 casos para cada 100 mil habitantes (3). No entanto, destacamos que, em geral, as capitais sediam centros de referência em tratamento, para onde muitos pacientes do interior são direcionados, como é o caso de Porto Alegre.

1.3. Fatores de Risco

Alguns fatores de risco estão bem estabelecidos para o desenvolvimento do câncer de mama, podendo ser divididos em três grandes grupos: genético, hormonal e ambiental (9-11).

a) genéticos: Associado a história familiar de câncer de mama, está relacionado a alterações principalmente nos genes supressores de tumor BRCA1 (Breast Cancer 1) e BRCA2 (Breast Cancer 2), localizados no cromossomo 17q21 e 13q12, respectivamente. Mulheres com presença da mutação têm aproximadamente 70% de chance de desenvolver o câncer de mama durante sua vida. Aumentando o risco na presença de familiares de primeiro grau, ou seja, mãe ou irmã.

b) hormonais: o envelhecimento é um dos fatores mais importantes para o câncer de mama pois a incidência está altamente relacionada ao aumento da idade, na qual também está correlacionada à participação hormonal e ainda a vida reprodutiva da mulher. O câncer de mama positivo para receptores de estrogênio (RE) é o mais diagnosticado. Postulou-se que essa relação é motivada por fatores de risco como, paridade baixa e/ou tardia, menarca precoce, menopausa tardia, bem como o uso de pílula anticoncepcional combinada e reposição hormonal (3, 11, 12)

c) ambientais: estilo de vida ,consumo excessivo de álcool, sedentarismo, exposição à radiação ionizante e ingestão excessiva de gordura na dieta, podem aumentar o risco de câncer de mama (13, 14).

Fatores de risco do tipo genético, não são passíveis de manipulação para fins de prevenção primária. Em virtude disto, estudos são realizados no intuito de identificar fatores de risco manipuláveis para tal prevenção. Como é o caso dos fatores ambientais, a prática de atividade física, alimentação saudável e manutenção do peso corporal, são capazes de reduzir o risco de desenvolver câncer de mama(15, 16).

A gordura corporal é um dos fatores de risco mais associado com câncer de mama. Estudos realizados recentemente, revelaram um aumento de 12% de risco em mulheres com sobrepeso na pós-menopausa, e de 25% nas mulheres obesas. Níveis elevados de gordura corporal também foram associados ao aumento do risco em mulheres com IMC normal (14, 17).

No entanto, um dos fatores de risco não passível de modificação (envelhecimento), está relacionado com o acúmulo de dano no ácido desoxirribonucleico (DNA) derivado de exposição crônica a agentes carcinogênicos, como fatores endógenos (variações genômicas) e fatores exógenos (estilo de vida e exposição ambientais) (18-20). Fatores estes, que podem ser monitorados e em alguns casos controlados. Estudos realizados em tumores conseguiram mostrar modificações no DNA causadas por estes fatores que implicam no equilíbrio de espécies reativas (21, 22).

1.4. Estadiamento e grau histológico

O sistema TNM, sob a direção do Comitê Conjunto Americano de Câncer (AJCC) e da União para o Controle Internacional do Câncer (UICC), é o sistema de estadiamento padrão usado para o câncer. O TNM é utilizado para verificar a extensão e disseminação da neoplasia. Essa avaliação permite estabelecer o prognóstico do paciente e o planejamento do tratamento **(quadro 1)** (23).

Quadro 1. Estágio clínico, conforme classificação AJCC

T	**N**	**M**	**Estágio clínico**
Tis	N0	M0	0
T1	N0	M0	IA
T0	N1mi	M0	IB
T1	N1mi	M0	IB
T0	N1	M0	IIA
T1	N1	M0	IIA
T2	N0	M0	IIA
T2	N1	M0	IIB
T3	N0	M0	IIB
T0	N2	M0	IIIA
T1	N2	M0	IIIA
T2	N2	M0	IIIA
T3	N1	M0	IIIA
T3	N2	M0	IIIA
T4	N0	M0	IIIB
T4	N1	M0	IIIB
T4	N2	M0	IIIB
Qualquer T	N3	M0	IIIC
Qualquer T	Qualquer N	M1	IV

Fonte: Adaptado de AJCC, 8° edição, 2018.

A oitava edição do AJCC TNM, fornece uma plataforma flexível para classificação de prognóstico com base em fatores anatômicos tradicionais, que podem ser modificados e aprimorados usando biomarcadores e dados de painéis prognósticos multifatoriais. O T está relacionado ao tamanho do tumor primário sendo, T0: Nenhuma evidência de tumor primário e T4: Tumor de qualquer tamanho, com extensão direta à parede torácica e / ou à pele (ulceração ou nódulos macroscópicos). O N está relacionado ao comprometimento de linfonodos. O N0 significa: Nenhuma metástase de linfonodo regional identificada; N3: Metástases em 10 ou mais linfonodos axilares. E o M se refere quanto a ocorrência de metástase. M0: Nenhuma evidência clínica ou radiográfica de metástases distantes; M1: Metástases distantes detectadas por meios clínicos e radiográficos (cM) e/ou metástases histologicamente comprovadas superiores a 0,2 mm (pM). A partir da análise tumoral desses três parâmetros (TNM), é dado a classificação do estágio clínico do paciente. (24).

O Grau Histológico (GH) do tumor é uma das características anatomopatológicas mais importantes. O sistema de classificação Nottingham, que é uma modificação do Scarff-Bloom-Richardson (SBR), é o sistema de classificação mais utilizado para determinar o GH em casos de câncer de mama. A avaliação é baseada em três características do tumor, formação de túbulos, atipia nuclear e pleomorfismo, e número de mitoses (**quadro 2**).

Quadro 2. Critérios utilizados para calcular o grau histológico do câncer de mama

Avaliação histológica do grau – Classificação de Nottingham	
Pontos glandulares (formação tubular)	Pontos
> 75% dos tumores que formam estruturas glandulares / tubulares	1
> 10% a 75% dos tumores que formam estruturas glandulares / tubulares	2
<10% dos tumores que formam estruturas glandulares / tubulares	3
Polimorfismo nuclear	
Núcleos pequenos, regulares e uniformes (semelhantes ao normal)	1
Núcleos moderadamente aumentados em tamanho e irregulares em forma	2
Núcleos vesiculares, geralmente nucléolos, variação acentuada em tamanho / forma	3
Contagem mitótica: número de mitoses/microscópio de área de campo*	
<7 mitoses / 10 HPFs 1	1
8-14 mitoses / 10 HPFs 2	2
> 15 mitoses / 10 HPFs 3	3
Nota geral (soma de cada recurso)	
G1 (bem diferenciado)	3 até 5
G2 (moderadamente diferenciado)	6, 7
G3 (pouco diferenciado)	8, 9

Fonte: Adaptada ESMO, 2019.

A avaliação final resulta em G1, G2 e G3, que é determinada pela soma das pontuações individuais. O grau 1 é o de melhor prognóstico e o grau 3 o pior, com características do tumor altamente invasivas (25).

1.5. Classificação molecular

Na prática clínica, painéis imunohistoquímicos são utilizados para identificar diferentes subtipos de tumores. Essa técnica tecidual *in situ* auxilia na busca de informações prognósticas e preditivas. A avaliação permite identificar características clínicas, patológicas e biológicas, usadas para estimar a probabilidade de resposta do paciente a um tipo específico de tratamento (6, 26).

A visão terapêutica do câncer de mama foi modificada com a publicação de Perou *et al.* (2000) a partir da análise de mais de oito mil genes por microarray, foram identificados quatro subtipos moleculares mais utilizados para o diagnóstico de câncer de mama, são eles: luminal A, luminal B (com suas variantes), HER2 (do inglês: *Human Epidermal growth factor Receptor-type 2*) super-expresso e "basal-like" (triplo-negativo) (27). Abaixo estão apresentadas as características de expressão de cada subtipo, **quadro 3**.

Quadro 3. Classificação molecular

Subtipo molecular	**Padrão de imunomarcação**
Luminal A	Receptores hormonais positivo (RE ou/e RP), Her-2-, Ki-67 <14%
Luminal B	***Luminal B (Her-2 negativo):*** Receptores hormonais positivo (RE ou/e RP), Her-2 negativo, Ki-67 >14%
	Luminal-B (Her-2 positivo): Receptores hormonais positivo (RE ou/e RP), Her-2 super-expresso ou amplificado, Ki-67 de qualquer índice

Her-2 super-expresso	Her-2 super-expresso ou amplificado, Receptores hormonais negativos
Basal	Receptor de estrógeno e progesterona negativos, Her-2 negativo.

Fonte: Adaptado de Goldhirsch *et al.* 2011; Yersal e Barutca, 2014.

De todos os casos diagnosticados com câncer de mama o subtipo luminal é o mais incidente com taxas de aproximadamente 70%. São receptores hormonais positivos (RH+). Cerca de 50% dos pacientes apresentam o subtipo luminal A e 10-20% luminal B (28).

O subtipo molecular luminal A, na sua maioria, apresenta um melhor prognóstico em relação aos outros subtipos. São classificados os tumores positivos para receptor de estrogênio (RE+) e/ou receptor positivo de progesterona (RP+), e negativos para amplificação e/ou superexpressão de HER2. Além disso, apresentam índice de Ki67 inferior a 14% de células neoplásicas imunomarcadas (6).

O subtipo luminal B, se diferencia por expressar maior proliferação (índice superior a 14% de Ki67) e em alguns casos, por expressar o gene HER2. Seu maior índice de proliferação celular resulta em um pior prognóstico em relação aos tumores luminal A (28).

As células neoplásicas dos receptores de estrogênio e progesterona apresentam similaridade das células normais da mama e estão em contato direto com o lúmen dos ductos mamários. Funcionando como fatores de transcrição, quando conectadas a seus respectivos ligantes (29).

O RE possui dois subtipos, os ERα e ERβ, que são codificados pelos genes presentes nos cromossomos 6 e 14, respectivamente. Cada um deles tem um papel específico na regulação de genes, como na expressão de células e tecidos e nas vias de sinalização jusante. A ativação do ERα pode promover a carcinogênese, pois induz a proliferação e invasividade das células de câncer de mama. Já o ERβ tem ação pouco conhecida. Algumas investigações

relacionaram ele à proliferação celular restringida e em outros casos mostrou aumentar a sensibilidade à hormonioterapia (30, 31).

O ERα está presente em cerca de 40 a 70% dos cânceres de mama e é considerado fator preditivo de resposta à terapia endócrina. Clinicamente, os pacientes com RE+ são tratados com terapia anti-hormonal, com várias moléculas moduladoras seletivas de receptores de estrogênio (MSREs), como o TMX, degradadores seletivos de receptores de estrogênio (DSREs) ou inibidores da enzima de aromatase (IA), que converte andrógenos em estrógenos(32, 33).

1.6. Terapia hormonal

Em pacientes pré-menopausa, os ovários são o principal local de produção hormonal. O reconhecimento da relação do ovário com o câncer de mama foi observado pela primeira vez por *Thomas William Nunn*, que notou regressão da doença, após 6 meses de interrupção da menstruação de uma paciente. Em 1972, foi realizado pela primeira vez a ooforectomia, concluindo que, após a remoção do ovário a doença seria reduzida (34).

Por alguns anos a abordagem cirúrgica foi utilizada como tratamento inicial. Atualmente, segundo as Diretrizes de Consenso Internacional de *St. Gallen* (2019), o tratamento padrão de mulheres com câncer de mama RE+ inclui terapia hormonal adjuvante. Algumas mulheres obterão benefícios adicionais com a quimioterapia, enquanto muitas das pacientes podem evitar com segurança. O estágio clínico da doença permanece um determinante importante de risco de recorrência e, portanto, a necessidade de quimioterapia é destinada para as pacientes com estágio III. Recomenda-se também quimioterapia em mulheres com quatro ou mais linfonodos afetados, incluindo as com carcinoma lobular e/grau 1 ou luminal A. Por outro lado, mulheres com nódulo-negativo e tumor menor que 1 cm

raramente requerem quimioterapia, sendo beneficiadas somente com tratamento anti-hormonal sistêmico (35, 36).

O TMX é um modulador seletivo do receptor de estrogênio oral, derivado do trifeniletileno não esteroide, sendo eficaz em pacientes pré-menopausa e pós-menopausa (37, 38). É o medicamento mais utilizado no tratamento adjuvante sistêmico, tanto em estágio inicial quanto em estágio avançado nos pacientes RH+. Uma revisão sistemática com meta-análise mostrou que o TMX adjuvante por 5 anos, resultou em uma redução do risco de recorrência e morte, após um acompanhamento de 15 anos (39).

O TMX atua em diferentes mecanismos, sendo agonista ou antagonista do receptor de estrogênio em diferentes tecidos. Na mama, o TMX atua como antagonista, competindo com estrogênio humano primário 17β-estradiol, que possui uma alta afinidade com o RE. Nas células normais a ligação do 17β –estradiol ao RE sela a bolsa hidrofóbica através da hélice-12, no qual, o RE desloca-se para o núcleo, onde vai ativar o elemento de resposta ao estrogênio e conduzir a transcrição de genes dependentes de estrogênio (40).

O TMX é metabolizado em 4-hidroxitamoxifeno (4HT), que foi desenvolvido para se ligar ao RE. Essa ligação resulta em uma alteração conformacional diferente do 17β-estradiol, na qual a bolsa hidrofóbica não é selada pela hélice-12. Consequentemente, o 4HT bloqueia a ativação do RE impedindo a ativação da cascata genica, e levando à morte celular, representado na (**figura 3)** (41, 42).

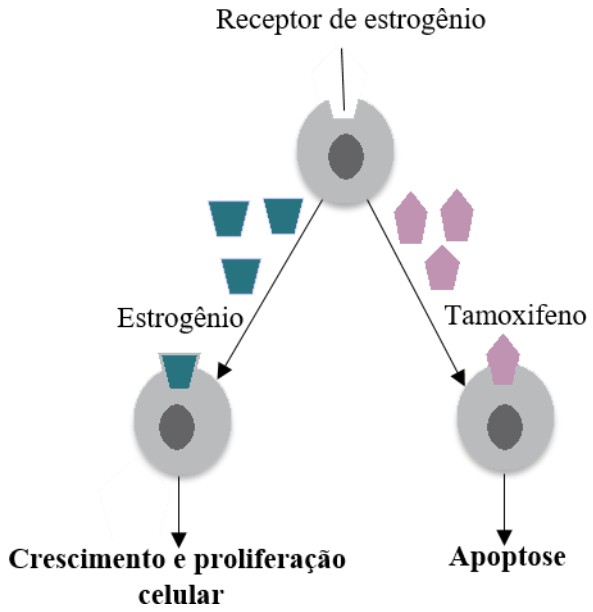

Fig. 3 Ação do tamoxifeno (Fonte: A autora)

O mecanismo de ação do TMX não está totalmente elucidado. Tal fato se deve ao TMX não ser um antagonista puro. Estudos vem demonstrando que o TMX pode estar envolvido na produção de espécies reativas de oxigênio (EROS). O aumento do EROS resulta em dano oxidativo nas células neoplásicas e induz a apoptose celular, tendo um efeito terapêutico positivo no câncer. Portanto, há evidências *in vitro* que o TMX não somente possui propriedades citostáticas, que estão relacionadas ao bloqueio do receptor de estrogênio, mas também possui mecanismos citotóxicos, que resultam no dano direto do DNA, tendo como consequência a morte celular(43-45).

1.7. Resistência ao TMX

A terapia endócrina melhorou consideravelmente a sobrevida em pacientes com câncer de mama nas últimas décadas, no entanto, a resistência a essa terapia continua sendo uma das principais causas de falha ao tratamento adjuvante (46).

Cerca de 30% das mulheres tratadas com TMX vão apresentar resistência intrínseca, presente no indivíduo antes do início de qualquer tratamento, ou resistência adquirida, após exposição prolongada ao medicamento. A resistência intrínseca ao TMX é rara, embora tenham sido relatados polimorfismos, como os de nucleotídeo único nas enzimas do citocromo p450 que afetam a metabolização do TMX à sua forma ativa (31, 47, 48).

A resistência adquirida é mais frequente e, embora ainda seja algo complexo, uma variedade de mecanismos tem sido relatada. Muitos deles centralizados na estrutura, ativação e funções complexas do RE, como, por exemplo, na substituição da sinalização pró-proliferativa do RE por vias alternativas de sinalização (conhecidas como *cross-talk*), que podem envolver caminhos diferentes, tais como, EGFR/HER2, IGFR entre outros (49-51).

Apesar dos efeitos do TMX na sinalização induzida por estrogênio, investigações anteriores relataram um mecanismo de ação associado ao dano oxidativo e apoptose em células tumorais de câncer de mama, independente dos níveis de expressão do RE (52, 53).

Uma investigação realizada recentemente por Tomková *et al.* (2019) mostrou que células de câncer de mama tratadas com TMX e cultivadas por um longo período de tempo, desenvolveram um fenótipo de resistência ao medicamento. No qual, foi observado níveis elevados de EROs e uma redução da função mitocondrial (54).

Evidencias adicionais realizada por Bekele *et al*. (2016) mostraram que o TMX se incorpora na bicamada lipídica e gera EROS (superóxido), o que resulta em uma peroxidação lipídica e subsequente formação de 4HNE, que ativa a caspase-3 e leva as células neoplásicas a apoptose. Porém, com o passar do tempo, o estresse oxidativo induzido pelo TMX gera um aumento dos níveis de fator nuclear eritróide 2 (NRF2), e dessa forma, ativa o elemento de resposta antioxidante (ERA) levando a expressão de genes antioxidantes e dos transportadores resistentes a múltiplas drogas, que vai defender as células neoplásicas do

estresse oxidativo gerado pelo TMX e impedir a apoptose celular, contribuindo para a resistência aos efeitos terapêuticos do TMX continuado, representado na (**figura 4)** (45).

