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ANÁLISE DOS POLIMORFISMOS POR DELEÇÃO DE *GSTM1* E *GSTT1* E NÍVEIS DE GLUTATIONA EM PACIENTES COM CARCINOMA MAMÁRIO: SUSCETIBILIDADE E PROGNÓSTICO

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LISTA DE ABREVIATURAS E SIGLAS

CDI	Carcinoma ductal invasivo
CDIS	Câncer ductal in situ
Ceonc	Hospital do Câncer
CLIS	Câncer lobular in situ
CYP1A1	gene 1A1 do citocromo P450
DNA	Ácido desoxirribonucleico
GSH	Glutationa reduzida
GST	Glutationas-S-transferase
GSTM1	Glutationa-s-transferase mu 1
GSTT1	Glutationa-s-transferase theta 1
HER2	Receptor do fator de crescimento epidermal humano 2 (Human epidermal growth factor receptor 2)
IARC	Agência Internacional para Pesquisa em Câncer
IMC	Índice de massa corpórea
INCA	Instituto Nacional do Câncer
Ki67	Índice de proliferação
LEAP	Laboratório de Estudos e Aplicações de Polimorfismo de DNA
NATs	N-acetiltransferases
OR	Odds Ratio
pb	Pares de base
PCR	Reação em cadeia da polimerase
RE	Receptor de estrógeno
RP	Receptor de progesterona
rpm	Rotações por minuto
SDS	Duodecil Sulfato de Sódio
SRQ	Self report questionaire
TCA	Ácido Tricloroacético
TCLE	Termo de Consentimento Livre e Esclarecido
TNM	Estadiamento Tumor-Nódulo-Metástase
UICC	União Internacional de Controle ao Câncer

Análise dos polimorfismos por deleção de *GSTM1* e *GSTT1* e níveis de glutationa em pacientes com carcinoma mamário: suscetibilidade e prognóstico

Resumo

O câncer de mama é uma doença heterogênea e de incidência mundial, sendo considerado uma questão de saúde pública. Os fatores externos ambientais, genericamente denominados de xenobióticos, impactam diretamente no desenvolvimento e no curso dessa doença. A metabolização de xenobióticos e de seus subprodutos garante a eliminação de compostos que poderiam ser danosos ao organismo e essa ação é desempenhada, também, pelas enzimas glutationas-S-transferases (GSTs). Os genes glutationa-s-transferase mu 1 (*GSTM1)* e glutationa-s-transferase theta 1 (*GSTT1)* pertencem às classes mu e theta, respectivamente. Indivíduos com variantes polimórficas das GSTs possuem menor eficiência na detoxificação de metabólitos provenientes de drogas ou carcinógenos e, consequentemente, esses indivíduos podem ser mais suscetíveis ao desenvolvimento de doenças como o câncer. Nesse sentido, o presente trabalho visou avaliar os polimorfismos por deleção de *GSTM1* e *GSTT1* e os níveis glutationa em pacientes com câncer de mama e suas correlações clinicopatológicas. Participaram do estudo 121 mulheres com câncer de mama ductal invasivo (CDI) e 151 mulheres livres de neoplasia. A extração do DNA das pacientes foi feita por *Salting out* e das mulheres controle pelo Kit de Extração Mini Spin Plus (BioPur, Curitiba, Paraná, BR). A análise dos polimorfismos foi realizada usando a técnica de Reação da polimerase em cadeia (PCR) Multiplex. As dosagens de glutationa foram realizadas com o reagente de Ellman, a partir das alíquotas de plasma das pacientes. De acordo com o estudo caso-controle, os polimorfismos *GSTT1* e *GSTM1* parecem não ser candidatos a marcadores de suscetibilidade ao câncer de mama. Além disso, eles não se mostraram marcadores prognósticos ao serem correlacionados com parâmetros da doença. Resultados significativos foram obtidos entre a presença de *GSTT1* e uma concentração reduzida de GSH em pacientes com histórico de estresse crônico. Também houve significância entre o genótipo duplo positivo e um aumento de GSH na presença de embôlos angiolinfáticos, bem como significância entre o genótipo duplo deletado e um alto valor de ki67 (> 14%). Estes resultados mostram que GSH

tem um comportamento diferente na presença ou ausência desses genes. Com esse trabalho espera-se poder contribuir para o desenvolvimento de estudos adicionais, levando em consideração a necessidade de estabelecer novos marcadores para essa neoplasia, dada a sua considerável prevalência entre as mulheres.

Palavras-chave: câncer de mama, polimorfismo genético, GSTs, xenobióticos, glutationa reduzida.

Analysis of *GSTM1* and *GSTT1* deletion polymorphisms and glutathione levels in patients with breast carcinoma: susceptibility and prognosis

Abstract

Breast cancer is a heterogeneous disease of worldwide incidence and is considered a public health issue. External environmental factors, generically called xenobiotics, have a direct impact on the development and progression of this disease. The metabolization of xenobiotics and their byproducts ensures the elimination of compounds that could be harmful to the body and this action is also performed by the glutathione-S-transferase enzymes (GSTs). The genes glutathione transferase mu 1 (*GSTM1*) and glutathione transferase theta 1 (*GSTT1*) belong to the mu and theta classes, respectively. Individuals with polymorphic variants of GSTs are less efficient in detoxifying metabolites from drugs or carcinogens and, consequently, these individuals may be more susceptible to the development of diseases such as cancer. Therefore, this study aimed to evaluate polymorphisms by deletion of *GSTM1* and *GSTT1* and glutathione levels in patients with breast cancer and their clinicopathological correlations. A total of 121 women with invasive ductal breast cancer (CDI) and 151 women free of neoplasia participated in the study. DNA extraction of patients was performed by Salting out and of control women by the Mini Spin Plus Extraction Kit (BioPur, Curitiba, Paraná, BR). Polymorphism analysis was performed using the Multiplex polymerase chain reaction (PCR) technique. Glutathione dosages were performed with Ellman's reagent from the patients' plasma aliquots. According to the case-control study, the *GSTT1* and *GSTM1* polymorphisms do not seem to be candidates for markers of susceptibility to breast cancer. In addition, they did not show prognostic markers when correlated with disease parameters. Significant results were obtained between the presence of *GSTT1* and a reduced concentration of GSH in patients with a history of chronic stress. There was also significance between the double positive genotype and an increase in GSH in the presence of angiolymphatic emboli, as well as significance between the double deleted genotype and a high ki67 value (> 14%). These results show that GSH has a different behavior in the presence or absence of these genes. This study is expected to contribute to the

development of additional studies, taking into account the need to establish new markers for this neoplasm, given its considerable prevalence among women.

Keywords: breast cancer, genetic polymorphism, GSTs, xenobiotics, reduced glutathione.