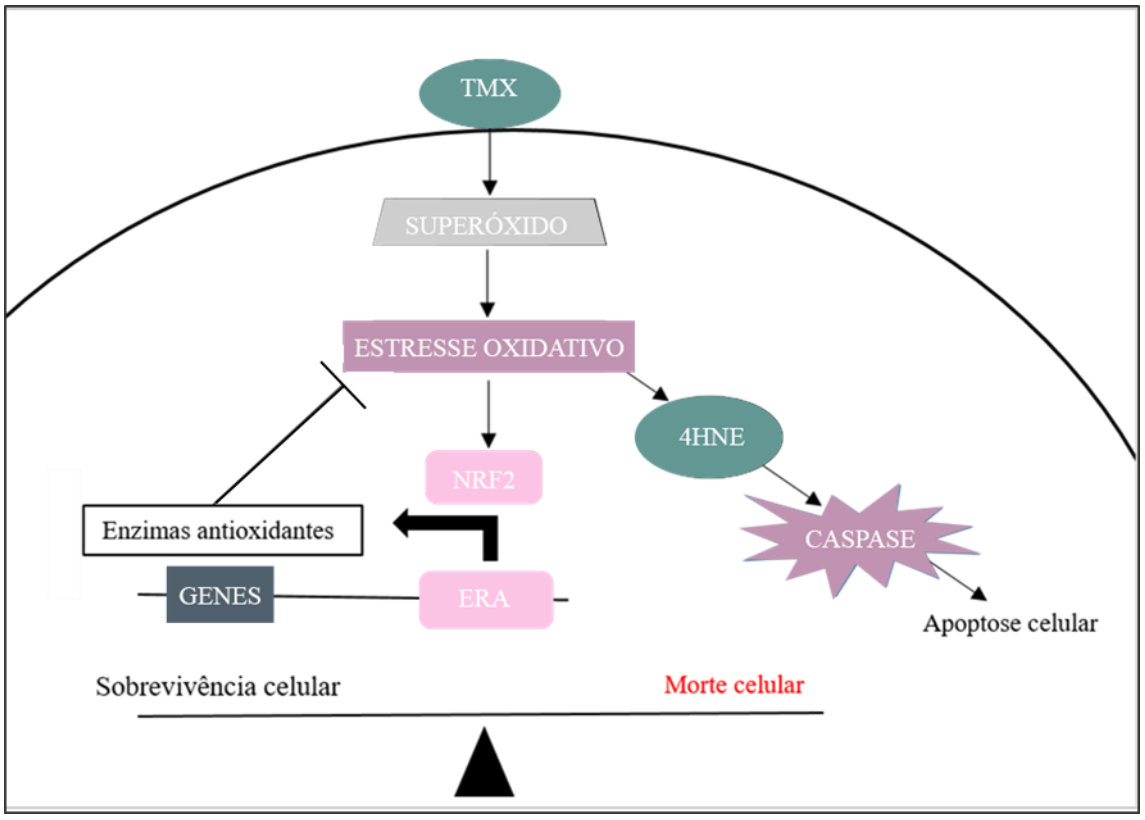

Fig.4 Esquema de sinalização dos efeitos do TMX no câncer de mama e no desenvolvimento da resistência (Fonte: A autora).

Sotgia *et al.* (2017) realizou uma busca em bases de dados e análise *in silico* para investigar genes associados à mitocôndria, que poderiam ser usados para prever a recorrência do tumor e a falha terapêutica do TMX. O estudo encontrou relação significativa com diversos genes, incluindo a superóxido dismutase 2 (SOD2), que foi notavelmente relacionada com valor prognóstico, valor de risco 2,94 ($p = 0,0001$). De acordo com os dados encontrados, pacientes com características de alto risco tumoral foram identificados com alta expressão de marcadores mitocondriais nos tumores primários da mama. O estudo sugere uma intervenção terapêutica (por exemplo, metformina em combinação com o TMX) para garantir a eficiência do TMX (55).

Além disso, um estudo *in vitro* e *in vivo* criou um modelo para reverter a resistência ao TMX silenciando a enzima mitocondrial SOD2. Ela desmuta as espécies reativas de oxigênio induzida por TMX (superóxido) para peroxido de hidrogênio, dificultando os efeitos terapêuticos. Com o modelo foi possível silenciar a enzima e restaurar a apoptose induzida por TMX e suprimido significativamente o crescimento *in vivo*, como confirmado por ensaios bioquímicos e observações histológicas (56).

Diante do exposto, percebe-se que os mecanismos de resistência são múltiplos, e podem se desenvolver através de diversos locais, sendo que a recorrência tardia e a morte por câncer de mama positivo para RE+ podem ocorrer por pelo menos 20 anos após o diagnóstico, mesmo após 5 anos de terapia endócrina adjuvante (57). Portanto, identificar mecanismos de resistência, estratégias e biomarcadores preditivos de resposta terapêutica é fundamental, principalmente no início do tratamento, para com isso, ajudar a prevenir a recorrência tumoral e consequentemente melhorar a sobrevida dos pacientes.

1.8. Espécies reativas

Radicais livres (RL) são moléculas ou átomos que possuem um ou mais elétrons desemparelhados em sua órbita mais externa da eletrosfera (58, 59), conferindo um alto poder de reatividade. Outras moléculas além do oxigênio, como o enxofre, o carbono e o nitrogênio também podem produzir RL ou espécies reativas, no entanto, o oxigênio é o que mais recebe atenção, dada sua importância nos processos metabólicos celulares (58, 60).

Os RL possuem uma vida curta (cerca de mili, micro ou nanosegundos), reagindo prontamente com lipídios, DNA e proteínas, e assim, causando danos e gerando produtos prejudiciais, como peróxido de lipídios. O dano causado nas proteínas, pode resultar em perda de atividade enzimática, enquanto que no DNA, o dano pode resultar em mutagênese e carcinogênese (61).

As EROs são produzidas de forma contínua pelas células como parte de seusprocessos metabólicos normais. EROs incluem radicais livres de oxigênio, como por exemplo, superóxido (O_2•-), radical hidroxila (OH•), radicais alcoxila (LO•), óxido nítrico (NO•) e peroxila (LOO•), assim como espécies não-radicais (ex. peróxido de hidrogênio, hidroperóxidos orgânicos e hipoclorido). No geral, elas são indispensáveis para diversos processos fisiológicos da célula, incluindo ciclo celular, proliferação, apoptose e senescência. Entretanto, um nível aumentado dessas espécies pode resultar em estresse oxidativo e criar um ambiente potencialmente tóxico para as células (62).

O estresse oxidativo pode também ativar fatores de transcrição como o fator nuclear κB (NF- κB), resultando na liberação de citocinas e promoção do processo inflamatório. Em condições fisiológicas normais, a homeostase celular (balanço entre geração de EROs e defesas oxidativas) estão presentes nas células para garantir o funcionamento normal sem causar danos (63, 64).

1.9. Defesas Antioxidantes

Células eucariotas têm a capacidade de desenvolver um elaborado mecanismo de defesa para combater a produção de EROs. Este mecanismo é chamado sistema de defesa antioxidante e atua na prevenção e reparo químico e físico dos danos oxidativos nos diversos sistemas orgânicos (21, 59).

As defesas antioxidantes podem ocorrer por mecanismos endógenos (antioxidantes enzimáticos), que são produzidos pelo próprio organismo humano. As principais enzimas envolvidas neste processo são, a glutationa peroxidase (GPx), o superóxido dismutase (SOD) e a catalase (CAT)(65-67). Mas também podem ocorrer por mecanismos exógenos (não-enzimáticos) que são antioxidantes derivados da alimentação. São eles a glutationa reduzida

(GSH), tióis não-proteicos, α-tocoferol (vitamina E), ácido ascórbico (vitamina C) entre outros (59, 68).

A respiração mitocondrial é a maior fonte de EROs, como resultado da produção de O_2•- pelos complexos I e III da cadeia de transporte de elétrons, o qual representa cerca de 1-2% do consumo de oxigênio pela célula. Com isso, as SODs são responsáveis por catalisara dismutação de O_2•- em peroxido de hidrogênio (H_2O_2), que serve de substrato para as próximas enzimas da via antioxidante, CAT e GPx. As mesmas irão transformar o H_2O_2 em água e oxigênio (O_2) (69-71).

Existem três isoformas conhecidas da SOD, que são caracterizadas de acordo com a sua localização e seu metal componente: SOD1 (CuZnSOD), SOD2 (MnSOD) e SOD3 (EcSOD) (72). Dentre as três, a SOD2 é a enzima mais significativa por atuar dentro da mitocôndria, onde ocorre a maior produção de espécies reativas. Além disso, têm sido amplamente relacionada com o risco de câncer e agressividade tumoral, sendo o polimorfismo Val16Ala-SOD2 o mais investigado (63, 73-75).

Em resumo, o manejo do estresse oxidativo é dependente do funcionamento de sistemas endógenos e exógenos de defesas antioxidantes, que é influenciado por variações genéticas individuais (**figura 5**). Polimorfismos que codificam enzimas antioxidantes, como o Val16Ala-SOD2, tem impacto direto no equilíbrio do estresse oxidativo, sendo positivamente ou negativamente em células neoplásicas (76, 77).

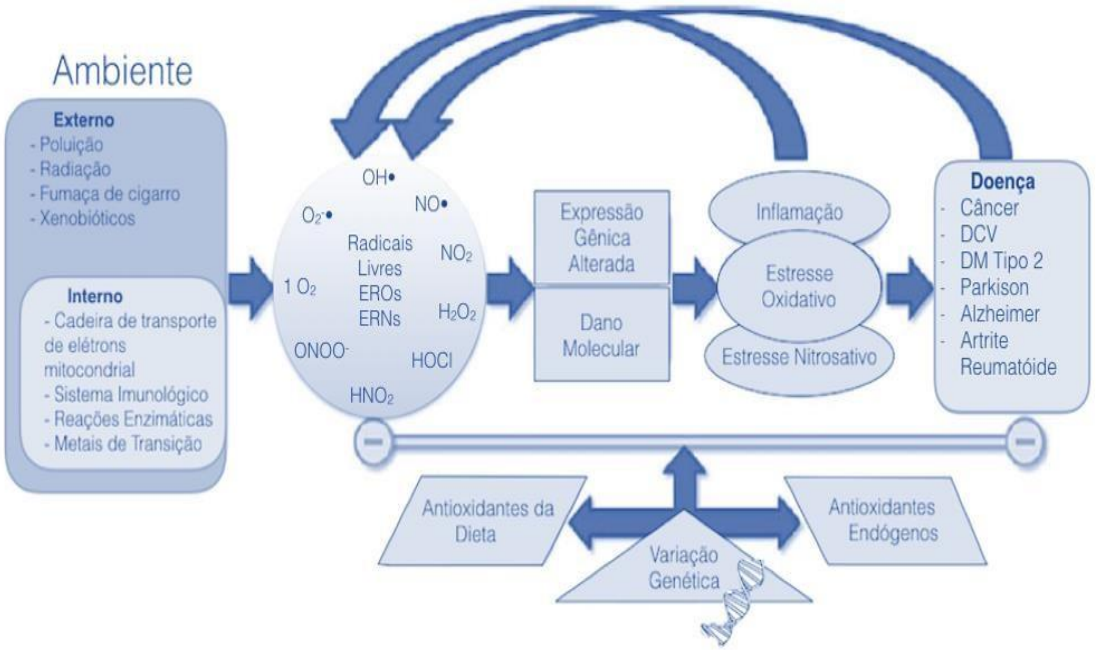

Fig. 5 Ilustração da relação entre produção de EROs, estresse oxidativo, desenvolvimento de doenças e o papel dos antioxidantes e da variação genética. Acúmulo de EROs a partir de estímulos externos e internos podem causar danos moleculares e resultar em estresse oxidativo e nitrosativo. As EROs ainda podem alterar a expressão gênica, conduzindo à liberação de citocinas e inflamação, que resulta em mais produção EROs e espécies reativas de nitrogênio (ERNs). A inflamação e o estresse oxidativo podem colaborar para o desenvolvimento de produção extra de espécies reativas e doenças crônicas. Com tudo, antioxidantes endógenos e exógenos trabalham juntos para reduzir o desenvolvimento de estresse oxidativo e o dano celular. No entanto, sua função pode ser modificada pela variação genética individual. DCV = Doença cardiovascular; T2DM = Diabetes Mellitus Tipo **2.** (Fonte: Adaptada de Costa *et al*. 2012).

1.10. SOD2 e Polimorfismo Val16Ala

A SOD2 é um homotetrâmero que contém um íon de manganês por subunidade, que está localizada no cromossomo 6 região q25.3. A SOD2 é essencial para a sobrevivência celular de mamíferos (78). Como esta enzima é sintetizada a partir de um gene nuclear, inicialmente é produzida uma proteína SOD2 inativa, que é sintetizada no retículo endoplasmático rugoso e enviada para o interior da mitocôndria, através de uma pequena sequência peptídica denominada sequência mitocondrial alvo (do inglês: *mitocondrial target sequence*, MTS). Ao passar pelos poros da membrana mitocondrial interna, o segmento peptídico MTS é clivado pelos lisossomos, e a proteína assume a sua forma SOD2 ativa, tornando-se uma enzima funcional (**figura 6**) (79, 80).

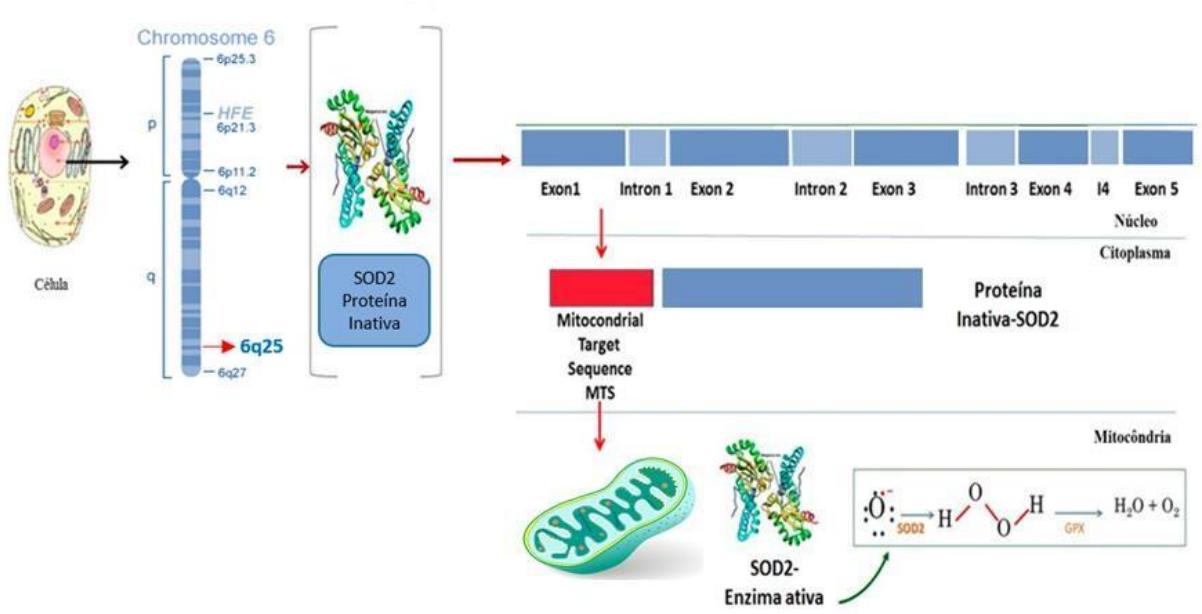

Fig. 6 Codificação a ativação da enzima SOD2 (Fonte: adaptada Tese Barbisan, 2017).

Investigações foram realizadas e variações genéticas foram encontradas que poderiam influenciar no transporte mitocondrial da enzima SOD2 e na estabilidade de seu RNAm, e com isso, modular mecanismos de apoptose, adesão celular e proliferação celular (63, 81).

Um estudo realizado por Ambosone *et al.* (1999) mostrou uma associação entre o polimorfismo localizado na região MTS e câncer de mama. O polimorfismo encontrado, que até hoje é o mais investigado, é do tipo polimorfismo de nucleotídeo simple (SNP), denominado Val16Ala-SOD2 (rs4880). Nele, ocorre a substituição de uma timina (T) por uma citosina (C) no exon 2, nucleotídeo 47. Esta substituição afeta o códon 16, que codifica o aminoácido 9, resultando na substituição do aminoácido valina (GTT) para alanina (GCT). Com isso, existem dois alelos Ala (alanina) e Val (Valina) (79, 82).

Ao herdarmos este polimorfismo, 3 possíveis genótipos podem ocorrer na população: Ala/Ala, Ala/Val e Val/Val (**figura 7**). A variante Ala/Ala-SOD2 possui uma estrutura α-hélice, que é facilmente importada para o interior da mitocôndria. A variante Val/Val-SOD2 possui uma estrutura parcial β-lâmina, que faz com que ela fique

parcialmente retida no poro da membrana interna mitocondrial. Já a variante Ala/Val-SOD2 apresenta uma estrutura helicoidal (63, 80).

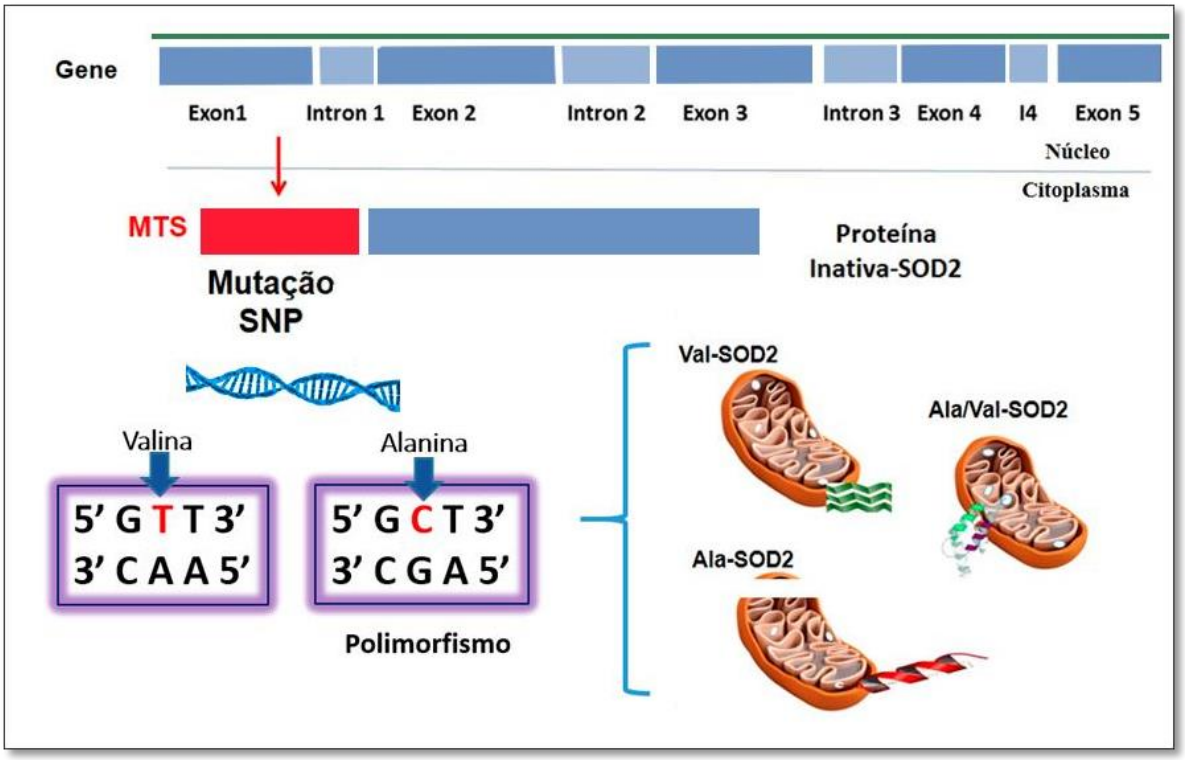

Fig. 7 Representação gráfica do Polimorfismo Val16Ala-SOD2 (Fonte: adaptada Tese Barbisan, 2017).

Estudos *in vitro* demonstraram que o Ala/Ala-SOD2 é capaz de gerar homotetrâmeros SOD2 com cerda de 30-40% mais atividade do que a matriz processada com o precursor Val-SOD2 (80, 83). No entanto, apesar da maior eficiência do genótipo Ala/Ala, investigações têm retratado associação entre essa variante genética ao câncer de próstata (84), linfoma não-Hodgkin (85), câncer de mama (86), pulmão e estômago (87), esôfago e câncer de colo do útero (88, 89). Uma hipótese para ocorrência desse fenômeno é devido a maior eficiência da SOD2, que se não for acompanhada por um aumento das outras enzimas CAT e GPx, ou até mesmo de compostos antioxidantes não enzimáticos, resulta na geração excessiva de H_2O_2. Sendo assim, o H_2O_2 pode reagir com metais de transição via reação de Fenton, formando o

radical OH•, que é o mais lesivo dos radicais e é fortemente mutagênico, e contra o qual o organismo não apresenta mecanismos de defesa (90).

O genótipo Val/Val-SOD2 tem sido associado a níveis elevados de LDL-oxidado (91), a um maior risco de desenvolvimento de obesidade (92), hipercolesterolemia (93) e maior agressividade tumoral (74, 86, 94). Uma das suposições é que o Val/Val-SOD2, por apresentar uma menor eficiência enzimática, pode gerar um acúmulo de O_2•- dentro da mitocôndria, e este acúmulo, rapidamente reage com o NO formando o peroxinitrito (ONOO). Essa reação resulta em uma extensa oxidação das membranas celulares pela alta afinidade com lipídios (95).

Segundo dados encontrados na literatura, o polimorfismo parece ainda interferir na farmacodinâmica e farmacocinética de fármacos como: quimioterápico metotrexato (96), citrato de clomifeno, um inibidor do receptor do estrogênio, que é amplamente utilizado como indutor da ovulação (76) e interferir na resposta aos agentes de fluoracil e platina em pacientes com câncer gástrico (97).

Dois estudos conseguiram desenvolver um modelo farmacológico de desbalanço da superóxido-peróxido de hidrogênio (S-PH). O paraquat foi utilizado para elevar os níveis de superóxido e a porfirina os níveis de peróxido de hidrogênio, visto que, essa molécula é considerada similar a SOD2 ou SOD-like. Este modelo foi mimetizado em células de câncer de próstata e colorretal, descrevendo que o desbalanço entre S-PH pode afetar diretamente a proliferação, sobrevida e resistência a quimioterápicos (74, 98).

Além disso, as investigações realizadas com câncer de mama encontraram uma associação significativa em relação à atividade da enzima antioxidante SOD2 e à resistência ao TMX, devido à redução da apoptose celular (54-56, 99). Entretanto, apenas um estudo foi encontrado nas principais bases de dados de artigos científicos da área médica e biomédica, associando o polimorfismo Val16Ala-SOD2 e a resistência ao TMX. Tal estudo foi realizado

com pacientes RE+ que usaram apenas TMX adjuvante e mostrou que indivíduos portadores do genótipo Val/Val-SOD2 tiveram uma maior sobrevida livre de recorrência. Esses pacientes tiveram uma diminuição das defesas antioxidantes e um aumento das EROs, características que segundo os autores, facilita a apoptose das células neoplásicas (100).

1.11. Biomarcadores

Biomarcadores são investigações biológicas mensuráveis que permitem conhecer, a partir de sua expressão, o estágio da doença ou até mesmo a resposta a um determinado tratamento (101). Novos anticorpos e metodologias (imuno-histoquímica, microdissecção a laser, microarranjos de DNA, sequenciamento de última geração) estão permitindo estratificar e classificar o câncer de mama de acordo com a sua expressão proteica e genômica, estabelecendo desta forma, assinaturas genéticas tanto preditivas quanto em relação à possibilidade de resposta a terapia (102).

Os biomarcadores podem ser usados para diversos parâmetros, os mais utilizados são de prognóstico e de diagnóstico (102, 103). Os de prognóstico dão informações de sobrevida e história natural da doença, amplamente utilizado na oncologia. Os de diagnóstico, também chamados de preditivos, são indicadores de resposta ao tratamento (101, 102).

Entre os diversos biomarcadores utilizados na oncologia personalizada do câncer de mama, destacam-se: Bcl-2, Caspase-3 e Ki67.

O gene Bcl-2 pertence a uma grande família de genes, também denominada Bcl-2, que são responsáveis pela manutenção do equilíbrio entre proliferação e morte celular programada (apoptose) (104). Este gene antiapoptótico codifica uma proteína mitocondrial, localizada no envelope nuclear, retículo endoplasmático e membrana mitocondrial externa, de vários tecidos normais e neoplásicos (105).

Células normais produzem níveis relativamente altos de Bcl-2, com o objetivo de preservar essas células cuja morte seria destrutiva para o organismo. No entanto, o excesso de proteção em células cancerosas pode gerar tumores mais agressivos, já que resistem mais à morte programada. Como encontrado no estudo Ellis *et al.* (1998), associação da expressão de Bcl-2 em células de câncer de mama e resistência a quimioterapia citotóxica (106). Além disso, um estudo sugere que o estrogênio promove resistência aos medicamentos TMX e cisplatina, através do aumento da proteína Bcl-2 (107).

Outro gene fundamental para a apoptose é a Caspase-3, que ao ser ativada atua como uma caspase efetora, clivando várias proteínas celulares da cascata apoptótica, e, portanto, apresenta um papel central na fase de execução da morte celular. Ativada na célula apoptótica por vias extrínseca (ligante da morte) e intrínseca (mitocondrial). (108). O papel da Caspase-3 permanece controverso na literatura. Uma investigação *in vitro* mostrou que a restauração da expressão da Caspase-3 pode sensibilizar a apoptose induzida por doxorrubicina, sugerindo que a deficiência de caspase-3 pode ser um mecanismo de quimioresistência (109). Zohny *et.al.* (2019) encontrou a mesma relação, pacientes com câncer de mama maligno tiveram baixa atividade de Capase-3 em comparação aos pacientes com câncer de mama benigno (110). Contrário a estes resultados, um estudo encontrou relação da baixa expressão de Caspase-3 e um melhor prognóstico (111).

Em relação à proliferação celular, o biomarcardor Ki-67 é um dos mais estudados atualmente. O aumento da proliferação celular está correlacionado com um pior prognóstico (112). O Ki-67 está presente em todas as células em proliferação, sendo uma proteína nuclear não histona ausente somente na fase G0 do ciclo celular. Os usos potenciais do Ki-67 incluem: prognóstico de responsividade relativa, resistência à quimioterapia ou terapia endócrina, estimativa de risco residual em pacientes em terapia padrão e como um

biomarcador dinâmico de eficácia do tratamento em amostras obtidas antes, durante e após a terapia neoadjuvante (113, 114).

Neste contexto, os biomarcadores são ferramentas poderosas que nos auxiliam a fornecer informações para caracterizar as diferentes assinaturas do câncer, as quais, são importantes para um tratamento personalizado.

1.12. Justificativa

Diante do exposto, existem muitos estudos mostrando a relação da enzima antioxidante SOD2 e a resistência ao TMX, mas poucos em relação ao polimorfismo Val16Ala-SOD2 que mostrou em estudos anteriores um importante papel frente ao desenvolvimento e agressividade do câncer e à resposta terapêutica. Além disso, a complexidade da função do RE nas células tumorais ressalta a heterogeneidade da biologia do câncer de mama e demonstra a necessidade de pesquisa básica continuada e demonstração clínica para atingir efetivamente os caminhos essenciais à sobrevivência das células tumorais. Com isso, justifica-se que entender os processos biológicos para identificar possíveis biomarcadores preditivos de resposta ao tratamento com TMX é fundamental para permitir uma conduta personalizada e assim, garantir uma melhor sobrevida livre de recorrência nas pacientes.

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3. OBJETIVOS

3.1. Objetivo Geral

Investigar biomarcadores e a influência do polimorfismo Val16Ala-SOD2 nas pacientes com câncer de mama em uso do TMX adjuvante.

3.2.Objetivo Específicos

- Revisar na literatura os atuais biomarcadores utilizados para predição de resistência ao TMX em pacientes com câncer de mama Luminal;
- Determinar a frequência genotípica dos alelos da SOD2 Val e Ala na população em estudo;
- Estimar o risco do desenvolvimento de recorrência da doença em relação aos genótipos investigados;
- Avaliar a expressão imunohistoquímica dos marcadores Bcl2 (anti-apoptose), Caspase-3 (Pró-apoptose) e Ki-67 (proliferação) e correlaciona-los com a recorrência da doença;
- Correlacionar os genótipos da SOD2 com estadiamento, grau e marcadores de apoptose e proliferação.

4. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

4.1.Artigo I

“MOLECULAR MARKERS ASSOCIATED WITH THE OUTCOME OF TAMOXIFEN TREATMENT IN ER-POSITIVE BREAST CANCER PATIENTS: ASCOPING REVIEW AND *IN SILICO* ANALYSIS”

Maiquidieli Dal Berto, Giovana Tavares dos Santos, Aniúsca Vieira dos Santos, Andrew Oliveira Silva, José Eduardo Vargas, Rafael José Vargas Alves, Fernanda Barbisan, Ivana Beatrice Mânica da Cruz, Claudia Giuliano Bica

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MOLECULAR MARKERS ASSOCIATED WITH THE OUTCOME OF TAMOXIFEN TREATMENT IN ESTROGEN RECEPTOR-POSITIVE BREAST CANCER PATIENTS: SCOPING REVIEW AND *IN SILICO* ANALYSIS

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ABSTRACT

Tamoxifen (TMX) is used as adjuvant therapy for estrogen receptor-positive (ER+) breast cancer cases due to its affinity and inhibitory effects. However, about 30% of cases show drug resistance, resulting in recurrence and metastasis, the leading causes of death. A literature review can help to elucidate the main cellular processes involved in TMX resistance. A scoping review was performed to find clinical studies investigating the association of expression of molecular markers profiles with long-term outcomes in ER+ patients treated with TMX. *In silico* analysis was performed to assess the interrelationship among the selected markers, evaluating the joint involvement with the biological processes. Forty-five studies were selected according to the inclusion and exclusion criteria. After clustering and gene ontology analysis, 23 molecular markers were significantly associated, forming three clusters of strong correlation with cell cycle regulation, signal transduction of proliferative stimuli, and hormone response involved in morphogenesis and differentiation of mammary gland. Also, it was found that overexpression of markers in selected clusters is a significant indicator of poor overall survival. The proposed review offered a better understanding of independent data from the literature, revealing an integrative network of markers involved in cellular processes that could modulate the response of TMX. Analysis of these mechanisms and their molecular components could improve the effectiveness of TMX.

Keywords: recurrence, tamoxifen, breast cancer, HR positive, biological processes, molecular targets.

DECLARATIONS

Funding

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Availability of data and material

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consent to publication.

Competing interests

The authors declare that they have no potential conflicts of interest.

Authors' contributions

Conceptualization; M.D.B. G.T.S, A.V.S., C.G.B.; Data curation; M.D.B, G.T.S., A.V.S.; Formal analysis; A.O.S, F.B., I.B.M.C., C.G.B.; Funding acquisition; J.E.V., R.J.V.A., C.G.B.; Investigation; M.D.B., G.T.S., F.B., I.B.M.C.; Methodology and Project administration; M.D.B., G.T.S., A.V.S., R.J.V.A., A.O.S., J.E.V., C.G.B.

Final review: All authors read and approved the final manuscript.

1. Introduction

Breast cancer (BC) is the most prevalent cancer among women in developed and developing countries

[1]. Due to its incidence and mortality, it is considered a serious public health problem worldwide [2,3]. This type of cancer is a complex and heterogeneous disease with several distinct histopathological and molecular subtypes. Among them, about 60 to 70% of all diagnosed BC are positive for hormone receptor expression (HR+), specifically estrogen receptor (ER+) and/or progesterone receptor (PR+).

The stimulation of both receptors by its ligand molecules – estrogen and progesterone – induces mainly transcriptional changes, which trigger biological processes such as cell survival, proliferation, and differentiation. Therefore, in breast cancer, its constant stimulation by sexual hormones could contribute to tumor increasing and disease progression [4,5], justifying anti-estrogen therapies as an adjuvant treatment for this type of neoplasia [6].

Tamoxifen (TMX) is one of the most used types of adjuvant treatment in ER+ breast cancer. Administered as a prodrug, TMX must be metabolized in the liver to form active metabolites that will perform their estrogen antagonist functions and thus referred to as a *selective estrogen receptor modulator* (SERM) which blocks the binding of estrogen to the estrogen receptor and consequently inhibit cell proliferation and survival [7-11]. For this reason, TMX is the standard hormone therapy for HR+ breast cancer patients in the premenopausal stage [12-14].

Whereas this hormone adjuvant therapy has considerably improved survival in HR molecularly subtyped BC patients in recent decades, the development of pharmacological resistance has been an increasing challenge for oncology, once the lack of responsiveness to treatment is a critical and limiting factor for therapeutic efficacy [15,16]. Around 30 to 50% of HR+ BC patients, regardless of the expression level of these receptors, presented intrinsic or acquired resistance to TMX [17,18]. Consequently, the five-year survival rate after resistance occurrence was less than 20% [19,20].

Several clinical works have been published in the literature bringing information about a new specific marker involved with this frame of resistance, associating its molecular profile with a better or poor outcome in BC patients. Therefore, a better comprehension of the interrelationship among these identified molecular markers associated with TMX resistance becomes increasingly relevant and necessary to strengthen the understanding of what molecular mechanisms and cell processes are changed in tumor cells when they acquire hormone resistance in an attempt to overcome it and improving therapeutic effectiveness.

A major meta-analysis carried out by Wirapati et al. (2008) analyzed the gene expression in breast cancer patients and associated it with important biological processes that could be present in all molecular subtypes [21]. Another meta-analysis by Mihály et al. (2013) investigated new independent biomarkers that could be related to the tamoxifen response in breast cancer patients [22].

This work presents a systematic review of recent papers in the literature, focusing only on translational studies with a minimum 3-year follow-up. It highlights how individual biomarkers related to TMX resistance impact the survival rate of ER+ breast cancer patients. Then, it employs a clustering technique to find groups of biomarkers whose biological processes show a strong interaction. Based on this clustering analysis, this work evaluates how these biological processes influence the overall survival rate in ER+ breast cancer patients, indicating which processes could be predictive of clinical results in response to treatment with TMX. To the best of the authors' knowledge, this is the first systematic review that presents such a clustering technique for the conjoint evaluation of multiple biomarkers in the survival rate of ER+ breast cancer patients.

2. Methodology

2.1 Identifying relevant studies

The manuscript selection was performed according to the PRISMA Extension for Scoping Reviews (PRISMA-ScR) methodology: Checklist and Explanation [23]. The literature review was performed to identify clinical studies assessing the correlation between the expression profile of a molecular biomarker and the long-term outcome of HR+ BC patients in response to TMX treatment. The initial inclusion criteria were original manuscripts, published from 2013/01/01 to 2018/06/30, full texts in any language, obtained from EMBASE or PUBMED databases. For manuscript search, three primary keyword descriptors were applied: “breast cancer”, “hormone therapy” and “resistance”, or their variations listed in Supplementary material 1. Case studies, reviews, and comments were not considered.

2.2. Selection of eligible studies

The manuscripts found in the initial search were analyzed in three stages: first, a title reading was performed, selecting only those presenting all primary keyword descriptors or their variations. Duplicates were removed. The second and third stages consisted of abstract and full-text reading, respectively. These stages ensure that the selected manuscripts respected the inclusion and exclusion criteria, according to Table 1. The essential items of the REMARK protocol [24] were considered in this review. Two independent reviewers performed the manuscript search and selection processes, while a third reviewer solved incongruities. Only manuscripts that assessed molecular biomarkers as a prognostic factor and not as a risk factor were considered. Studies only evaluating patient expression databases, in vivo or in vitro experimentation were also excluded.