SUMÁRIO

1. INTRODUÇÃO

O câncer é uma doença heterogênea e de incidência mundial, sendo considerado uma questão de saúde pública. Conforme mostra o panorama da Agência Internacional para Pesquisa em Câncer (IARC), para 2018, estimou-se 18,1 milhões de novos casos e 9,6 milhões de óbitos. Ressalta-se, que o carcinoma mamário está em segundo lugar no ranking de causas de morte por câncer no mundo, com 11,6% do total, seguido pelo de próstata em terceiro, com 7,1% (Bray *et al.*, 2018). Segundo dados do Instituto Nacional do Câncer (INCA), no Brasil, há estimativas de aproximadamente 60 mil novos casos de neoplasia mamária para os anos de 2018 e 2019. Além disso, desconsiderando os tumores de pele não melanoma, esse é o tipo mais incidente na região Sul do país, revelando um risco estimado de 73,07 casos a cada 100 mil mulheres (Inca, 2017).

Assim como os demais tipos de câncer, o desenvolvimento do carcinoma mamário é complexo e é influenciado não somente pela genética, mas também por variáveis ambientais como o estilo de vida, idade (Anders *et al.*, 2009; Fredholm *et al.*, 2009), fatores hormonais (Yerushalmi et al., 2010), menopausa (Ahmed *et al.*, 2016), tabagismo (Catsburg *et al.*, 2015), exposição à radiação ionizante (Boice *et al.*, 1991) e sobrepeso (Sebastiani *et al.*, 2016).

Entre esses fatores, destaca-se a idade ao diagnóstico sendo mais incidente em mulheres acima de 50 anos de idade. Quando diagnosticado em mulheres jovens essa neoplasia pode ser mais agressiva e com prognóstico menos favorável nesses casos se comparadas àquelas diagnosticadas no grupo de mulheres com mais idade (Anders *et al.*, 2009; Fredholm *et al.*, 2009). A menopausa tardia (após os 55 anos de idade) também consiste em um fator de risco importante, devido a maior exposição hormonal que essa mulher sofre (Winters *et al.*, 2017).

Fatores externos como o histórico de estresse emocional crônico tem sido associados ao desenvolvimento do câncer de mama (Kornblith *et al.*, 2001). Além disso, em estudo brasileiro, foi demonstrado que esse parâmetro estava associado a uma coorte de pior prognóstico, caracterizados por presença majoritária de superexpressão do Receptor do fator de crescimento epidermal humano 2 (HER2) e obesidade (Cormanique *et al.*, 2015).

A carcinogênese é acompanhada de diversos fatores desencadeantes como o acúmulo de mutações celulares que marcam o início de uma neoplasia, levando a manutenção da proliferação celular, inibição dos supressores de crescimento, resistência à apoptose, aumento da angiogenêse e capacidade em realizar metástase (Hanahan e Weinberg, 2000). O desenvolvimento dessa patologia também é influenciado pela evasão ao sistema imunológico e pelo conjunto de fatores que regulam o metabolismo energético do indivíduo, mostrando que o microambiente em que o tumor está inserido é crucial para o entendimento do desenvolvimento e progressão dessa doença (Hanahan e Weinberg, 2011).

Para entender a origem e o desenvolvimento do câncer de mama é preciso compreender que esta doença abrange diferentes tipos histológicos, os quais recebem uma classificação de acordo com sua origem, localização e características morfológicas. Os carcinomas correspondem aos tumores malignos de origem epitelial e compreendem a maioria dos cânceres de mama, sendo divididos em duas classes: *in situ* e invasivos. A primeira é caracterizada por células tumorais localizadas no ducto, câncer ductal *in situ* (CDIS) ou no lóbulo, câncer lobular *in situ* (CLIS) das glândulas mamárias. Já os carcinomas invasivos compreendem a classe em que a membrana basal é parcial ou totalmente destruída, sendo o tipo mais comum o carcinoma ductal invasivo (CDI) (Hondermarck et al., 2001).

Os CDIs não possuem especificação que permita uma classificação em subtipos especiais, dessa forma, parâmetros como o grau histopatológico são utilizados para subclassificar esses tumores conforme o pleomorfismo nuclear, quantidade de mitoses e índice de formação dos componentes funcionais da mama, os túbulos e as glândulas. A partir disso, o tumor de mama pode ser dividido em graus I, II e III, conforme a diferenciação do carcinoma em bem diferenciado, moderadamente diferenciado e pouco diferenciado, respectivamente. Este parâmetro é tido como um fator prognóstico significante, uma vez que a sobrevida piora em uma relação direta com o aumento do grau histológico da paciente, visto que quanto menos diferenciado, menos semelhante são as células tumorais em relação às células normais da mama (Elston e Ellis, 1991; Rakha *et al.*, 2010).

Para auxiliar no direcionamento do tratamento do câncer de mama, é preconizado pela União Internacional de Controle ao Câncer (UICC), o uso do sistema de estadiamento Tumor-Nódulo-Metástase (TNM). Este sistema visa estratificar o tumor conforme sua extensão, levando em consideração o tamanho do tumor primário

(T), o acometimento ou não dos linfonodos regionais (N) e a presença ou ausência de metástase à distância (M), permitindo a classificação do tumor em categorias de 0 a IV (Sobin *et al.*, 2009). Mais recentemente, adicionou-se fatores prognósticos a esse sistema, visando avaliar mais especificamente cada tumor. Desta forma, a classificação atual inclui, além dos parâmetros já citados, o grau tumoral, análise de expressão de HER2 e dos receptores hormonais de estrogênio e de progesterona, além de testes genômicos (Byrd e Greene, 2018) como o *Oncotype* Dx (Mcveigh e Kerin, 2017) e o *Mammaprint* Dx (Xin *et al.*, 2017).

Com relação aos receptores hormonais, aproximadamente 80% das mulheres diagnosticadas com câncer de mama possuem tumores positivos para a expressão de receptores de estrógeno e progesterona, o que lhes confere melhor prognóstico (Piccart-Gebhart, 2011). Pacientes com tumores positivos para estes receptores apresentam melhor resposta terapêutica a tratamentos endócrinos quando comparados com pacientes com tumores sem a expressão desses receptores (Henderson e Patek, 1998; Salles, M. D. A. *et al.*, 2009). Assim como pacientes que apresentam a superexpressão de HER2 respondem bem ao tratamento com anticorpo monoclonal específico, o trastuzumab. Por outro lado, pacientes portadoras de tumores com ausência de expressão desses receptores cursam com doença mais agressiva e de pior resposta ao tratamento (Goldhirsch *et al.*, 2011b; Liu, Z. *et al.*, 2014).