Table 1. Study inclusion and exclusion criteria

Study characteristics	Inclusion criteria	Exclusion criteria

Design	Cohort Human	● Trial ● Review
Publication	Any language Abstract and full text available	● Dissertation ● Conference proceeding, abstract or poster
Participants	Breast cancer female Hormone therapy	● < 35 samples ● Databases
Intervention	Tamoxifen (TMX)	● Hormone therapy non described
Outcome	Correlation with survival or recurrence	● Just prognosis with clinical pathological parameters

2.3. Data extraction

After the full-text review stage of the selected manuscripts included in this scoping review, the following information was plotted in Table 2:

- Population: number of participants and follow-up of only those HR+ BC patients treated with Tamoxifen.
- Marker profile: the marker symbol used by each selected manuscript; the genetic alteration (deletion, mutation, or polymorphisms) when altered; the type of molecule assessed (DNA, RNA, or protein); the molecular status of the analyzed marker in relation to activity, expression, subcellular localization, or post-translational modification, which could alter its functionality.
- Clinical outcomes and statistical analysis: clinical profile of tested BC patients (survival or recurrence) associated with the alteration of analyzed molecular markers; the statistical analysis of the association between the molecular status of markers and clinical outcome, prioritizing multivariate analysis; the significance level of statistical analysis when available. In order to standardize the collected information, it was plotted molecular profile of markers, which was associated with a poor clinical outcome, except for those presenting polymorphisms or specific subcellular localization.

2.4. In silico analysis

2.4.1. Design of Networks

After a full-length analysis of manuscripts and data extraction, the official symbol of all selected molecular markers was confirmed in the HUGO Gene Nomenclature Committee (HGNC) website for *in silico* analysis (Supplementary Table 1). Those presenting statically significant associations ($p < 0.05$) with some clinical outcomes in HR+ BC patients were assessed together using the ©STRING CONSORTIUM 2019 (version 11.0) web tool. The minimum interaction score adopted in this analysis is 0.700 (high confidence) for

each connection (edges), including all active interaction sources used by the tool, excluding text mining. The resulted network was presented with an interaction score of each connection between two markers for further modular analysis of networks. Posteriorly, the unconnected markers were separately analyzed. Only those markers whose molecular profile of expression/activity direct influence the clinical outcomes of HR+ BC patients were considered, excluding micro RNAs. For polymorphisms, only the affected biomarker was included for network design, not considering the polymorphic variant itself.

2.4.2. Modular Analysis of Network

From the STRING resulting network, it was used ClusterONE plugin [25] of Cytoscape software. The cluster establishment was assessed considering the following criteria: at least three proteins compounding a cluster, density and interaction quality values above 0.5, and significant p-value ($p < 0.05$). Molecular markers that were not included in any cluster were called unclustered markers.

2.4.3. Gene Ontology Analysis

Once the clusters within a molecular marker network were identified, it was performed the gene ontology (GO) analysis using the Biological Network Gene Ontology (BiNGO) plugin (version 3.0.3) in Cytoscape software to assess the possible biological processes that could be involved with the specific set of markers grouped in each cluster. Statistically significant clusters were assessed, considering only their members or added to their direct neighbor markers (networked markers directly linked with clustered markers). Statistical significance was measured using a hypergeometric test with the multiple testing correction of Benjamini & Hochberg False Discovery Rate (FDR). Only biological processes with a p-value < 0.05 were considered significant. Based on biological knowledge about BC resistance, the five biological processes often involved with this event were selected to be illustrated in the results and further discussed.

2.4.4 The predictive power of different sets of TMX-resistance markers

In order to assess the predictive power of each set of markers among those significantly associated with any clinical outcome in TMX-treated HR+ BC patients, the Kaplan Meier-plotter web software [26] was used. It was assessed a general analysis for all selected markers, those connected and unconnected markers from STRING analysis, and those clusters obtained from ClusterOne plugin (with or without their direct neighbor markers). The data extracted from the original papers showing high expression/activity of molecular markers associated with poor outcome were directly related in the KM-plotter analysis settings, inverting those presenting low or absence expression/activity associated with poor prognosis status (Supplementary Table 1). Equal weights were attributed to all tested markers. The Kaplan-Meier graphs represent overall survival (OS) with a follow-up threshold of 240 months, splitting patients by median, using all probe sets per gene, without any restriction of breast cancer subtypes or selected cohorts studies as dataset source.

3. **Results**

It was identified 2.487 articles in EMBASE and PUBMED databases from the keyword descriptors and their variations. The flow diagram of literature analysis, manuscript selection, and in silico analysis is illustrated in Figure 1.

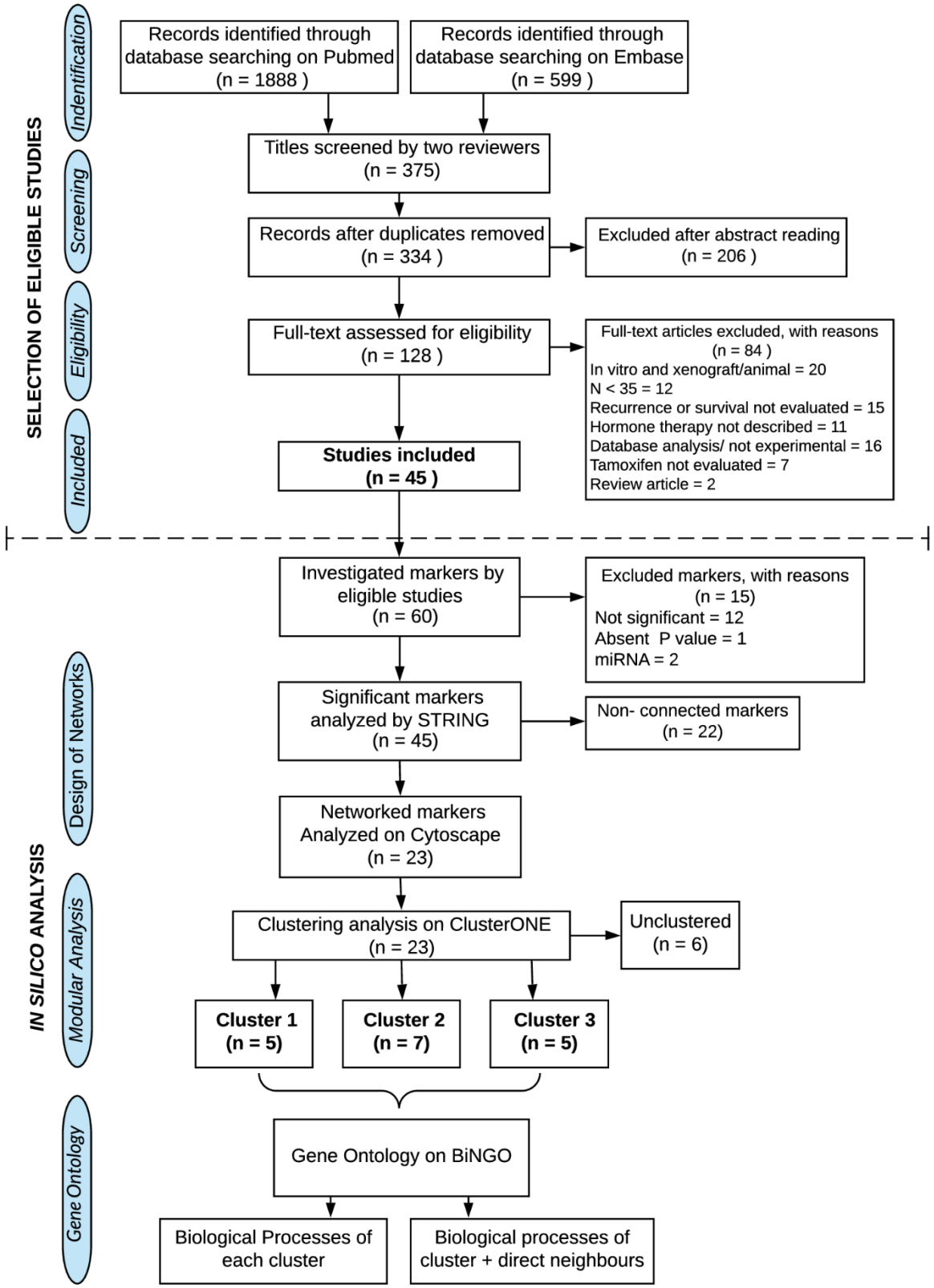


Figure 1- PRISMA diagram of selection of studies and *in silico* analysis. The upper section corresponds to the step-by-step manuscript selection process according to the established inclusion and exclusion criteria. The Lower section includes the sequence of *in silico* analysis performed with all selected biomarkers to search a better and integrated understanding of the influence of each protein in TMX resistance.

The application of the eligibility criteria resulted in the exclusion of 2.443 papers that were not adequate to one or more selection factors or did not provide the needed information. The main excluding factors were: (1) in vitro experimentation; (2) in vivo assays; (3) clinical studies assessing less than 35 patients (4) the absence of prognostic factor assessing in clinical studies; (5) the absence of hormone therapy with TMX; (6) manuscripts using of transcription or genomic sequencing data deposited in public databases; (7) works testing other types of hormone therapy, differently of TMX; (8) and review manuscripts. Thereby, 45 manuscripts were selected from the eligibility analysis, analyzing 60 different molecular targets, as shown in Table 2.

Table 2. Selected publications accessing the influence of molecular markers on clinical outcomes of Tamoxifen-resistant breast cancer patients

Author (Year)	N	Follow-up (Years)	Marker Symbol	Marker Alteration	DNA, RNA or Protein	Marker status	Outcome	Statistical Analysis of Association	*P* value
Browne BC. et al. (2013)	171	12.5	MARCKS	--	Protein	Positive Expression	Poorer OS	Multivariate Analysis	***0.045***
Margolin S. et al. (2013)	313	13	CYP2D6	--	Protein	Decreased Activity	Poorer RFS	Multivariate Analysis	***0.018***
							Poorer OS	Multivariate Analysis	***0.030***
Zhang L et al. (2013)	37	10	NCOR2	BQ323636.1 Splice variant	DNA	Overexpression	Poorer OS	Multivariate Analysis	***0.030***
							Poorer DFS	Multivariate Analysis	***0.038***
Ijichi N. et al. (2013)	100	16.4	EBAG9	--	Protein	Positive Expression	Poorer DFS	Multivariate Analysis	***0.035***
Piva M. et al. (2013)	55	8	SOX2	--	Protein	Overexpression	Increased Recurrence	Multivariate Analysis	***< 0.001***
							Poorer DFS	Multivariate Analysis	***< 0.001***
Elzawahry HM. et al. (2013)	70	6	ki-67	--	Protein	Overexpression	Increased recurrence	Multivariate Analysis	***0.007***
Hrstka R. et al. (2013)	61	10.8	AGR2	--	mRNA	Low Expression	Increased PFS	Univariate Analysis	***0.036***
Chen M. et al. (2013)	104	10	ERα	Phosphorylation at serine 118	Protein	Phosphorylation at Specific Site	Poorer DFS	Univariate Analysis	***0.022***
							Poorer OS	Univariate Analysis	***0.013***
				Phosphorylation at serine 167	Protein	Phosphorylation at Specific Site	DFS	Univariate Analysis	*0.515*
							OS	Univariate Analysis	*0.300*
Thrane S. et al. (2014)	244	18 - 19	AURKA	--	Protein	Overexpression	Poorer DFS	Multivariate Analysis	***0.006***
							Poorer OS	Univariate Analysis	***0.038***
Reijm EA. Et al. (2014)	250	3	EZH2	--	Protein	Positive Expression	Poorer PFS	Multivariate Analysis	***0.017***
						% Positive Cells	Poorer PFS	Multivariate Analysis	***0.002***
Huang R. et al. (2014)	N/A	28	STAT3	--	mRNA	Low Expression	Poorer DMFS	Univariate Analysis	***< 0.001***
					Protein	Low Expression	Poorer DFS	Univariate Analysis	***0.006***
			STAT1	--	Protein	Overexpression	DMFS	Univariate Analysis	*0.067*
							DFS	Univariate Analysis	*0.256*
Wong PP. et al. (2014)	144	26	MAGEA2	--	Protein	Positive Expression	Poorer OS	Univariate Analysis	***0.006***
Putluri N. et al. (2014)	45	14	RRM2	--	Protein	Overexpression	Poorer RFS	Univariate Analysis	***0.04***
Lehn S. et al. (2014)	101	16-17	YAP1	--	Protein	Absent Expression	Poorer RFS	Univariate Analysis	***< 0.001***
Wei C. et al. (2014)	129	6.6	TBK1	--	Protein	Overexpression	Poorer DFS	Univariate Analysis	***0.003***
Winder T. et al. (2014)	219	10	IGF1R	Polymorphism (rs2016347)	DNA	Presence of Polymorphic Form	Poorer DFS	Multivariate Analysis	***0.024***
							OS	Multivariate Analysis	*0.14*

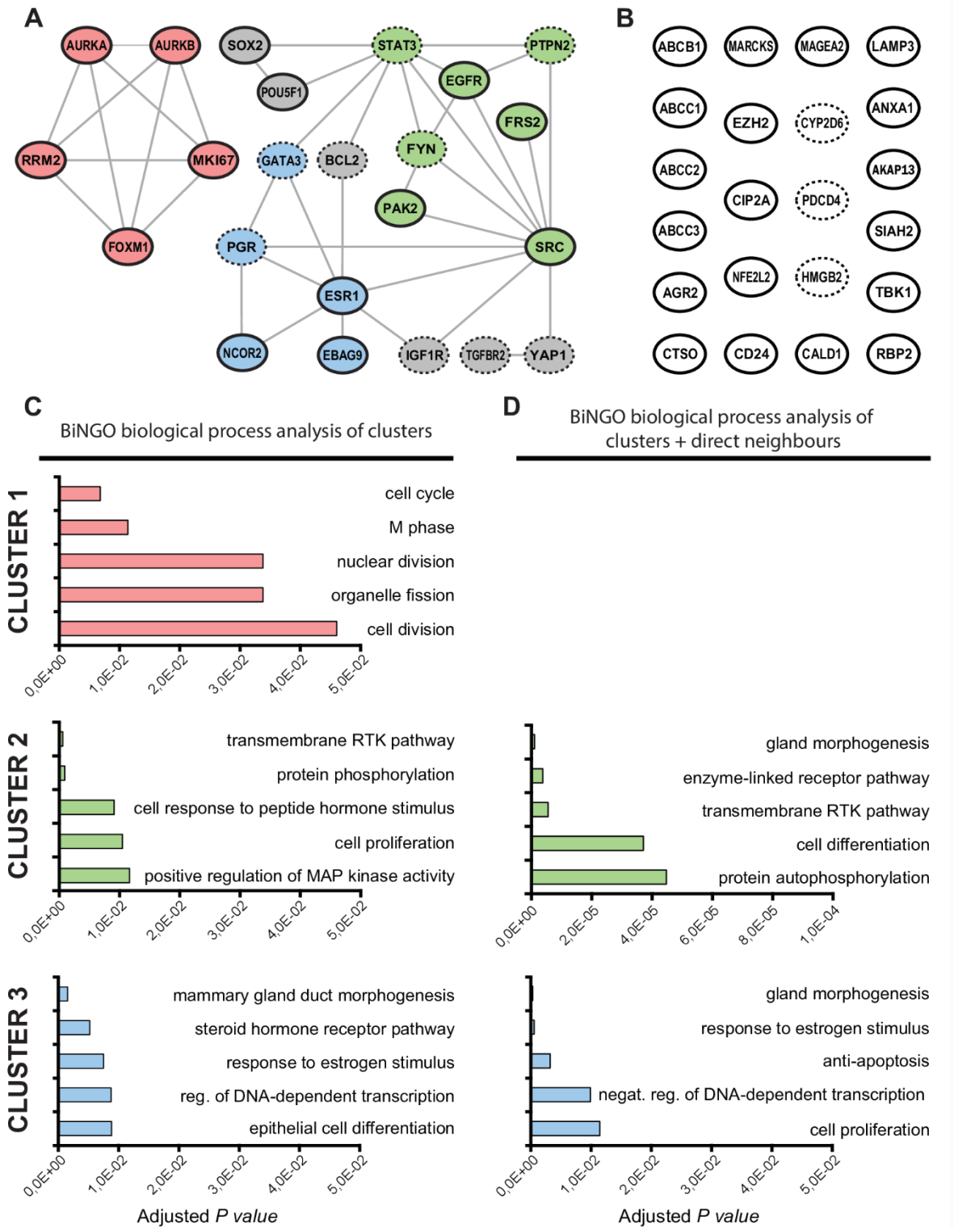
	221	*10*	IGF1	Polymorphism (rs6214)	DNA	Presence of Polymorphic Form	DFS	Multivariate Analysis	*0.95*
							OS	Multivariate Analysis	*0.86*
				Polymorphism (rs7136446)	DNA	Presence of Polymorphic Form	DFS	Multivariate Analysis	*0.74*
							OS	Multivariate Analysis	*0.73*
				Polymorphism (rs2946834)	DNA	Presence of Polymorphic Form	DFS	Multivariate Analysis	*0.58*
							OS	Multivariate Analysis	*0.77*
	208	*10*	IGFBP3	Polymorphism (rs2854744)	DNA	Presence of Polymorphic Form	DFS	Multivariate Analysis	*0.58*
							OS	Multivariate Analysis	*0.79*
	220	*10*	IRS1	Polymorphism (rs1801123)	DNA	Presence of Polymorphic Form	DFS	Multivariate Analysis	*0.93*
							OS	Multivariate Analysis	*1.0*
Redmond AM. et al. (2014)	N/A	5	HMGB2	--	Protein	Absent Expression	Poorer DFS	Univariate Analysis	***0.006***
Bergamaschi A. et al. (2014)	501	16	FOXM1	--	Protein	Overexpression	Poorer DFS	Univariate Analysis	***0.003***
Nagelkerke A. et al. (2014)	304	3	LAMP3	--	mRNA	Overexpression	Poorer PFS	Multivariate Analysis	***0.032***
							Poorer PROS	Univariate Analysis	***0.04***
Karlsson E. et al. (2015)	73	19	PTPN2	deletion	Protein	Absent Expression	Poorer DRFS	Univariate Analysis	***0.011***
Elias D. et al. (2015)	76	6-7	FYN	--	Protein	Plasma Membrane-associated Expression	Increased MFS	Univariate Analysis	***< 0.003***
		12-13					Increased OS	Univariate Analysis	***< 0.001***
Hato Y. et al. (2015)	335	29	CTSO	Polymorphism (rs10030044)	Protein	Presence of Polymorphic Form	Poorer DFS	Univariate Analysis	***0.005***
							Poorer OS	Multivariate Analysis	***< 0.001***
Honma N. et al. (2015)	447	20	Bcl-2	--	Protein	Positive Expression	Increased OS	Univariate Analysis	***0.024***
							DFS	Multivariate Analysis	*0.743*
Busch S. et al. (2015)	87	17	TGFBR2	--	Protein	Low Expression	Poorer RFS	Univariate Analysis	***0.008***
	99	17	SMAD2	Phosphorylation at serine 465/467	Protein	Phosphorylation at specific site	RFS	Univariate Analysis	*0.878*
Larsen SL. et al. (2015)	259	19	SRC	--	Protein	Plasma Membrane-associated Expression	Poorer DFS	Multivariate Analysis	***0.004***
							Poorer OS	Multivariate Analysis	***0.026***
Argalácsová S. et al. (2015)	71	16-17	CYP2D6	Several polymorphisms	Protein	Enzymatic Activity	DFS and TTP	Univariate Analysis	*N/A*
			ABCB1	Polymorphism (rs2032582)	DNA	Presence of Polymorphic Form	DFS	Univariate Analysis	*N/A*
				Polymorphism (rs1045642)	DNA	Presence of Polymorphic Form	Increased DFS	Univariate Analysis	***0.012***
Bentin TC. et al. (2015)	94	36	AKAP13	--	mRNA	Overexpression	Poorer PFS	Multivariate Analysis	***0.044***

Zhong X. et al. (2016)	88	12	FRS2	--	Protein	Overexpression	Poorer DFS	Multivariate Analysis	***0.03***
			miR-4653-3p	--	miRNA	Low Expression	Poorer DFS	Multivariate Analysis	***0.004***
Ahern TP. et al. (2016)	911	--	Pak1	--	Protein	Cytoplasmic Expression Level	Recurrence	Univariate Analysis	*N/A*
	962					Nuclear Expression	Recurrence	Univariate Analysis	*N/A*
Babyshkina N. et al. (2016)	97	8-9	EGFR	--	Protein	Positive Expression	Poorer PFS	Univariate Analysis	***0.006***
Liu J. et al. (2016)	148	2.9	GATA3	Mutation	Protein	Presence of Mutated Form	PFS	Univariate Analysis	*0.800*
				--	mRNA	Low Expression	ORR	Univariate Analysis	*0.31*
							Poorer PFS	Multivariate Analysis	***0.008***
De Marchi T. et al. (2016)[a]	317	3	ANXA1	--	Protein	Overexpression	Shorter TTP	Multivariate Analysis	***0.016***
	259	3	CALD1	--	Protein	Overexpression	Shorter TTP	Multivariate Analysis	***0.001***
De Marchi T. et al. (2016)[b]	294	3	PDCD4	--	Protein	Overexpression	Longer TTP	Multivariate Analysis	***0.009***
			OCIAD1	--	Protein	Expression	TTP	Univariate Analysis	*N/A*
			CGN	--	Protein	Expression	TTP	Univariate Analysis	*N/A*
			G3BP2	--	Protein	Expression	TTP	Univariate Analysis	*N/A*
Sensorn I. et al. (2016)	73	14.3	ABCC2	Polymorphism (rs717620)	DNA	Presence of Polymorphic Form	Increased DFS	Multivariate Analysis	***0.040***
Bekele RT. et al. (2016)	94	8	Nrf2	--	Protein	Overexpression	Poorer OS	Univariate Analysis	***0.002***
			ABCC1	--	Protein	Overexpression	Poorer OS	Univariate Analysis	***0.04***
			ABCC3	--	Protein	Overexpression	Poorer OS	Univariate Analysis	***0.01***
			NQO1	--	Protein	Expression	OS	Univariate Analysis	*0.12*
Van der Willik KD. et al. (2016)	245	3	SIAH2	--	Protein	Overexpression	Poorer PFS	Multivariate Analysis	***0.015***
Thistle, J. E. et al. (2017)	1082	10	14-3-3ζ	--	Protein	Nuclear and Cytoplasmic Overexpression	Poorer RFS	Univariate Analysis	***N/A***
Gwak JM. et al. (2017)	129	10.5	Oct4	--	Protein	Overexpression	Poorer DFS	Multivariate Analysis	***< 0.001***
Abudureyimu K. et al. (2017)	76	13-14	AURKB	--	Protein	Positive Expression	Poorer OS	Univariate Analysis	***0.001***
Snell CE. et al. (2017)	63	10	PR	--	Protein	Absent Expression	Poorer RFS	Multivariate Analysis	***0.005***
Baldacchino S. et al. (2017)	187	16-20	CIP2A	--	Protein	Plasma Membrane-associated Expression	DFS	Multivariate Analysis	***0.002***
Han SH. et al. (2017)	95	12	miR-222	--	miRNA	Expression	DFS	Univariate Analysis	*0.286*
Zhang Y. et al. (2018)	291	3	PAK2	--	Protein	Overexpression	Poorer PFS	Multivariate Analysis	***0.008***
	101			--	mRNA	Overexpression	Poorer PFS	Univariate Analysis	***0.033***
Moon YW. et al. (2018)	99	12.9	CD24	--	Protein	Positive Expression	Poorer PFS	Multivariate Analysis	***0.006***
			CD44	--	Protein	Expression	PFS	Univariate Analysis	*1.0*
			ALDH1	--	Protein	Expression	PFS	Univariate Analysis	*0.090*
Choi HJ. et al. (2018)	85	12.5	RBP2	--	Protein	Overexpression	Poorer DFS	Univariate Analysis	***0.04***

OS = Overall Survival; **DFS** = Disease-Free Survival; **RFS** = Relapse-Free Survival; **PFS** = Progression-Free Survival; **DMFS** = Distant Metastasis Free Survival; **PROS** = Post-Relapse Free Survival; **DRFS** = Distant Recurrence Free Survival; **MFS** = Metastasis Free Survival; **TTP** = Time To Progression; **ORR** = Overall Response Rate

The total number of analyzed patients in each selected study has varied between 37 [29] and 1082 [62]. The follow-up of most studies was 5 years or more, and 6 other studies presented a follow-up of 3 years [68,61,58,59,45,36] or less [57]. The main reported alterations in assessed molecular targets were changes in their expression level or activity. However, some structural modifications at the DNA, RNA, or protein level were also found, such as polymorphisms, post-translational modifications (phosphorylation), and mutations (deletion or splicing variant).

Fifteen of the 60 selected markers were excluded from the in silico analysis (Figure 1). Thereby, only 45 of 60 selected markers were eligible for in silico analysis. STRING analysis resulted in a functional association among 23 of 45 tested markers, forming two networks with 5 and 18 nodes, respectively (Figure 2a).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STAT3
PTPN2
POU5F1
EGFR
FRS2
RRM2
MKI67
GATA3
BCL2
FYN
PAK2
FOXM1
PGR
SRC
ESR1
NCOR2
EBAG9
IGF1R
TGFBR2
YAP1
B
ABCB1
MARCKS
MAGEA2
LAMP3
ABCC1
EZH2
CYP2D6
ANXA1
ABCC2
AKAP13
CIP2A
PDCD4
ABCC3
SIAH2
NFE2L2
HMGB2
AGR2
TBK1
CTSO
CD24
CALD1
RBP2
C
BiNGO biological process analysis of clusters
CLUSTER 1
cell cycle
M phase
nuclear division
organelle fission
cell division
0,0E+00
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2,0E-02
3,0E-02
4,0E-02
5,0E-02
CLUSTER 2
transmembrane RTK pathway
protein phosphorylation
cell response to peptide hormone stimulus
cell proliferation
positive regulation of MAP kinase activity
CLUSTER 3
mammary gland duct morphogenesis
steroid hormone receptor pathway
response to estrogen stimulus
reg. of DNA-dependent transcription
epithelial cell differentiation
Adjusted P value
D
BiNGO biological process analysis of clusters + direct neighbours
gland morphogenesis
enzyme-linked receptor pathway
transmembrane RTK pathway
cell differentiation
protein autophosphorylation
0,0E+00
2,0E-05
4,0E-05
6,0E-05
8,0E-05
1,0E-04
gland morphogenesis
response to estrogen stimulus
anti-apoptosis
negat. reg. of DNA-dependent transcription
cell proliferation
Adjusted P value

Figure 2- *In silico* analysis. A) 3 clusters were formed with strongly linked molecular markers, cluster 1(red), cluster 2 (green), and cluster 3 (blue). The markers in gray were not included in the clusters, remaining just interconnected proteins among clusters inside the network. B) unconnected markers. A and B still reveal the functional status of each molecular marker. 12 markers (dashed borderline in the molecular markers) decrease in functional activity associated to poor outcomes in BC patients, the other 33 markers presented an elevated functional activity associated to worst outcomes (full borderline in the molecular markers), accordingly information of their original manuscripts. C) Gene ontology analysis for biological processes: CLUSTER 1 presented significant involvement with molecular events related to cell proliferation, CLUSTER 2 with the modulation of proliferative mechanisms, and CLUSTER 3 with the mammary gland development and its hormone stimulation. D) Genetic ontology analysis of clusters 2 and 3, taking into account not only its constituent markers but with its direct neighbors.

Furthermore, to find densely associated subgroups of molecular markers inside the obtained networks, ClusterONE analysis revealed the presence of 3 clusters with strongly linked molecular markers (red, green, and blue markers). Five proteins (AURKA, MK167, AURKB, FOXM1, RRM2) constituted the CLUSTER 1 (red markers) that presented a density value = 1,000 and interaction quality = 1000, displaying a high interactive power among these markers, represented by a *p-value* = 0.002. The CLUSTER 2 (green markers) was configured with 7 proteins (STAT3, PTPN2, FYN, SRC, PAK2, FRS2, EGFR), presenting a density value = 0.571 and quality = 0.600 and a *p-value* = 0.022. The CLUSTER 3 (blue markers) was established with 5 proteins (ESR1, GATA3, PGR, NCOR2, EBAG9), presenting density = 0.600, interaction quality = 0.545 and *p-value* = 0.055, considered a borderline, accordingly the established criteria.

Not all networked molecular markers were included in the clusters, remaining just interconnected proteins among clusters inside the network (gray markers). This subgroup was called unclustered markers and was constituted by six proteins (SOX2, POU5F1, BCL2, IGF1R, TGFBR2, YAP1). Among these, SOX2 and POU5F1 were directly linked only with CLUSTER 2, and TGFBR2 and YAP1 only with CLUSTER 3. On the other hand, BCL2 and IGF1R seem to establish a link between CLUSTER 2 and 3, acting as an intermediate node between them. The other remaining 22 markers presented no interaction among them (Figure 2b).

Figures 2a and 2b reveal the functional status of each molecular marker that is associated with a worsening outcome in patients, according to the information of their original manuscripts. From 45 tested markers, 12 of them presented a decrease in functional activity associated with poor outcomes in BC patients (dashed borderline in the molecular markers), which may be due to an inactivating polymorphism, a gene deletion, a gene/protein expression decrease, or still an activity reduction. Further, the other 33 markers presented an elevated functional activity associated with the worst outcomes due to an activating polymorphism, elevated gene/protein expression, or increasing functional activity.

In the gene ontology analysis (Figure 2c), CLUSTER 1 presented significant involvement with molecular events related to cell proliferation, strongly suggesting that this subset of molecular markers could directly influence the mitotic mechanism regulation. The analysis of CLUSTER 2 presented significant implications in biological processes directly connected with signaling pathways involved with the modulation of proliferative mechanisms. Lastly, CLUSTER 3 showed a significant relationship with processes related to the development of the mammary gland and its hormone stimulation, suggesting a possible involvement with mechanisms of differentiation and signaling pathways of sexual hormone response in the breast tissue.

CLUSTER 2, together with its direct neighbors (BCL2, SOX, POU5F1, GATA3, YAP1. ESR1, PGR, IGF1R) (figure 2d), has shown significant implication with biological processes as “gland morphogenesis,” “enzyme-linked receptor pathway,” “transmembrane RTK pathway,” “cell differentiation” and “protein autophosphorylation.” The same approach was followed for CLUSTER 3 and its direct neighbors (BCL2, STAT3, SRC, IGF1R), resulting in significant involvement with cellular mechanisms such as “gland morphogenesis,” “response to estrogen stimulus,” “anti-apoptosis,” “negative regulation of DNA-dependent transcription” and “cell proliferation.” It was found that some cell processes had changed between CLUSTER 2 and 3 when direct neighbors were added in analysis, highlighting their proximity inside the network and the possible involvement of both clusters in similar or the same cellular processes.

In order to complement our data, a more in-depth analysis was carried out to test if these clusters would have a predictive power of overall survival in BC patients when treated with TMX (Figure 3).

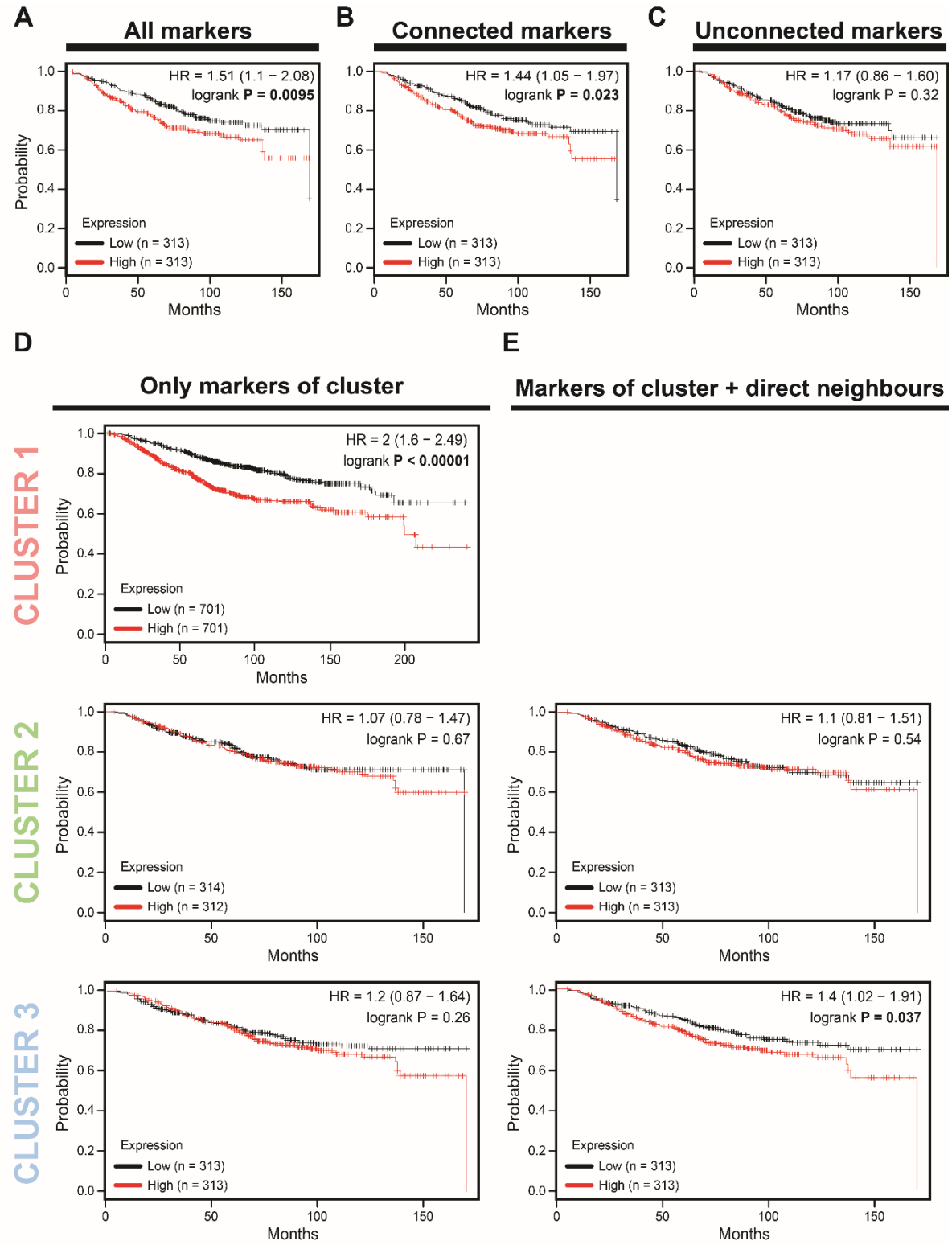

Figure 3 – Predictive analysis of overall survival for each set of markers in BC patients using the Kaplan-Meier plotter tool. A) Analysis of all 45 selected markers. B) Only the networked markers (23 markers). C) Unconnected markers (22 markers). D) Analysis of clusters 1, 2, and 3. E) Cluster 2 and 3 with their direct neighbors. Significant p-values (< 0.05) are highlighted in bold.