A análise do índice de proliferação (Ki67), o qual é expresso ciclicamente nas células em fases G1, S, G2 e M, porém ausente em células no período G0, possibilita uma detecção rápida e fácil para a determinação do nível de crescimento celular (Gerdes et al., 1984). Com isso, além da avaliação dos receptores de estrógeno e progesterona e da expressão e/ou amplificação do oncogene HER2, a avaliação desse marcador intranuclear desempenha um importante papel como fator prognóstico e preditivo na neoplasia mamária, uma vez que quanto mais a célula tumoral prolifera, pior é o prognóstico da paciente (Yerushalmi et al., 2010).

A análise desses fatores em conjunto permite realizar a classificação molecular do câncer de mama conforme a expressão dos receptores hormonais de estrógeno (RE) e progesterona (RP), análise da superexpressão de HER2 e do Ki67 (Salles, M. A. *et al.*, 2009; Hammond *et al.*, 2010; Lloyd *et al.*, 2010; Vallejos *et al.*, 2010). A mais recente classificação é proveniente da conferência internacional 15th St. *Gallen International Breast Cancer Consensus Conference*, na qual foram definidos os cinco

subtipos principais de câncer de mama mostrados na tabela a seguir, adaptado de Curigliano *et al.* (2017).

Tabela 1 – Classificação molecular dos tumores mamários.

Classificação	Perfil de expressão	Ki67
Luminal A	RE+ / RP+/ HER2-	<14%
Luminal B	RE+ e/ou RP+ / HER2-	>14%
Luminal HER	RE+ / RP+ / HER2 +	Qualquer valor
HER2+	RE- / RP- / HER2+	Qualquer valor
Triplo negativo	RE- / RP- / HER2-	Qualquer valor

RE: Receptor de estrógeno; RP: Receptor de progesterona; HER2: receptor do fator de crescimento epidermal humano 2.

Fonte: adaptado de Curigliano *et al.* (2017).

Entre os parâmetros prognósticos, o tamanho tumoral tem fundamental importância, uma vez que mulheres com tumores menores tem melhor sobrevida em comparação àquelas com tumores de tamanhos maiores (Fletcher, 2013). Além disso, é importante avaliar o acometimento de linfonodos, uma vez que algumas células malignas podem invadir preferencialmente linfonodos em vez de órgãos viscerais (Fidler, 2003). Dessa forma, compreender melhor o contexto da carcinogênese tem contribuído para mudar as concepções de um tratamento mais eficaz, visando atingir o maior número de células tumorais que possam estar em outros locais do corpo. Logo, fatores como a detecção precoce da neoplasia mamária têm contribuído para uma redução no acometimento de linfonodos regionais (Group, 2007).

A presença de êmbolos angiolinfáticos também deve ser avaliada no contexto do câncer de mama. Esse fator é relacionado à uma resposta do endotélio devido ao processo inflamatório presente no microambiente tumoral (Hasebe *et al.*, 2008; Samad e Ruf, 2013), visto que essa patologia é caracterizada por um estado pró-trombótico e pró-inflamatório, sendo a formação de êmbolos relacionada ao crescimento tumoral, formação de novos vasos e invasão de novos sítios, além disso, esse processo é favorecido em caso de presença de obesidade (Rubio-Jurado *et al.*, 2018).

1.1 Polimorfismos genéticos em genes de metabolização

Sabe-se que além do acúmulo de mutações celulares que marcam o início de uma neoplasia, outros processos têm sido listados como a manutenção da proliferação celular, inibição dos supressores de crescimento, resistência à apoptose, aumento da angiogênese e capacidade em realizar metástase (Hanahan e Weinberg, 2000). O desenvolvimento do câncer também é influenciado pela evasão ao sistema imunológico e pelo conjunto de fatores que regulam o metabolismo energético do indivíduo, mostrando que o microambiente em que o tumor está inserido é crucial para o entendimento do desenvolvimento e progressão dessa doença (Hanahan e Weinberg, 2011).

Os fatores externos ambientais, genericamente denominados de xenobióticos, impactam diretamente no desenvolvimento e no curso do câncer de mama. A metabolização de xenobióticos e de seus subprodutos garante a eliminação de compostos que poderiam ser danosos ao organismo e essa ação é desempenhada pelas enzimas de detoxificação de fase I, componentes do sistema do citocromo P450 (Mcfadyen *et al.*, 2004), e pelas enzimas de detoxificação de fase II, que englobam as as glutationas-S-transferases (GSTs) (Hayes *et al.*, 2005) e as N-acetiltransferases (NATs) (Windmill *et al.*, 2000). As GSTs constituem uma superfamília de enzimas citosólicas, mitocondriais e microssomais, atuando de forma a proteger o organismo contra o estresse oxidativo (Daly *et al.*, 1994; Hayes *et al.*, 2005).

Essa superfamília é dividida nas classes Alpha, Mu, Omega, Pi, Sigma, Theta e Zeta, conforme características estruturais, químicas e físicas. A ação dessas enzimas compreende a interação entre o resíduo N-terminal das GSTs, com o grupo tiol da glutationa reduzida (γ-L-glutamil-L-cisteína-glicina, GSH), facilitando sua conjugação com os compostos a serem excretados, incluindo carcinógenos, drogas e produtos do metabolismo (Dirr *et al.*, 1994; Oakley, 2011).

São chamados de polimorfismos genéticos as mutações no DNA (ácido desoxirribonucleico) hereditárias que estão presentes na população com uma frequência superior a 1% (Schafer e Hawkins, 1998), podendo gerar variações na atividade das enzimas codificadas por esses genes, como as deleções completas, as quais conferem um fenótipo nulo (Townsend, Danyelle M. e Tew, Kenneth D., 2003; Singh, 2015). Estudos mostram que polimorfismos genéticos podem funcionar como

preditores da suscetibilidade a alguns tipos de câncer, como pulmão (Yuan, Z. *et al.*, 2015), colorretal (Kakkoura *et al.*, 2017), mama (Krishna *et al.*, 2018) e cervical (Pandey *et al.*, 2019).

Os genes glutationa-s-transferase mu 1 (*GSTM1)* e glutationa-s-transferase theta 1 (*GSTT1)* pertencem às classes mu e theta, respectivamente. O primeiro está localizado no cromossomo 1 e possui 3 variantes, das quais duas são funcionais e uma é nula por deleção (Widersten *et al.*, 1991). Já o gene *GSTT1* está localizado no cromossomo 22, apresentando um alelo funcional e outro nulo por deleção (Arruda *et al.*, 1998). Essas variantes polimórficas nulas por deleção são também chamadas de genótipos deletados ou *null*, em relação ao genótipo selvagem, que caracterizaria a presença do gene e, portanto, da expressão proteica (Di Pietro *et al.*, 2010).