For this analysis, *Kaplan Meier-plotter web software* [26] was used, assessing the overall survival (OS) with a follow-up of 240 months. A first analysis of all 45 selected markers shown Hazard Ratio (HR) = 1.51 (95% CI = 1.1 – 2.08) and a p-value = 0.0095 (Figure 3a). A significant predictive power was found when only those networked markers were analyzed, with an HR = 1.44 (95% CI = 1.05 – 1.97), p-value = 0.023 (Figure 3b).. As expected, the joint analysis of non-connected markers was not significant in predicting overall survival (Figure 3c).

Furthermore, it was assessed if the specific molecular clusters would present predictive power for overall survival. Figure 3d shows Kaplan-Meier for each analyzed cluster, and, among them, only CLUSTER 1 has presented significant predictive capability, displaying HR = 2.0 (95% CI = 1.6 – 2.49), p-value < 0.00001. Conversely, knowing the proximity and possible functional overlap between CLUSTER 2 and 3, it was assessed the predictive power of specific clusters together with their direct neighbors (Figure 3e). Only CLUSTER 3 with its direct neighbors displayed significant results, with HR = 1.4 (95% CI = 1.02 – 1.91), p-value = 0.037.

4. Discussion

This review found evidence in the literature about possible molecular targets capable of individually predicting TMX resistance in patients with BC, demonstrating a strong association between their molecular status and specific clinical outcome, such as tumor survival and/or recurrence. Therefore, an *in silico* analysis was performed to aid in data analysis. An interrelation between some molecular targets was found, forming three distinct clusters, showing significant involvement in biological processes. Our method, based on a dense analysis from literature datasets, showed that there might be groups of cellular processes that could be more involved with resistance to TMX in HR+ BC patients. It is worth highlighting that the same weight was assigned for all analyzed markers in the KM-plot analysis since this information is not commonly evaluated in the study models of the original data sources. Therefore, it might represent a limitation in the present analysis since each marker can have different influence degrees within a signaling pathway to control a specific biological process.

The markers MKI67, AURKA, AURKB, FOXM1 in CLUSTER 1 presented significant involvement with molecular events related to cell proliferation (Figure 2c), as observed by their original manuscripts [32,35,39,44,64]. However, this cellular process is highly complex, involving several components that could regulate it positively or negatively, and it has been widely studied as it is crucial for resistant subpopulation rising, not only to TMX but also to other drugs [72-75].

Analysis of overall survival of CLUSTER 1 showed a significant association with a worse outcome of the TMX-treated BC patients (Figure 3d) when strongly expressed or activated. *Bergamaschi A. et al.* showed that the analysis of a gene signature related to mitosis control, orchestrated by marker 14-3-3ζ, was able to control the resistance profile to endocrine therapy in BC, influencing the expression of markers involved with CLUSTER 1, such as FOXM1 and AURKB [76]. Perhaps, if the *Thistle JE. et al.*, who analyzed the involvement of 14-3-3ζ with a worse outcome in TMX + treated ER + patients, had presented statistical correlation values, this marker would be highly related to CLUSTER1 and could result in an increase of predictive power for this set of genes for TMX therapy response [62].

The uncontrolled cell cycle is considered a hallmark of cancer [77], as shown in the meta-analysis that evaluated three key biological processes for the development of breast cancer (cell proliferation, ER, and HER2 signaling). This analysis highlighted cell proliferation as an important prognostic role. Further, the results showed that the subtypes ER + / HER2- with low proliferation showed a higher DRFS, whereas the ER

- / HER2 - and HER2 + showed a higher level of cell proliferation and worse outcomes. However, there was no analysis of the markers with regard to the different hormonal treatments [21].

Besides, there are studies providing information about possible combined effects among these markers associated with a worse prognosis in patients with other BC subtypes [78,79]. However, no studies simultaneously evaluate the expression profile of five markers composing CLUSTER 1 in tumor cells, which is a promising molecular target to be better explored in future research about this predictive power in response to TMX treatment.

No significant correlation was found with the clinical outcomes for CLUSTERS 2 and 3 (considering only the constituent markers) (Figure 3d). However, individually, each molecular marker has presented an association with worse outcomes in their original studies. This controversial effect of joint analysis demonstrates the importance of simultaneously analyzing as many markers as possible and jointly correlating with clinical outcomes. The more extensive and more complete the molecular profile of tumor cells is, the closer to reality this analysis will be, increasing the representativeness of the experimental data.

Reinforcing this concept, when only constituting markers of CLUSTER 3 were analyzed, no association was found with some clinical outcome. However, when other close markers were included in this analysis, a significant association with overall survival was observed (Figure 3e). The combination of CLUSTER 3 and its direct neighbors have shown involvement with cellular processes linked to mammary gland development and differentiation, in addition to sexual hormone response and gene transcription regulation (Figure 2d). The latter has been associated with Decitabine (DAC) chemoresistance, also in BC [80]. However, some of these cellular processes are quite generic and could be related to several molecular markers. Therefore, more evidence about this subset of markers is required to increase the number of molecular components integrating this cluster, restrict the involvement with generic cellular processes, and improve its predictive power concerning clinical outcomes.

The investigation of gene signatures relating a specific cell profile with specific clinical outcomes has gained traction, not only for BC but also for other tumor types. Toshimitsu et al. revealed a specific molecular profile of cisplatin resistance in esophageal cancer based on in vitro studies using drug-resistant cell lineage [81]. Other works have discovered gene signatures related to BC resistance for the most varied treatments, such as neoadjuvant therapy [82], inhibitory molecules[83], and hormone therapy [84], among others. These studies make use of multiple strategies, such as transcriptome sequencing and microarray, to simultaneously investigate a range of markers in the same tumor sample and compare it with the resistant condition.

The study used a gene expression assay to quantify the likelihood of recurrence in patients with positive breast cancer, negative nodule, and ER+ treated with TMX. The study managed to identify 16 genes that were related to cancer recurrence that belonged to either of the following biological processes: proliferation (such as Ki-67 and cyclin B1), estrogen receptor (including genes such as ER and PR), HER2 (including HER2 and GRB7), and cell invasion (including genes like Stromolysin 3 and Cathepsin L2) [85]. A follow-up study was developed to investigate the ESR1 marker, which has been reported as a strong predictor of resistance to TMX in ER-positive patients with low levels of ESR1 [86].

Mihály et al. (2013) performed a meta-analysis looking for studies that evaluated expression-based markers to provide independent information regarding TMX treatment. Out of 68 biomarkers with probable connection to the TMX resistance, three (PGR, MAPT, and SLC7A5) were the most promising observed in patients treated with TMX. However, only independent markers were evaluated without making a joint analysis to investigate their main biological processes. According to our review, we can also observe that many markers remain under study [22].

In conclusion, this systematic review is the first of its kind to adopt a clustering technique to evaluate

the independent data on the literature regarding TMX resistance in ER+ breast cancer patients. This analysis revealed three networks of biomarkers involved in biological processes: cell cycle, signal transduction of proliferative stimuli, and hormone response involved in morphogenesis and differentiation of mammary gland. As expected, CLUSTER 1 corroborated that the cell proliferation pathway is strongly linked to the prediction of TMX response. Further, CLUSTER 2 and 3 indicate that there are other resistance pathways that must be thoroughly investigated as currently available data are not sufficient to reach a statistically significant conclusion. When the direct cluster neighbors are considered, CLUSTER 3 showed a strong link between TMX resistance and the development of the mammary gland and its hormone stimulation. Thus, our data found are hypothesis generators, which suggest promising mechanisms and biomarkers for predicting resistance to TMX and contributing to future studies and personalized medicine.

Abbreviations

TMX: Tamoxifen; ER+: Estrogen of receptor-positive; BC: Breast cancer; HR+: hormone receptor expression; PR+:Progesterone receptor; SERM: Selective estrogen receptor modulator; REMARK: Reporting Recommendations for Tumor Marker Prognostic; GO: Gene ontology; BiNGO: Biological Network Gene Ontology; FDR: False Discovery Rate; OS: overall survival; AURKA: Aurora Kinase A; MK167:Marker Of Proliferation Ki-67; AURKB: Aurora Kinase B; FOXM1: Forkhead Box M1; RRM2:Ribonucleotide Reductase Regulatory Subunit M2; STAT3: Signal. Transducer and Activator of Transcription 3; PTPN2: Protein Tyrosine Phosphatase Non-Receptor Type 2; FYN: protein-tyrosine kinase oncogene family; SRC: Proto-Oncogene, Non-Receptor Tyrosine Kinase; PAK2: Serine/threonine protein kinase; FRS2: Fibroblast Growth *Factor Receptor Substrate 2;* EGFR: *Epidermal growth factor receptor;* ESR 1: Estrogen Receptor 1; GATA3: Transcription factor; PGR: Progesterone Receptor; NCOR2: Nuclear Receptor Corepressor 2; EBAG9: Estrogen Receptor Binding Site Associated Antigen 9; SOX2: SRY-Box Transcription Factor 2; POU5F1: POU Class 5 Homeobox 1; BCL2: B-cell lymphoma 2; IGF1R: Insulin Like Growth Factor 1 Receptor; TGFBR2: Transforming Growth Factor Beta Receptor 2; YAP1: yes-associated protein 1;

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4.2. Artigo II:

"VAL16ALA-SOD2 POLYMORPHISM MAY BE A PREDICTOR OF RECURRENCE RISK OF BREAST CANCER IN PATIENTS TREATED WITH ADJUVANT TAMOXIFEN"

Maiquidieli Dal Berto, Aniúsca Vieira dos Santos, Giovana Tavares dos Santos, Gabriela Krüger da Costa, Pettala Rigon, Rafael José Vargas Alves, Claudia Giuliano Bica

VAL16ALA-SOD2 POLYMORPHISM MAY BE A PREDICTOR OF RECURRENCE RISK OF BREAST CANCER IN PATIENTS TREATED WITH ADJUVANT TAMOXIFEN

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ABSTRACT

Introduction: About 30% of breast cancer (BC) patients with hormonal receptor positive (HR+) show resistance to tamoxifen (TMX) during adjuvant treatment, which may result in local or distant recurrence. From previous evidences is possible to infer that TMX-sensibility could be influenced by oxidative genetic imbalance. **Objective:** To evaluate the association between genotypes of SOD2 single nucleotide polymorphism (SNP, rs4880) and risk of recurrence in patients with luminal BC using adjuvant TMX. **Material and methods:** a retrospective cohort study of patients with BC (HR + / Her2-) using adjuvant TMX was performed (n = 72). Biopsies samples from tumors previously fixed with formalin were used to perform Val16Ala-SNP real-tie PCR genotyping. Other potential apoptosis and proliferation markers were also analyzed by immunohistochemistry. The survival of women carrying different genotypes was follow-up by a minimum of 60 months and compared by Cox-regression multivariate analysis adjusted for grade, clinical staging and Bcl-2 and Ki67 markers. **Results:** 36% patients relapsed, with a mean age of diagnosis of 46 ± 12.68 years, 35% presenting histological grade 3 and 29.6% presenting clinical stage III. The genotypic frequencies of SOD2 were Ala/Ala = 33.3%, Val/Val = 36.1% and Ala/Val = 30.6%. Although it was not significant, V-allele women trended to be present a risk for BC recurrence than others (RR = 2.14 (95% CI 0.84-5.47). Patients with Val allele had a 15% reduction in relapse-free survival between one year and three years of treatment, whereas Ala /Ala this reduction was only 8%. Of the 26 patients with recurrence, 19 (73.1%) had a positive expression for the marker Bcl2 (p = 0.015), indicating a reduced number of cell apoptotic in primary tumor. **Conclusion:** Despite not being significantly significance, the presence of the Val allele in patients with BC using adjuvant TMX showed a trend to increase the risk of relapse.

Keywords: Breast cancer, relapse, Val16Ala-SOD2 polymorphism, Tamoxifen.

INTRODUCTION

BC is the most prevalent among women in both developed and developing countries (1). Due to its incidence and mortality, it is considered a serious public health around the World (2, 3). About 60 to 70% of all diagnosed BC are HR+ expression, estrogen receptor positive ER+ and/or progesterone receptor positive (PR+) (4) .

In these cases, the use of TMX is highly prevalent in order to decrease the risk of cancer recurrence, since a selective estrogen receptor modulator (SERM) composed of triphenylethylene backbone structure that block ERα actions(5, 6). A previous study showed that 5 years of adjuvant TMX could safely reduce 15-year risk of BC recurrence and death associated with this cancer (7). However, despite TMX improves survival in HR molecularly subtyped BC patients, some patients can develop pharmacological resistance (8, 9). Evidences suggested that approximately 30% of HR+ BC patients are TMX resistantresulting in recurrence and metastasis, the leading causes of death (10, 11). In this context, investigations that identify potential factors that increase resistance to TMX are relevant.

There are previous evidences suggesting that oxidative stress triggered by increase of reactive oxygen species (ROS) is an important risk to carcinogenesis by promotion of DNA mutations (12). Moreover, despite, moderate ROS concentrations are required for several biological functions including kill cancer cells (13), excess of some ROS molecules can contribute to tumor promotion and progression (14). Some *in vitro* investigations described that TMX was able to increase ROS production inducing apoptosis in BC and ovarian cancer cells (15-17).

However, an *in vitro* study performed by Tonková et al suggested that BC-TMX resistant cells obtained from MCF7 line exhibit mitochondrial dysfunction and higher superoxide levels. Therefore, authors suggested that this ROS imbalance could contributes to TMX resistance. These results corroborated previous investigations suggesting that an oxidative imbalance associated with manganese superoxide antioxidant gene (SOD2 or MnSOD) could has influence on BC risk or BC-aggressiveness. In fact, Val16Ala-SOD2 SNP (rs4800) presents two alleles (Val and Ala) resulting from a structural mutation that replaces a thymine (T) with a cytosine (C) is the most common genetic variation found in the SOD2 gene (rs4880). The substitution of valine (Val) by alanine (Ala) in codon 16, changing the valine amino acid (GTT) into alanine (GCT) (9, 10). The Val-SOD2 protein has a β-

sheet secondary structure whereas the Ala-SOD2 produces a α-helix protein structure. These protein specificities interfere directly in the SOD2-transport from cytoplasm into mitochondria that is more efficient in Ala-SOD2 than Val-SOD2 (18, 19).

Despite to be more efficient, specially women carrying Ala/Ala-genotype present higher risk of some cancer types including BC probably due increase of hydrogen peroxide (HP) levels catalyzed by SOD2 enzyme (20, 21) Val/Val-genotype seems to increase the risk of BC metastasis (22) also decreasing BC survival and relapse-free survival than Ala-alleles (23). However, it is not clear whether the Val/Val genotype that generates an increase in superoxide levels could be a causal factor associated with the risk of resistance to TMX.

In addition, apoptosis and cell proliferation markers can be great allies in this investigation. Ki-67 is present in all proliferating cells, being a non-histone nuclear protein absent only in the G0 phase of the cell cycle. It is generally used, mainly in BC, for prognosis of relative responsiveness, resistance to chemotherapy or endocrine therapy and residual risk estimation in patients on standard therapy (24, 25).

Studies suggest that estrogen promotes resistance to the drugs tamoxifen and cisplatin, increasing the proportion of Bcl-2 (26). In addition, was found increased expression of Bcl2 in Luminal A and B subtypes (Hwang (27). The pro-apoptotic Caspase-3 marker, although it has been extensively investigated, its role in breast cancer remains controversial. Some studies show that its low expression in breast cancer is related to more aggressive tumors (28, 29). In contrast, studies show that its high expression is related to a worse prognosis (30, 31).

In order to test these issues, the present study investigation potential association between the different genotypes of Val16Ala-SOD2 polymorphism and of markers of apoptosis and cell proliferation in luminal patients in relation to the failure of adjuvant therapy with TMX.

METHODOLOGY

Study design and sample

We carried out a retrospective cohort study of patients with BC (HR+/Her2-) using adjuvant TMX. This investigation was carried out with a limited and selected number of patients, considering that the cancer subtype and the various treatments could interfere in the

analysis conducted. The patients enrolled here were diagnosed and treated in a regional referral cancer center from 2008 to 2013. According to the eligibility requirements, we kept in the study only the patients who had a minimum of 60 months' follow-up data and those who had biological material available. All cases that lacked immunohistochemistry of the surgery material were excluded. Medical records were reviewed to collect clinical, pathological, and immunohistochemical information.

Genotyping

As this is a retrospective study, SOD2-genotyping was performed using biopsies samples of subjects included in the investigation. Therefore, genomic DNA was extracted from 10 micrometers of tissue slices of formalin-fixed paraffin-embedded (FFPE) BC-samples using Magnetic Method to Purify DNA from FFPE Tissue/ MagneSil® Genomic, Fixed-Tissue System (PROMEGA) kit following the manufacturer specifications. DNA sample dilution was standardized at 1:10μL for the polymerase chain reaction (PCR), and isolated DNA was subjected StepOnePlus Real-Time PCR System in final volume of 10μL, containing 1μL of DNA sample dilution, 0,25μL of probe TaqMan™ SNP Genotyping Assay, human rs:4880 (Sequence: CTGCCTGGAGCCCAGATACCCCAAA**[A/G]**CCGGAGCCAGCTGCCTGCTGGTGCT), 5μL of TaqMan™ Genotyping Master Mix and 3,75μL of H2O MilliQ. The reaction consisted of 60ºC of an initial denaturation step of 30s at 95ºC for 10min, followed by 50 cycles of 92ºC for 15s, 60ºC for 1min30s and a final extension step at 60ºC for 30s. The negative control consisted of a reaction in which water was used in the place of the DNA. Genotypes were determined for the Val16Ala-SOD2 polymorphism as Val/Val (TT), Val/Ala (TC), Ala/Ala (CC).

Immunohistochemistry

In addition to the immunohistochemical markers that are routinely performed on breast biopsies, two other emerging markets have also been investigated here by immunohistochemistry analysis. We used Anti-Bcl-2, Abcam (Rabbit Monoclonal Antibody, Clone E17, 1: 100 dilution; Control: B-cell lymphoma) and Cleaved Caspase-3 Asp174, Cell Signaling (Rabbit Monoclonal Antibody Anti-Human, 1: 100 dilution; control: human tonsil) to classify breast tumors. The expression data of the Ki67 marker were collected from the

medical records of each patient. Tissue sections were heated (60°C, 30 min), deparaffinized (two washes in xylenes), and rehydrated by successive washes in absolute ethanol and deionized water according to laboratory protocol. For immunohistochemical staining, antigen retrieval was performed at 98°C for 40 min in pH 9.0 TRIS-EDTA. After heating and cooling for 20 min, endogenous peroxidase activity was blocked by immersing the slidein hydrogen peroxide 5% in H2O (3 × 10 min). The slides were washed twice with phosphate-buffered saline (PBS) and incubated in a solution to block non-specific binding (bovine serum albumin to 1% for 1 h).

A negative control (bovine serum albumin 1%) was substituted for the primary antibody. All primary antibodies and controls were incubated for 1 h at room temperature and then submitted to temperatures of 4°C overnight. After that, the blades were kept at room temperature for 1 h, in sequence, and were washed thrice with PBS. The sections were incubated with the DAKO AdvanceTM link HRP for 40 min, then washed again with PBS. Staining was completed by incubating the specimens with 3,3'-diaminobenzidine+ substrate-chromogen for 5 min. Finally, tissue sections were washed twice with water, counterstained with hematoxylin-harris.

All cases were evaluated by a pathologist and analyzed at the microscope 200× magnification. To analyze the expression of Bcl2 were identified: negative - lack of immunostaining (no BCL-2 expression) or heterogeneous staining within tumor area, regardless of the intensity and positive-intense staining in all tumor cells (BCL-2 overexpression). Examined tissues for Caspase-3's were classifieds in two categories according to the average number of apoptotic cells: low levels of apoptosis (5 cells)/moderate levels (>5 to 10) and high levels (>10), shown in figure 1 [32,33].

Figure 1 here

Statistical analysis

The statistical analyses were conducted with SPSS version 21.0. The data on survival and causes of death were collected from hospital registries. BC-specific survival (BCSS) were assessed as the time from date of diagnosis to the date of last follow- up or death. Relapse free survival (RFS) was calculated from time of diagnosis to time of the first relapse (local relapse, contralateral BC, or metastatic disease). Variables significantly associated

with the outcome ($P \leq 0.20$) in univariate analyses were included in the multivariate analysis. The multivariate Cox regression method were applied using stepwise backward selection of variables, starting with all the eligible ones and removing those with p value greater than 0.05. The results of the regression analysis of Val16Ala-SOD2 genotypes on RFS were expressed as relative risk (RR) and confidence intervals of 95 % (CI 95 %). Chi-square ($\chi 2$) analysis was used to estimate the Hardy–Weinberg equilibrium.

RESULTS

From 460 patients BC-diagnosed were evaluated 72 patients with early-stage BC (HR +/Her2-) who only used TMX for their adjuvant treatment and that met our eligibility requirements. The clinical characteristics of the patients are shown in **table 1**.

Table 1 here

As seen in Table 1, the highest incidence of the disease among the 72 patients was between 40 and 60 years old. Grade II and the staging II was the most prevalent. The molecular subtypes Luminal A and Luminal B were estimated at 47 (65.3%) and 25 (34.7%), respectively. The frequencies of the Val16Ala polymorphism genotypes were the same in the Val / Val and Ala / Ala homozygous each with 25 (34.7%) patients. Of the 69 patient records, which informed the usage of radiotherapy treatment, 62 of them (86%) showed that the patients underwent the treatment. Conversely, of 61 patient records informing the adoption of chemotherapy, only 16 patients (22%) were subject to the treatment. Among the 72 patients, 26 had recurrence of the disease, representing a percentage of 36.1%. In addition, of the 72 patients, 13 (18 %) died.

Clinical data, genotyping and immunohistochemistry of patients with relapse and no relapse receiving adjuvant TMX therapy for at least five years of follow-up are available in **table 2.**

Table 2 here

The mean age of patients who had a recurrence was 46.1 ± 12.68. Patients with tumors of grade III, stage III had an increased risk of relapse with p= 0.011 and p = <0.001,

respectively. All patients who underwent chemotherapy had recurrence of the disease (p = 0.001). Patients with luminal subtype A (34/73.9%) had a lower chance of recurrence p = 0.037. In the analysis of the Val16Ala polymorphism, the genotypes had little difference between them and when analyzed in relation to relapse and non-relapse patients. Of the 26 patients with recurrence, 19 (73.1%) had a positive expression for the marker Bcl2 (p = 0.015), indicating a reduced number of cell apoptosis in these patients. The other markers were not significant in our group of patients.

Survival analysis revealed a lower recurrence-free survival for ER + patients with at least one Val allele. In the multivariate analysis adjusted for Grade, staging, Bcl2 and Ki67, even without significance, a trend for increased relapse risk RR = 2.14 (95% CI 0.84-5.47) was shown in patients with Val allele in comparison to patients with Ala/Ala genotype (**Figure 2**).

Figure 2 here

According to the results, in approximately three years of treatment, 84% of patients with Ala/Ala-genotype in comparison with 72% of patients with Val genotype did not relapse. After 5 years, patients with the Ala / Ala genotype that did not relapse were 72% while the patients with the Val genotype that did not relapse were 63.8%. Moreover, patients with Val allele had a 15% reduction in relapse-free survival between one year and three years of treatment, whereas patients with Ala / Ala had an 8% reaction in relapse-free survival.

DISCUSSION

The biological role of the SOD2 enzyme in healthy cell metabolism is unquestionable because under oxidative stress conditions this enzyme is considered the first mitochondrial line of defense against oxidative damage by removing excessive superoxide anion [34,35].

In our study we observed a reduced relapse-free survival in TMX-treated ER+ patients with at least one Val allele of the Val16Ala polymorphism, compared to individualswith Ala/Ala genotype, which suggests a strong association of the polymorphism and the effect of endocrine therapy with TMX.

The role of this enzyme in cancer cells is not fully understood and several conflicting results have been published in the literature, including genetic investigations of the Val16Ala-SOD2 polymorphism. Cheng et al. (2009) found a significantly higher risk of

developing esophageal squamous cell carcinoma in individuals with the Ala allele compared with those with Val allele [36]. The same result was found in cisplatin-treated breast cancer [37]. However, other studies have found an association with the Val allele and an increased risk of disease progression and death in lung cancer and BC [38,39].

Beside its action on estrogen-induced signaling, TMX has also some beneficial effects for tumors with low ERα2 levels [40,17]. Studies have shown that TMX is able to facilitate apoptosis in non-ER expressing cancer cells [15,41,42] such as pancreatic cancer, malignant glioma and melanoma [43,44].

Bekele et al. (2016) showed a relationship between cancer cell oxidative response and TMX treatment. Oxidative damage caused by TMX had a positive effect in therapy by killing cancer cells. On the other hand, the authors realized that the accumulation of TMX generates an increase in the levels of erythroid nuclear factor 2 (NRF2), which activates the antioxidant response element leading to the expression of antioxidant genes, which will protect cells from further oxidative damage, producing resistance to the continued therapeutic effects of TMX [17]. These findings corroborate with ours data presented in figure 1, showing that the longer the use, the greater the number of patients with disease progression. To the best of our knowledge, we have found a single study evaluating Val16Ala polymorphism in ER + patients treated exclusively with TMX [23]. However, their data were different from ours, presenting a better prognosis for patients with Val allele.

Our hypothesis regarding the Val allele association with patients a shorter disease-free survival compared to Ala/Ala is that the TMX that is retained in the lipid bilayer produces superoxide ($O_2^{\bullet-}$) free radicals [17]. That is, the Ala/Ala genotypes of the Val16Ala polymorphism have a super efficiency of the enzyme that acting directly of the hydrogen peroxide anion (H_2O_2), serving as a substrate for the CAT and GPX enzyme that generate water and oxygen. On the other hand, despite its reduced action of up to 30% in relation to Ala, the Val/Val genotype acts directly on the radical $O_2^{\bullet-}$ [45,18]. Thereby, the prolonged action of TMX generates an accumulation of $O_2^{\bullet-}$, that can increase the activity of the SOD2 enzyme, especially for patients with the Val allele, which acts directly on the superoxide, and so increasing the chances of TMX failure.

Our hypothesis is similar by Cho et al., (2013), whose model was created to reverse the resistance to TMX by silencing the enzyme SOD2. TMX generates an increase in superoxide production and this increase will generate the activation of the SOD2 enzyme, protecting tumor cells from apoptosis. By creating the model, they were able to silence the

enzyme and restore TMX-induced cell apoptosis as well as significantly suppress growth in vivo [10].

As a complementary analysis, we evaluated the expression of proteins involved in apoptosis and cell proliferation. In which, was found no correlation of the Ki-67 marker with recurrence. As it is an important marker to classify luminal A (ER-and/or PR-positive/HER2-negative /14 %< Ki-67) and luminal B (ER- and/or PR-positive/HER2-negative/ 14% Ki-67) subtypes, we used it in the multivariate analysis of disease-free survival [46,47].

A compromised apoptotic mechanism allows cancer cells to survive and promote tumor proliferation and invasion. In addition, tumor cells can prevent apoptotic mechanisms, acquiring resistance against treatments [48,49]. Bcl2-positive individuals had worse disease-free survival, corresponding previous studies that found tumor-suppressing effects on stomach, lung and intestinal-type gastric cancer [50-52] and other study that suggests that estrogen promotes resistance to tamoxifen and cisplatin, by increasing Bcl-2 [26].

The Caspase-3 marker was not significant in our analyzes. However, the role of Caspase-3 remains is controversial in the literature. An in vitro investigation has shown that restoring Caspase-3 expression can sensitize doxorubicin-induced apoptosis, suggesting that caspase-3 deficiency may be a mechanism of chemoresistance [31]. Zohny et.al. (2019) found the same relationship patients with malignant breast cancer had low Capase-3 activity compared to patients with benign breast cancer [28]. Contrary to these results, a study found a relationship between low Caspase-3 expression and a better prognosis [30]. For further clarification, the ideal would be to test in other groups of patients or in a larger group.

In conclusion, this study emphasizes the importance of the oxidative response in cancer cells in the treatment with TMX. On the other hand, the continued use of this drug activates antioxidant enzymes that will protect neoplastic cells from cell death, instigating a double action of the SOD2 enzyme. In normal cells it fights oxidative damage, protecting cells from future undergoing changes, whereas in the tumor it seems to prevent the apoptosis of neoplastic cells from occurring, which facilitates resistance to TMX. Although our study did not show statistically significant results, it did show a strong trend that can be considered a hypothesis generator. It presented another TMX action path that can be measured according to the different genotypes of the Val16Ala-SOD2 polymorphism acquired from each patient. It can be explored in larger studies and extended to other BC subtypes.

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COMPLIANCE WITH ETHICAL STANDARDS

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FIGURE LEGENDS

Fig. 1 Markers expression A) Bcl2 negative and positive B) Caspase-3 low levels of apoptosis (5 cells)/moderate levels (>5 to 10) and high levels (>10). Original magnification x200.

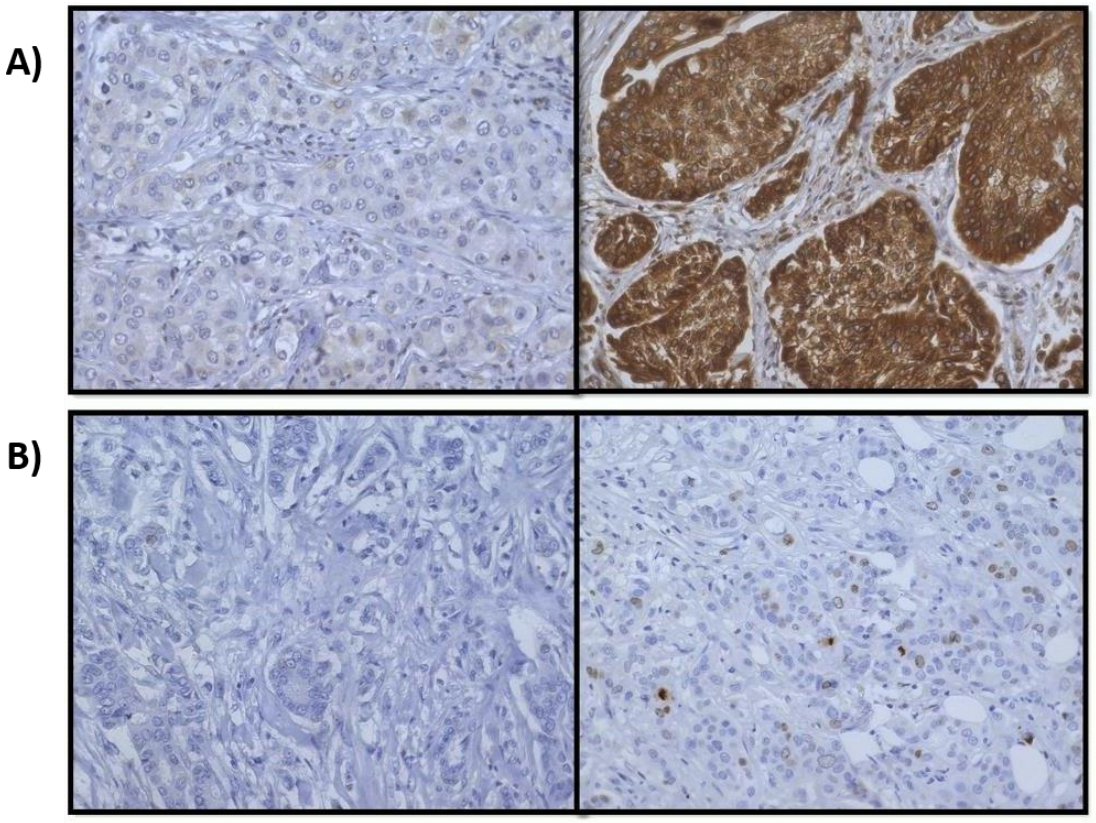

Fig. 2 Risk analysis of recurrence in clinical with breast cancer. Cox-regression multivariate analysis (backward wald) adjusted for grade, clinical staging and BCL-2 and KI 67 markers. The presence of the Val allele shows a risk trend for recurrence (RR = 2.14 (CI 95% 0.84- 5.47).

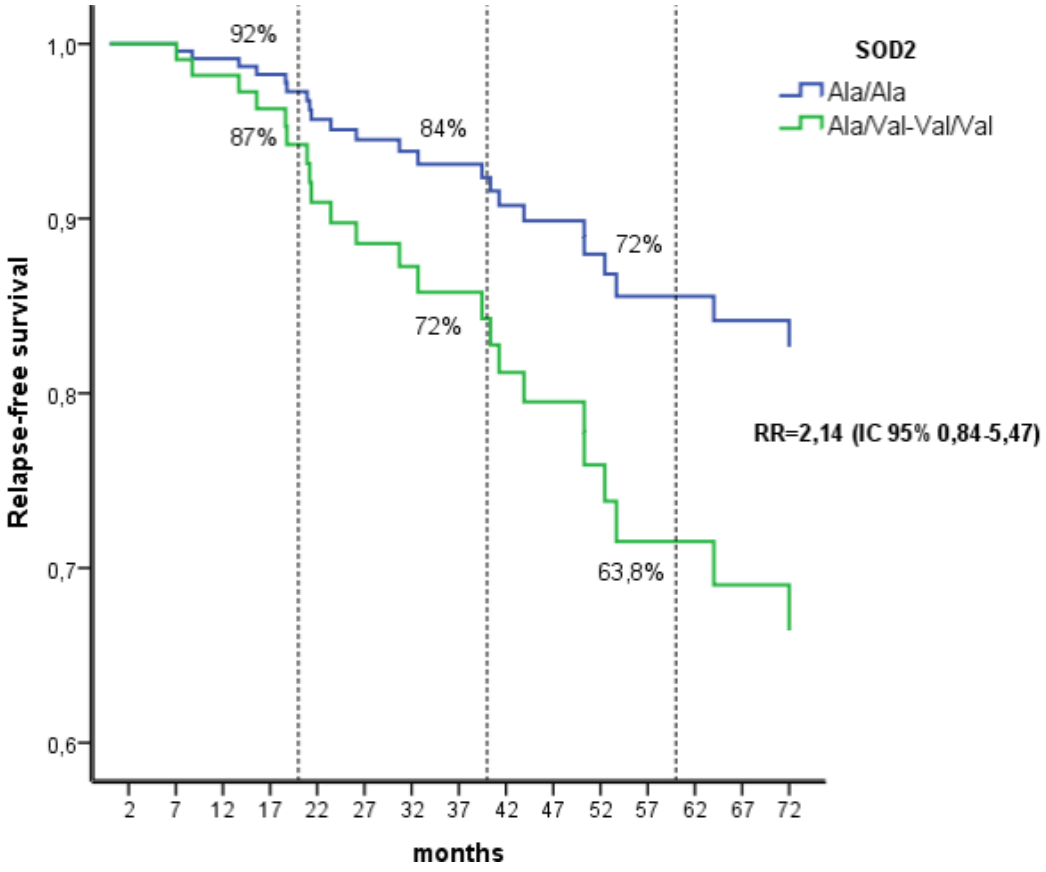

Table 1 – Clinical and pathological characteristics of patients.

Clinical profile	n (%)
Age (years)	
≤ 40	32/72 (44)
40 to 60	30/72 (42)
≥ 60	10/72 (14)
Tumoral grade	
I	12/72 (16.7)
II	35/72 (48.6)
III	25/72 (34.7)
Clinical staging	
I	19/71 (26.8)
II	31/71 (43.6)
III	21/71 (29.6)
Molecular subtype	
Luminal A	47/72 (65.3)
Luminal B	25/72 (34.7)
Frequency of the genotypes	
Ala/Ala	24/72 (33.3)
Val/Val	26/72 (36.1)
Ala/Val	22/72 (30.6)
Radiotherapy	
Yes	62/69 (86.1)
No	7/69 (9.7)
Chemotherapy	
Yes	54/70 (77.1)
No	16/70 (22.9)
Relapse	
Yes	26/72 (36.1)
No	46/72 (63.9)
Death	
Yes	13/72 (18.1)
No	59/72 (81.9)

Table 2. Characterization of the patients in relation to the relapse.