Indivíduos com variantes polimórficas das GSTs possuem menor eficiência na detoxificação de metabólitos provenientes de drogas ou carcinógenos e, consequentemente, esses indivíduos podem ser mais suscetíveis ao desenvolvimento de doenças como o câncer. Nesse sentido, diversos estudos têm sido publicados a respeito da influência dos polimorfismos de deleção de *GSTT1* e *GSTM1* em associação com diversas patologias como leiomioma de útero (Mostafavi *et al.*, 2016), hipertensão (Eslami e Sahebkar, 2014), psoríase (Hruska *et al.*, 2017), carcinoma de próstata (Malik *et al.*, 2016) e leucemia mieloide crônica (Weich *et al.*, 2016). desenvolvimento de algumas malignidades como a leucemia mieloide aguda (He *et al.*, 2014), câncer de pulmão (Liu, Kui *et al.*, 2014) e carcinoma hepatocelular (Sui *et al.*, 2014), entretanto, os resultados desses estudos mostram uma relação contraditória e ainda não bem compreendida a respeito da influência desses polimorfismos.

Deve-se, portanto, ressaltar a necessidade da realização de estudos envolvendo possíveis marcadores moleculares, sobretudo, na área das diferentes etapas do metabolismo de compostos endógenos e exógenos, devido à importância do conhecimento sobre a presença destes elementos para nortear o diagnóstico e prognóstico das pacientes. Desse modo, o presente trabalho visou avaliar a influência das mutações nos genes *GSTT1* e *GSTM1* e sua relação com fatores prognósticos do câncer de mama, assim como correlacionar os níveis de GSH entre os diferentes genótipos observados.

2. OBJETIVOS

2.1 Geral

Analisar os polimorfismos por deleção de *GSTM1* e *GSTT1* e os níveis glutationa em pacientes com câncer de mama e suas correlações clinicopatológicas.

2.2 Específicos

-Estratificar o perfil clinicopatológico das pacientes com câncer de mama estudadas.

-Avaliar a associação entre os polimorfismos de *GSTM1* e *GSTT1* e o câncer de mama por meio de um estudo de associação.

-Avaliar a associação entre os polimorfismos de *GSTM1* e *GSTT1* com os parâmetros clinicopatológicos das pacientes.

-Quantificar a glutationa reduzida nas amostras de sangue periférico das pacientes com câncer de mama e comparar os valores encontrados entre os diferentes genótipos de presença e ausência de *GSTM1* e *GSTT1.*

-Verificar a associação entre a concentração de glutationa reduzida e os parâmetros clinicopatológicos, conforme os genótipos de *GSTM1* e *GSTT1.*

3. METODOLOGIA

3.1 Aspectos éticos

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa envolvendo Seres Humanos da Universidade Estadual do Oeste do Paraná, o qual está de acordo com a resolução 196/96 – CNS e aprovado sob o número CAAE 35524814.4.0000.0107 (anexo A) e pelo Comitê de Ética em Pesquisa envolvendo Seres Humanos da Universidade Estadual de Londrina (5231/CAAE N_ 171231134.0000.5231) (anexo B). Todas as participantes assinaram o Termo de Consentimento Livre e Esclarecido (TCLE) – anexos C e D.

3.2 Caracterização da amostra

A amostra foi composta por 147 mulheres diagnosticadas com câncer de mama operável em estadiamento inicial, das quais foram coletados 10 mL de sangue periférico em tubos contendo EDTA, atendidas no Hospital do Câncer de Francisco Beltrão (Ceonc, Francisco Beltrão - PR) no período de janeiro de 2015 a agosto de 2018. No dia da cirurgia as pacientes responderam a um questionário (anexo E) e também ao Self report questionaire (SRQ) (Cormanique *et al.*, 2015) (anexo F). O plasma e o *buffy coat* foram obtidos por meio de centrifugação à 4000 rotações por minuto (rpm), durante 5 minutos, com posterior armazenamento à -20°C até o momento das análises. Após confirmação do diagnóstico pela biópsia, foram incluídas no estudo apenas aquelas com câncer de mama ductal invasivo, resultando em 121 mulheres.

A partir de consulta em prontuários foram coletados os seguintes dados: idade ao diagnóstico; status menopausal; índice de massa corpórea (IMC), obtido por meio do cálculo da divisão do peso da paciente pela altura elevada ao quadrado (Misra e Dhurandhar, 2019), o qual estratifica as pacientes em baixo peso (<18.5), eutróficas (18.5 – 24.9) e sobrepeso/obesidade (>25); expressão de receptores hormonais (estrógeno e progesterona), superexpressão de HER2, valor de Ki67, subtipos, tamanho tumoral; grau histológico do tumor; presença de êmbolos angiolinfáticos; presença de metástases em linfonodos e recorrência.

A partir da entrevista da paciente, obtiveram-se os seguintes dados: exposição a agrotóxicos, seja por meio da aplicação ou pelo contato indireto; histórico de estresse crônico; histórico familial de câncer de mama, positivas para o histórico familial da síndrome câncer de mama e/ou ovário hereditária segundo instrumento SFH-7.

Para compor o grupo controle foram coletados 5 mL de sangue periférico heparinizado de 151 mulheres que tiveram como critério de inclusão não possuir história de neoplasia de mama atual ou anterior, de acordo com exame clínico e mamografia atualizadas para o momento da coleta, no período de 2010 a 2011 em uma Unidade Básica de Saúde da cidade de Londrina, Paraná, em parceria com o Laboratório de Estudos e Aplicações de Polimorfismo de DNA (LEAP) da Universidade Estadual de Londrina. Estas amostras foram processadas e armazenadas conforme descrito anteriormente para as pacientes.

3.3 Delineamento da pesquisa

Inicialmente realizou-se um estudo do tipo caso-controle com o intuito de avaliar uma possível associação dos polimorfismos de *GSTT1* e *GSTM1* com a suscetibilidade ao desenvolvimento do câncer de mama. Em seguida realizou-se um estudo do tipo transversal na busca por uma correlação entre os polimorfismos estudados e os parâmetros clinicopatológico das pacientes: idade ao diagnóstico; status menopausal; exposição a agrotóxicos; histórico familial de câncer de mama; expressão de receptores hormonais (estrógeno e progesterona), superexpressão de HER2, índice de proliferação Ki67; grau histológico do tumor; presença de êmbolos angiolinfáticos; presença de metástases em linfonodos; recorrência e óbito. Por fim, foram realizadas análises também no sentido de avaliar a relação entre a concentração glutationa e os polimorfismos estudados, em relação aos parâmetros clinicopatológicos.

3.4 Extração do DNA genômico

A extração do DNA genômico das pacientes foi realizado a partir de sangue periférico por meio do protocolo extração *Salting-out* (Miller *et al.*, 1988). Primeiramente o tubo com anticoagulante foi centrifugado, após isso o plasma foi

separado e armazenado e para o presente trabalho foi utilizado o *buffy coat* para prosseguir a extração. Fez-se uma sequência de duas lavagens com água destilada gelada, intercaladas com centrifugação. Após o descarte do sobrenadante, ressuspendeu-se o *pellet* em uma solução de lise de leucócitos, adicionou-se proteinase K (2mg/mL) e solução de Duodecil Sulfato de Sódio (SDS) a 20%. Após homogeneização entre cada etapa, incubou-se os frascos em banho-maria a 37ºC durante 3 horas. Em seguida, acrescentou-se NaCl 6M e agitou-se no vórtex, centrifugou-se por 5 minutos a 4000 rpm e, por fim, separou-se o sobrenadante em tubos cônicos, com posterior adição de 2 volumes de etanol absoluto 100% gelado para precipitar o DNA.

O DNA genômico das mulheres controle foi extraído a partir de amostras de 200 uL de sangue periférico total pelo Kit de extração *Mini Spin Plus* (BioPur, Curitiba, Paraná, BR) de acordo com as instruções do fabricante.

As amostras de DNA das pacientes e das mulheres controles foram quantificadas por espectrofotometria, a 260 nm, no aparelho Nanodrop 2000 (NanoDrop Technologies, Wilmington, DE, EUA). A pureza dos analitos foi avaliada através da razão entre absorbâncias obtidas nos comprimentos de 260nm e 280nm, a qual fornece um parâmetro de avaliação da qualidade do DNA (valores inferiores a 1,8 podem indicar contaminação com proteína).

3.5 Reação em cadeia da polimerase (PCR)

A análise dos polimorfismos foi realizada no LEAP através da técnica de PCR *Multiplex* para verificar a presença ou ausência dos polimorfismos nos genes *GSTM1* e *GSTT1* (Abdel-Rahman *et al.*, 1996), modificado (Tabela 2). O gene 1A1 do citocromo P450 (*CYP1A1)* foi usado como controle interno da reação. Os iniciadores para este gene amplificam um fragmento não polimórfico de 312 pares de base (pb). Os fragmentos de 215 e 480 pb são observados, respectivamente, nos indivíduos *GSTM1* e *GSTT1* positivos (tabela 3). A ausência de amplificação *GSTM1* (215 pb) ou *GSTT1* (480 pb), na presença de controle interno, indica os respectivos genótipos nulos para cada gene ou para ambos.

Os fragmentos foram amplificados em termociclador PTC-100 (MJ *Research, Inc*), submetidos à eletroforese (3V/ml) em gel de poliacrilamida 10% e corados com nitrato de prata. Para a confirmação do tamanho dos produtos amplificados, foram

utilizados marcadores de DNA (*Ladder*) de 100 pb, bem como controles de pureza dos reagentes (branco da reação) em cada reação de amplificação. O perfil eletroforético dos polimorfismos *GSTM1* e *GSTT1* está representado na Figura 1.

Tabela 2 – Condições da reação de amplificação dos polimorfismos dos genes *GSTT1*, *GSTM1* e CYP1A1.

	REAGENTES	**VOLUME**
	H_2O Milli-Q q.s.p.	6,8 µL
	Buffer 10x*	2,5 µL
	$MgCl_2$ 50mm*	1,0 µL
	dNTP 1,25 mm*	4,0 µL
	Primer sense 2,5 µm (T1, CYP, M1)*	1,5 µL
	Primer anti-sense 2.5 µm (T1, CYP, M1)*	1,5 µL
	Taq (1:10)*	0,2 µL
	DNA template	1,5 µL
	TOTAL	**25,00 µL**

*INVITROGEN *Life Technologies* - Brasil

Tabela 3 – Sequências dos iniciadores e respectiva ciclagem usados na reação de amplificação dos genes *GSTT1*, *GSTM1* e CYP1A1.

Genes	Iniciadores	Condições da reação
GSTM1	5'GAACTCCCTGAAAAGCTAAAGC3' 5'GTTGGGCTAAATATACGGTGG3'	
CYP1A1:	5'GAACTGCCACTTCAGCTGTCT3'	94°C for 5 min, 30 cicles (94°C for 1 min, 59°C for 1 min, 72°C for 1 min) 72°C for 5 min
	5'CAGCTGCATTTGGAAGTGCTC3'	
GSTT1:	5'TTCCTTACTGGTCCTCACATCTC3' 5'TCACCGGATCATGGCCAGCA3'	

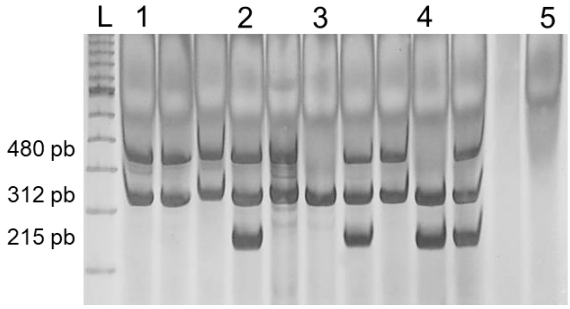

Figura 1 - Perfil eletroforético para os polimorfismos dos genes *GSTM1* e *GSTT1*. Legenda: L - Marcador de tamanho de fragmento de 100pb (*Ladder*); 1 - *GSTT1* presente; 2 – *GSTT1* e *GSTM1* presentes; 3 - *GSTT1* e *GSTM1* ausentes; 4 - *GSTM1* presente; 5 - Controle negativo (livre de DNA) para ambos os alelos.

3.6 Dosagem de glutationa reduzida

Foram realizadas dosagens de GSH a partir de alíquotas de 100uL de plasma descongelado a temperatura ambiente, que em seguida foi desproteinizado com 100uL de Ácido Tricloroacético (TCA) 50% e centrifugado por 5 minutos em 4000 rpm. Um volume de 50uL desse sobrenadante foi adicionado à microplaca e misturado a 200 uL de solução tampão Tris-HCl (0,4 Molar; pH 8,9). Para controle da reação foi realizada uma curva padrão com diluição seriada da solução de GSH 0,03 M. Por fim foi adicionado 50uL de DTNB (reagente de Ellman) 0,01 M (preparado em metanol) em todas as amostras e na curva. Houve a formação de um composto amarelo que foi detectado por espectrofotometria em comprimento de onda de 405 nm, adaptado de (Sedlak e Lindsay, 1968).