Sample characteristics	**No relapse (n=46)**	**With relapse (n=26)**	**P value***
Profile clinical			
Age at diagnosis (mean ± SD)	47.0 ±12.24	46.1±12.68	
Grade III	11 (23.0%)	14 (56.0%)	0.011*
Staging III	5 (10.9%)	16 (64.0%)	<0.001*
Radiotherapy	39 (87.0%)	23 (92.0%)	0.656
Chemotherapy	28 (59.0 %)	26 (100%)	<0.001*
Death	1 (2.2 %)	12 (46.2%)	<0.001*
Molecular subtype			
Liminal A	34 (73.9 %)	13 (50.0 %)	0.037*
Luminal B	12 (26.1 %)	13 (50.0 %)	0.037*
Genotypes			
Ala/Ala	17 (37.0%)	7 (26.9%)	0.274
Ala/Val	13 (28.3%)	9 (34.6%)	0.380
Val/Val	16 (34.8%)	10 (38.5%)	0.475
Immunohistochemistry			
Bcl2	20 (43.5 %)	19 (73.1 %)	0.015*
Caspase 3	5 (11.1%)	5 (22.7)	0.193
Ki67 >14	14 (30.4%)	13 (50.0%)	0.082

* SD standard deviation; rs4880 - genotype was available for 72 patients. Expression of Bcl2: positive; Expression Caspase-3

5. CONCLUSÕES

O presente estudo faz parte de um projeto maior intitulado “Estudo dos marcadores tumorais no câncer de mama”, aprovado pelos Comitês de Ética em Pesquisa (CEP) da UFCSPA (Parecer N° 066/2018) e do ISCMPA (Parecer Nº 525.170). Deste projeto maior estão sendo desenvolvidos diversos outros trabalhos no âmbito de iniciação cientifica, mestrado e doutorado.

O Serviço de Mastologia do Hospital Santa Rita da ISCMPA é um centro de referência no tratamento de pacientes com câncer de mama, assim como o serviço de Patologia, na excelência do diagnóstico. A parceria para o desenvolvimento deste trabalho teve início em 2016. Primeiramente foi feito a captação das pacientes, seguido da busca das informações clinicas e de tratamento e por fim, da busca do material biológico.

O vínculo entre um hospital de referência e uma universidade (UFCSPA), é extremamente promissor para a pesquisa. Com isso, a partir desse acolhimento, nosso grupo se propôs a aprimorar os conhecimentos na linha de marcadores tumorais preditivos de resistência ao TMX em pacientes com câncer de mama luminal, e assim, elucidar mecanismos e biomarcadores que possam estar envolvidos neste processo.

Como resultado desta pesquisa, o Artigo I, encontrou diversos biomarcadores relacionados a resistência ao TMX. No qual, alguns deles se mostraram estar interligados através de processos biológicos como, ciclo celular, transdução de sinal de estímulos proliferativos e resposta hormonal. Com isso, ressalta-se a importância de quanto mais alvos moleculares forem investigados em conjunto, mais representativo será da realidade e assim, maiores serão as chances de encontrar mecanismos fortemente envolvidos na predição da resistência ao TMX.

O Artigo II, foi selecionado 72 pacientes, diagnosticados com câncer de mama e que fizeram exclusivamente uso do TMX na terapia adjuvante. Sendo que destes, 36%

apresentaram recidiva da doença. Na nossa análise, a presença do alelo Val mostrou uma tendência a ser um fator de risco preditivo para resistência do TMX. Assim, o TMX além de agir como antagonista do RE, também pode ter sua ação através de danos oxidativos, no qual, ele produz superóxido para matar as células neoplásicas, agindo independentemente da ligação do RE. No entanto, conseguimos perceber que ao longo do tratamento está ação se torna prejudicial, a exposição prolongada de superóxido ativa as enzimas antioxidantes, que estas, vão proteger as células neoplásicas de apoptose. Com isso, pacientes portadores do alelo Val terão uma atividade da enzima antioxidante mais elevada, pois, o Val atua diretamente no superóxido. Aumentando as chances de risco de resistência das células neoplásicas ao TMX.

Contudo, mais investigações da relação dos genótipos do polimorfismo Val16Ala-SOD2 são necessárias, principalmente em estudos maiores. Os dados encontrados são geradores de hipótese, tanto do artigo I quanto do artigo II, que sugerem mecanismos e biomarcadores promissores para predição de resistência ao tratamento com TMX. Contribuindo para uma medicina mais personalizada e consequentemente, no aumento da sobrevida dos pacientes.

6. TRAJETÓRIA DOUTORADO

Em 2016 iniciei meu doutorado, voltado para a linha de pesquisa de marcadores tumorais, com ênfase em mecanismos de resistência para o tratamento adjuvante com tamoxifeno. Neste primeiro ano foi realizado a captação das pacientes no ambulatório de mastologia do hospital Santa Rita. Junto a isso, realizei pesquisas com o laboratório de Biogenômica da UFSM, no qual, foi publicado o artigo:

- AZZOLIN, VERÔNICA FARINA ; CADONÁ, FRANCINE CARLA ; MACHADO, ALENCAR KOLINSKI ; **BERTO, MAIQUIDIELI DAL** ; DORNELLES, EDUARDO BORTOLUZZI ; GLANZNER, WERNER GIEHL ; GONÇALVES, PAULO BAYARD ; BICA, CLAUDIA GIUGLIANO ; DA CRUZ, IVANA BEATRICE MÂNICA . Superoxide-hydrogen peroxide imbalance interferes with colorectal cancer cells viability, proliferation and oxaliplatin response. Toxicology in Vitro[JCR], v. 32, p. 8-15, 2016.

Também fui colaboradora do laboratório EPICLIN de 2016-2017. Participando desde a fase inicial (compra dos equipamentos, reagentes; organização e logística do laboratório), até o recebimento e processamento das amostras que chegavam de todo o Brasil. Do qual foi publicado o artigo:

- WENDLAND, E. M. ; VILLA, L. L. ; UNGER, E. R. ; DOMINGUES, C. M. ; BENZAKEN, A. S. ; MARANHAO, A. G. K. ; KOPS, N. L. ; BESSEL, M. ; CAIERAO, J. ; HOHENBERGER, G. F. ; HORVATH, J. D. ; Santos, G.T. ; MELLO, B. P. ; SANTOS, A. V. ; **Dal Berto, M.** ; BICA, C. G. ; PEREIRA, G. F. M. ; MORENO, F. C. . Prevalence of HPV infection among sexually active adolescents and young adults in Brazil: The POP-Brazil Study. Scientific Reports, v. 10, p. 4920, 2020

Em 2017, fiz a coleta do material biológico das pacientes que assinaram o Termo de Consentimento Livre e Esclarecido e a coleta dos dados clínicos e de acompanhamento do tratamento. Concomitante a isso, outros estudos foram publicados:

- FLORES, E. R. ; **DAL BERTO, M.** ; CADONÁ, FRANCINE CARLA ; MACHADO, ALENCAR KOLINSKI ; Santos, G.T. ; DA CRUZ, IVANA BEATRICE MÂNICA ; MARRONE, F. B. ; GRAZIOTTIN, T. ; BICA, C. G. . Effect of guaraná extract (Paullinia cupana), an amazonian fruit richest in caffeine on human bladder cancer cell line. REVISTA AMAZONENSE DE GERIATRIA E GERONTOLOGIA, v. 8, p. 88, 2017.
- Santos, G.T. ; **Dal Berto, M.** ; CRUZ, I. B. M. ; ROEHE, A. V. ; BRACKMANN, R. L. ; REITER, K. C. ; BICA, C. G. . Impact of Her-2 Overexpression on Survival of Patients with Metastatic Breast Cancer. Asian Pacific Journal of Cancer Prevention[JCR], v. 18, p. 2673-2678, 2017.

E trabalho apresentado:

- Augusto Marques Moreira, **Maiquidieli Dal Berto**, Tales Shinji Sawakuchi Minei, Nathalia Trindade Cardozo, Giovana Tavares dos Santos, Claudia Giuliano Bica. Projeto de Iniciação Científica, Mostra UFCSPA – 2017. **Associação das características clinicopatológicas na agressividade do câncer de mama em pacientes jovens.**

A parte laboratorial iniciou em 2018, com testes de imunohistoquimica, extração de DNA e genotipagem (PCR-real time). Também foi iniciado a produção do artigo de revisão de escopo I. Além disso, foram apresentados trabalhos na IV Mostra de trabalhos de Ensino, Pesquisa e Extensão da Universidade Federal de Ciências de Saúde de Porto Alegre:

- Maria Eduarda Wenczenovicz, **Maiquidieli Dal Berto**, Giovana Tavares dos Santos, Gabriela Krüger da Costa, Rafael José Vargas Alves. Orientadora: Claudia Giuliano Bica**. Características clinicas, demográficas e imunohistoquimicas de pacientes com recidiva de câncer de mama no uso de Tamoxifeno adjuvante.** Ganhando como Destaque.
- Raquel dos Santos Ramos, Giovana Tavares dos Santos, **Maiquidieli Dal Berto**, Camila Tombini da Silveira, Rafael José Vargas Alves, Claudia Giuliano Bica. **Análise de biomarcador associado a dormência do câncer de mama.**
- Tatiane Andressa Gasparetto, Giovana Tavares dos Santos, **Maiquidieli Dal Berto**, Camila Tombini da Silveira, Rafael José Vargas Alves, Claudia Giuliano Bica. **Análise imunoístoquímica associada a metástase pulmonar do câncer de mama.**

E no congresso do Hospital Santa Rita:

- Gabriela Krüger da Costa**, Maiquidieli Dal Berto,** Giovana Tavares dos Santos, Maria Eduarda Wenczenovicz, Rafael José Vargas Alves, Claudia Giuliano Bica. **Características clínicas e demográficas dos pacientes com recidiva de câncer de mama na vigência de tamoxifeno adjuvante em um ambulatório especializado de um centro de referência do sul do brasil.**
- Gabriela Krüger da Costa; **Maiquidieli Dal Berto**; Fernanda Barbisan; Verônica Azzolin; Giovana Tavares dos Santos; Ivana Beatrice Mânica da Cruz; Claudia Giuliano Bica. **Efeito do polimorfismo val16ala-sod2 no desequilíbrio do câncer de próstata.**

E outros trabalhos foram publicados:

- DA CRUZ, IVANA BEATRICE MÂNICA ; MOTTA, J. R. ; LENZ, A. F. ; FLORES, T. G. ; AZZOLIN, VERÔNICA FARINA ; **DAL BERTO, M.** ; MACHADO, ALENCAR KOLINSKI ; RIBEIRO, E. A. M. ; RIBEIRO, E. E. ; BARBISAN, F. **The antioxidant effect of Brazil nut (Bertholletia excelsa) is influenced by a genetic superoxidehydrogen peroxide imbalance in healthy humans.** Journal of Nutritional Health & Food Engineering, v. 8, p. 244-251, 2018.
- BARBISAN, F. ; PRADO-LIMA, P. A. S. ; AZZOLIN, VERÔNICA FARINA ; **DAL BERTO, M.** ; BICA, C. G. ; TEIXEIRA, C. F. ; CAPELETO, D. ; JUNG, I. E. C. ; RIBEIRO, E. E. ; DUARTE, M. M. M. F. ; CRUZ, I. B. M. **Antidepressant Drugs Modulate Differentially Anti-inflammatory Lithium?s Property: An in Vitro and in Vivo Study.** JOURNAL OF PHARMACY AND PHARMACOLOGY, v. 6, p. 287-304, 2018.

Em 2019 a parte laboratorial foi finalizada e as análises estatísticas e escrita do artigo II foram iniciadas. Dados preliminares foram apresentados nos congressos: *Next Frontiers To Cure Cancer* (A.C. Camargo), XXI Congresso Brasileiro de Oncologia Clínica e Congresso UFCSPA: conectando saúde e sociedade.

Durante os meus 4 anos de doutorado, além de trabalhar no meu projeto, tive a oportunidade de atuar em diversas áreas e grupos de pesquisa. Participei de orientações e coorientações de trabalhos de conclusão de curso, apoio e treinamento de bolsistas de iniciação cientifica e extensão. Além de atividades de docência com aulas e seminários em disciplinas de graduação e residência e cursos de Biossegurança.

Fui membro do Grupo de Pesquisa Translacional em Oncologia (GPTO) e colaboradora do projeto de extensão Mulheres em Ação. Atuando nas comunidades, empresas e universidade.

7. APÊNDICES

7.1. Parecer do Comitê de Ética da UFCSPA

-------- Mensagem encaminhada --------
Assunto: Registro de projeto de pesquisa
Data: Fri, 17 Aug 2018 17:46:20 -0300
De: compesq@ufcspa.edu.br
Para: claudia@ufcspa.edu.br

Prezado(a),

Informamos que o projeto de pesquisa intitulado 'Estudo dos marcadores tumorais no câncer de mama' foi registrado na Comissão de Pesquisa da UFCSPA sob o número 066/2018.

Atenciosamente,

ComPesq - UFCSPA

•••

7.2. Parecer do Comitê de Ética da ISCMPA

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Plataforma Brasil

Continuação do Parecer: 525.170

Objetivo Secundário:
descrever o perfil sócio-demográfico das pacientes com câncer de mama;
verificar os fatores ambientais, hormonais e hereditários das pacientes com câncer de mama;
comparar os fatores ambientais, hormonais e hereditários das pacientes no subtipo triplo-negativo e não triplo-negativo;
#estudar marcadores tumorais relacionados ao subtipo triplo negativo;
correlacionar a expressão dos marcadores tumorais ao tamanho tumoral, tipo histológico, TNM;
associar os marcadores tumorais ao prognóstico e sobrevida das pacientes com câncer de mama;
correlacionar à expressão dos marcadores tumorais com o risco câncer de mama metastático;
identificar marcadores do tumor primário e do sítio metastático, e correlacionar
sua expressão à taxa de sobrevida;
correlacionar os marcadores com os possíveis sítios de metástases e verificar o valor preditivo no desenvolvimento das metástases;
acompanhar a evolução e o desfecho da doença nas pacientes por 5 anos.

Avaliação dos Riscos e Benefícios:
Riscos: Os riscos associados a esta pesquisa são coleta de sangue, onde poderá haver dor ou incomodo decorrente da punção venosa e complicações rotineiras, como deslocamento da agulha, extravasamento de sangue, podem ocasionar algum desconfortos e hematomas.

Benefícios: As pacientes não terão nenhum benefício imediato com esta pesquisa, entretanto os dados científicos gerados contribuirão para o maior conhecimento desta importante neoplasia que acomete mulheres em todo o mundo.

Comentários e Considerações sobre a Pesquisa:
A proposta da presente pesquisa apresenta dois desenhos, subdivididos de acordo com a metodologia a ser adotada, sendo um estudo transversal e um estudo de coorte prospectiva.

Considerações sobre os Termos de apresentação obrigatória:
Foi apresentado uma declaração de responsabilidade de utilização dos materiais biológicos que expresse um especial cuidado para não exterminar com a amostra durante os estudos.

Recomendações:
Sem comentários adicionais.

Endereço: R. Profº Annes Dias,285 Hosp.Dom Vicente Scherer
Bairro: 6º andar - Centro **CEP:** 90.020-090
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo dos marcadores tumorais no câncer de mama.
Pesquisador: Claudia Giuliano Bica
Área Temática:
Versão: 2
CAAE: 22396713.1.0000.5335
Instituição Proponente: Irmandade da Santa Casa de Misericordia de Porto Alegre - ISCMPA
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 525.170
Data da Relatoria: 22/01/2014

Apresentação do Projeto:
A proposta da presente pesquisa apresenta dois desenhos, subdivididos de acordo com a metodologia a ser adotada, sendo um estudo transversal e um estudo de coorte prospectiva. O câncer de mama trata-se de uma doença heterogênea que possui uma etiologia complexa. Nos últimos anos o câncer de mama foi considerado um problema de saúde pública, sendo o segundo tipo de neoplasia mais frequente no mundo e o de maior frequência entre as
mulheres. As alterações no estilo de vida, bem como a adoção de hábitos não saudáveis da população, podem ajudar a responder o aumento de casos de câncer na população mundial. O advento da tecnologia de microarranjos de DNA complementar, com a análise paralela de milhares de genes, tem permitido correlacionar perfis de expressão gênica dos cânceres de mama com a evolução clínica das pacientes e com as respostas às terapias utilizadas. Painéis imunohistoquímicos têm sido propostos para a identificação desses subtipos, buscando reproduzir com certa aproximação os perfis de expressão gênica. Essas descobertas têm proporcionado importantes informações prognósticas e preditivas e uma melhor percepção sobre os complexos mecanismos biológicos da tumorigênese. Tamanho da amostra de 1320 pacientes.

Objetivo da Pesquisa:
Objetivo: Investigar marcadores preditivos do câncer de mama.

Endereço: R. Profº Annes Dias,285 Hosp.Dom Vicente Scherer
Bairro: 6º andar - Centro **CEP:** 90.020-090
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Conclusões ou Pendências e Lista de Inadequações:

Após análise do projeto acima descrito, recomenda-se aprovar. Os demais itens atendem a Resolução 466/2012 do CNS/MS.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Após reavaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer.

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA. Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 - Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

PORTO ALEGRE, 10 de Fevereiro de 2014

Assinador por:
Claudio Teloken
(Coordenador)

Endereço: R. Profº Annes Dias,285 Hosp.Dom Vicente Scherer
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