3.7 Análise Estatística

Os dados foram expressos como médias±erros das médias, e os resultados foram comparados por Mann-Whitney (dados não paramétricos) ou teste t de Student (dados paramétricos). O teste ANOVA também foi realizado quando comparados mais de 2 grupos. A associação caso-controle foi realizada pelo cálculo do Odds Ratio (OR) com intervalo de confiança de 95%. Outras análises estatísticas foram realizadas utilizando as estatísticas SPSS 25.0 (SPSS inc., Chicago, Illinois, EUA) e GraphPad Prism versão 7.00 para Windows (GraphPad Software, San Diego, Califórnia, EUA). Para todos os dados, o nível de significância adotado foi $p \leq 0.05$.

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5. ARTIGO 1

Polymorphisms in *GSTT1* and *GSTM1* genes as possible risk factors for susceptibility to breast cancer development: systematic review

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Abstract

Breast cancer (BC) is a heterogeneous and multifactorial disease. There is a system formed by glutathione-S-transferases (GSTs) that act in order to protect the organism against the oxidative stress generated by xenobiotics and their products. Glutathione transferase mu 1 (*GSTM1*) and glutathione transferase theta 1 (*GSTT1*) present null polymorphic variants by complete deletion and the absence of these enzymes may influence the susceptibility to several diseases such as BC. This study aimed to systematically review and evaluate studies regarding the occurrence of polymorphisms in the GST system in patients with this neoplasia, and investigate the existence of a possible correlation between the presence/absence of these genetic variants and the development of this pathology. In this review, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol was used and the searches were performed in the portal of the Virtual Health Library (BVS) and in the PUBMED, resulting in a total of 15 articles. Of these, a possible risk association was observed between *GSTT1* and/or *GSTM1* polymorphisms in relation to BC, while some studies showed a protective association, with better response to chemotherapy in patients with the presence of *GSTM1* variants. On the other hand, some studies concluded that there was no significant association between these polymorphisms and susceptibility, as well as in relation to response to chemotherapy. It is important to observe that a higher prevalence of studies showed association of risk between the deletions of *GSTT1* and *GSTM1* and this neoplasia. On the other hand, it should be noted that the response to treatment can also be influenced by these null genotypes.

Keywords: xenobiotics, glutathione, polymorphism.

1 INTRODUCTION

Cancer is a heterogeneous disease that affects individuals worldwide. The International Agency for Research on Cancer (IARC) expected, for 2018, 18.1 million new cases and 9.6 million deaths by neoplasia. Female breast cancer is in the second place in the ranking of causes of death by cancer, with 11.6% of all deaths in the world, followed by prostate tumors (7.1% of cases) (Bray *et al.*, 2018). According to the National Cancer Institute (INCA), in Brazil, there will be about 60 thousand new cases of breast cancer for the biennium 2018-2019. Regardless of non-melanoma skin tumors, this malignancy is the most incident in the Brazilian Southern, resulting in an estimated risk of 73.07 cases per 100,000 women (Inca, 2017).

Similar to other types of malignant tumors, the development of breast carcinoma is complex, environmental variables such as lifestyle and intrinsic characteristics of the patient also influence the development and evolution of this malignant disease, such as age (Anders *et al.*, 2009; Fredholm *et al.*, 2009), hormone factors (Yerushalmi et al., 2010), menopause (Ahmed *et al.*, 2016), smoking (Catsburg *et al.*, 2015), exposure to ionizing radiation (Boice *et al.*, 1991) and overweight (Sebastiani *et al.*, 2016). Thus, the importance of understanding these elements to determine the diagnosis and prognosis of patients should be emphasized.

Most of cancers are associated with external risk factors. Therefore, a wide of substances are constantly entering our organism by several routes, and need to eliminate in a secure manner to avoid any kind of injury. In this context, there is a complex system in the body formed by phase I detoxification enzymes, components of the P450 cytochrome system *(Mcfadyen et al., 2004)*, and by phase II detoxification enzymes, that includes the glutathione S-transferases (GSTs) (Hayes *et al.*, 2005) and the N-acetyltransferases (NATs) (Windmill *et al.*, 2000). This system, altogether with GSTs, is responsible for metabolize environmental and xenobiotic factors that can be potentially associated with the rise of carcinogenesis *(*Hayes *et al., 2005)*.

The GSTs represent a superfamily of cytosolic, mitochondrial and microsomal enzymes, which act to protect the body against the oxidative stress generated by the presence of xenobiotics and their products (Daly *et al.*, 1994; Hayes *et al.*, 2005). This superfamily is divided into Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta classes, according to its structural, chemical and physical characteristics. The action of these

enzymes includes the interaction between their N-terminal residue with the thiol group of the glutathione peptide in its reduced form (γ-L-glutamyl-L-cysteine-glycine, GSH), providing the conjugation of compounds to be excreted, including carcinogens, drugs and products of metabolism *(Dirr et al., 1994; Oakley, 2011).*

Genetic polymorphisms can lead to variations in the activity of the enzymes, resulting in combinations ranging from partial to complete deletions, which can result even in a null phenotype (Townsend, Danyelle M. e Tew, Kenneth D., 2003; Singh, 2015). The glutathione transferase mu 1 (*GSTM1*) and glutathione transferase theta 1 (*GSTT1*) genes belong to the mu and theta classes, respectively, present null polymorphic variants by complete deletion also called null genotypes, in relation to the wild genotype, and are characterized by the total absence of the proteins (Di Pietro et al., 2010).

It is known that these deletion polymorphisms are associated with the development of several diseases, such as uterine leiomyoma (Mostafavi *et al.*, 2016), hypertension (Eslami e Sahebkar, 2014), psoriasis (Hruska *et al.*, 2017), prostate carcinoma (Malik *et al.*, 2016) and chronic myeloid leukemia (Weich *et al.*, 2016), among others. In this context, studies show that genetic polymorphisms of this system may act as predictors of susceptibility to some types of cancer, such as lung (Yuan, Z. *et al.*, 2015), colorectal (Kakkoura *et al.*, 2017), breast (Krishna *et al.*, 2018) and cervical (Pandey *et al.*, 2019).However, few studies have discussed the clinical meaning of such variants, as well its correlation with the parameters that are determinant of poor prognosis.

This study aimed to systematically review and evaluate studies regarding the occurrence of polymorphisms in the GST system in patients with breast cancer, and investigate the existence of a possible correlation between the presence/absence of these genetic variants and the susceptibility, as well as in relation to the determinants of prognosis of this pathology.

2 MATERIALS AND METHODS

Review protocol

This review used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol, adapted from Moher *et al.* (2009).

Literature search

From April to May 2018, the regional portal of the Virtual Health Library (BVS) was used and from August to November 2018, the PUBMED portal was used to perform the searches. In the BVS, the descriptors were "glutathione transferase", "cancer", "neoplasia" and "polymorphism". In the search performed at PUBMED, the descriptors used were "glutathione transferase", "breast cancer" and "genetic polymorphism". Then, the filters were selected: Portuguese and English language, type of document (article), published between 2013 and 2018. In addition, complete readings of literature reviews and systematic reviews with similar subjects were performed.

Eligibility

The studies were pre-selected by reading of their titles and abstracts, and separated for further analysis and extraction of data.

Inclusion and exclusion criteria

Inclusion criteria were: 1 - influence of *GSTT1* and/or *GSTM1* polymorphisms on breast cancer; 2 - association of *GSTT1* and/or *GSTM1* polymorphisms with carcinogens; 3 - evolution of the disease in relation to these polymorphisms; 4 - response to treatment. Is was considered as exclusion criteria: 1 - review studies, meta-analyses, case reports or comments; 2 - association studies with chemotherapy toxicity; 3 - other types of cancer or hematological malignancies; 4 - clinical trials of drugs; 5 - studies containing incomplete data.

Biases

The possible biases of the eligible studies were analyzed according to the limitations of each study, such as sample size, statistics of the results and the parameters involved.

3 RESULTS

Elegible studies

From the first searching in the BVS database, 760 articles were obtained. After applying the filters of interest, a total of 130 articles were found and after reading the titles and abstracts, 17 articles were selected for complete analysis. Of this, 12 articles (Martinez-Ramirez *et al.*, 2013; Possuelo *et al.*, 2013; Sohail *et al.*, 2013; Tulsyan *et al.*, 2013; Zgheib *et al.*, 2013; Liu, J. *et al.*, 2014; Jaramillo-Rangel *et al.*, 2015; Soto-Quintana *et al.*, 2015; Wang *et al.*, 2015; Yuan, P. *et al.*, 2015; Zhou *et al.*, 2015; Kakkoura *et al.*, 2017) were selected to integrate this systematic review.

In Pubmed database, 235 articles were found and, after selecting the filters, 48 articles remained, of which 21 were selected for full reading. At the end, 12 articles were judged satisfactory to compose this study, 9 of them (Possuelo *et al.*, 2013; Tulsyan *et al.*, 2013; Liu, J. *et al.*, 2014; Jaramillo-Rangel *et al.*, 2015; Soto-Quintana *et al.*, 2015; Wang e Huang, 2015; Yuan, P. *et al.*, 2015; Zhou *et al.*, 2015; Kakkoura *et al.*, 2017) were common to those found in the BVS search and the others were different (Wang, 2015; Campos *et al.*, 2017; Garcia-Martinez *et al.*, 2017). Other articles from literature review readings were not included, because these articles did not satisfy the inclusion criteria, were not publications from the last 5 years, according to the interest of the present study or were already included. Thus, a total of 15 articles were included in this review.

Characteristics of the studies

The studies selected for systematic analysis are presented in Table 1, of which 12 are available on the Medline platform (Martinez-Ramirez *et al.*, 2013; Sohail *et al.*, 2013; Tulsyan *et al.*, 2013; Zgheib *et al.*, 2013; Liu, J. *et al.*, 2014; Jaramillo-Rangel *et al.*, 2015; Soto-Quintana *et al.*, 2015; Wang *et al.*, 2015; Wang e Huang, 2015; Yuan, P. *et al.*, 2015; Zhou *et al.*, 2015; Kakkoura *et al.*, 2017), one is available on the Lilacs platform (Possuelo *et al.*, 2013), one on the Scielo platform (Garcia-Martinez *et al.*, 2017) and another on the Pubmed platform (Campos *et al.*, 2017).

In addition, 9 are case-control studies (Martinez-Ramirez *et al.*, 2013; Possuelo *et al.*, 2013; Sohail *et al.*, 2013; Zgheib *et al.*, 2013; Jaramillo-Rangel *et al.*, 2015; Soto-

Quintana *et al.*, 2015; Campos *et al.*, 2017; Garcia-Martinez *et al.*, 2017; Kakkoura *et al.*, 2017), and 6 are cohort studies (Tulsyan *et al.*, 2013; Liu, J. *et al.*, 2014; Wang *et al.*, 2015; Wang e Huang, 2015; Yuan, P. *et al.*, 2015; Zhou *et al.*, 2015). Most studies (33.3%) are from China, followed by Mexico (26.7%). The sample size ranged from 49 to 1109 participants (5340 cases and 3075 controls in the total study).

Table 2 summarizes the main results found by the included studies for *GSTT1* and *GSTM1* polymorphisms, and their correlation with the development of breast cancer and response to chemotherapy. Some studies also analyzed other polymorphisms as shown in the second column of the table. Among these studies, 7 found a susceptibility association between at least one or both of the *GSTT1* and *GSTM1* polymorphisms in relation to breast cancer (Possuelo *et al.*, 2013; Sohail *et al.*, 2013; Jaramillo-Rangel *et al.*, 2015; Wang e Huang, 2015; Campos *et al.*, 2017; Garcia-Martinez *et al.*, 2017; Kakkoura *et al.*, 2017). Also, 3 studies showed a protective association, indicating a better response to chemotherapy in patients with the presence of *GSTM1* variant (Tulsyan *et al.*, 2013; Soto-Quintana *et al.*, 2015; Wang *et al.*, 2015). On the other hand, some studies concluded that there is no significant association between the polymorphisms of GSTs of classes M1 and T1 and the susceptibility to breast cancer (Martinez-Ramirez *et al.*, 2013; Zgheib *et al.*, 2013). Significant results were also not observed when analyzing the *GSTM1* and *GSTT1* polymorphisms and the response to chemotherapy, as well as their effect on the overall survival of patients (Liu, J. *et al.*, 2014; Yuan, P. *et al.*, 2015; Zhou *et al.*, 2015).

4 DISCUSSION

Polymorphic variants of GSTs influence the effectiveness of detoxification of the cytotoxins from drugs or carcinogens and can becoming some people more susceptible to cancer development. Several studies discuss the influence of *GSTT1* and *GSTM1* deletion polymorphisms in some malignancies such as acute myeloid leukemia (He *et al.*, 2014), lung cancer (Liu, Kui *et al.*, 2014) and hepatocellular carcinoma (Sui *et al.*, 2014). However, the role of these polymorphisms is not clear in relation to the susceptibility to breast cancer development, as well as their correlation with the factors that determine the prognosis of this disease.

The simultaneous deletion of *GSTM1* and *GSTT1* has been associated with a higher risk of developing breast cancer, which increases when it is correlated with

exposure to environmental factors such as pesticides, as demonstrated by Sohail et al., (2013) in a case-control study conducted in 200 Pakistani women. In addition, the authors reported an association between these GST variants and a higher risk of developing breast cancer in women who smoke or have a positive family history for the disease (Sohail *et al.*, 2013). Another article published by the group of Garcia-Martinez and collaborators (2017) observed an association of susceptibility to the development of breast cancer in Mexican women with deletion in *GSTM1* and an inverse borderline association between the development of breast cancer and the deletion of *GSTT1*, indicating this deletion as a protective factor, in a case-control study with 1882 women (Garcia-Martinez *et al.*, 2017). In other studies, significant results were obtained in relation to the deletion of *GSTM1*, suggesting that it would be associated with a higher risk of developing breast cancer (Possuelo *et al.*, 2013; Jaramillo-Rangel *et al.*, 2015). In a study performed in the population of Cyprus, it was concluded that the null variant for *GSTT1* was positively associated with the development of breast cancer, in relation to the wild variant, according to a study of 2286 women (Kakkoura *et al.*, 2017).

Breast tumor can be divided into grades I, II and III, according to the differentiation of the carcinoma cells into well differentiated, moderately differentiated and less differentiated, respectively. This parameter is considered a significant prognostic factor, considering that the less differentiated the tumor cells are, less similar to the normal breast cells (Elston e Ellis, 1991; Rakha *et al.*, 2010). Brazilian researchers showed that the deletion of *GSTT1* was positively associated with increased risk of disease recurrence, local and/or from distance, as well as deleted *GSTM1* was correlated with a worse prognosis of patients, because a higher percentage of patients with histopathologic grade III tumors was observed in the presence of this polymorphism (CAMPOS, *et al*. 2017).

Individuals carrying the null genotype for *GSTT1* and/or *GSTM1*, may have a better response to treatment with chemotherapy. According to an Indian cohort study, when the null *GSTM1* genotype was evaluated combined with the Ile/Val *GSTP1* genotype (another polymorphism of the GST family), an association was found with the presence of a response to neoadjuvant chemotherapy (Tulsyan *et al.*, 2013). It was also found an association of the null genotype of *GSTM1* as a protective factor in relation to the response to chemotherapy in patients with breast cancer who had high plasma levels of glucose (Soto-Quintana *et al.*, 2015). A similar association was

described in a study conducted in 262 women in the Chinese population, showing a better response to chemotherapy among patients with null *GSTM1* genotype (Wang *et al.*, 2015). In contrast, Wang and Huang (2015) demonstrated that the null *GSTM1* genotype was more associated with a worse response to chemotherapy and lower survival (Wang e Huang, 2015).

Some limitations were identified by the authors of the analyzed studies. Tulsyan and collaborators (2013) highlight the fact that they did not analyze variants in the genes that regulate the detoxification phase I, as well as the sample size used. Another study performed by Yuan et al., (2015) highlights that the disagreements found in the literature about polymorphisms of the GSTs family can be attributed to the different ethnic groups analyzed, as well as to the sample universe used (Yuan, P. *et al.*, 2015). Also, there is the possibility of biases in some correlations, such as in the assessment of exposure to environmental factors such as pesticides, due to the fact that exposure is reported verbally by patients, without the application of a structured quantitative instrument (Sohail et al., 2013).

5 CONCLUSIONS

The present study shows that the role of *GSTT1* and *GSTM1* polymorphisms is not clear in the literature regarding breast cancer development and prognosis. It was possible to note the higher prevalence of studies showing a possible risk association between the deletions of *GSTT1* and *GSTM1*, revealing that the absence of expression of enzymes encoded by these genes may increase in the susceptibility to breast cancer. On the other hand, it should be emphasized that the response to treatment can also be influenced by these null genotypes, since some of the studies have shown this fact as a protective factor for chemotherapy.

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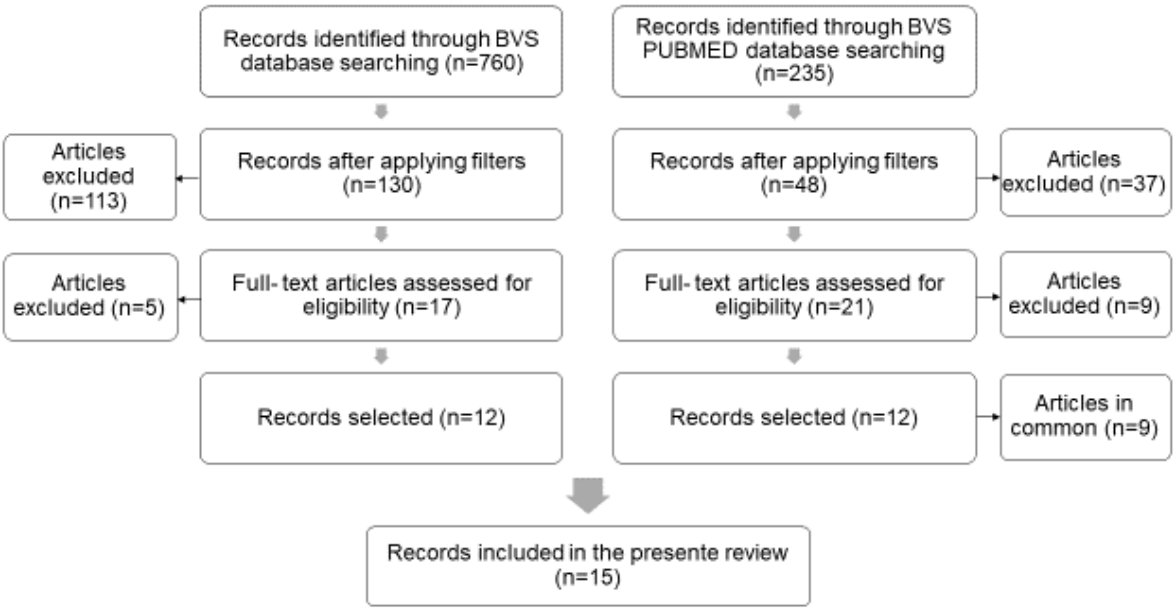

Figure 1 - Identification process of eligible studies. BVS: Virtual health library.

Table 1 - Distribution of *GSTT1* and *GSTM1* polymorphisms in the studies.

Study	Cases				Controls			
	GSTT1+	***GSTT1-***	***GSTM1+***	***GSTM1-***	***GSTT1+***	***GSTT1-***	***GSTM1+***	***GSTM1-***
Campos *et al*., 2017	104 (84,5%)	19 (15,5%)	55 (44,7%)	68 (55,3%)	118 (78,2%)	33 (21,8%)	77 (50,9%)	74 (49,1%)
García-Martínez *et al*., 2017	682 (73,5%)	245 (26,5%)	472 (50,9%)	455 (49,1%)	662 (69,3%)	293 (30,7%)	533 (55,8%)	422 (44,2%)
Kakkoura *et al*., 2017	620 (59,0%)	420 (40,4%)	441 (41,8%)	613 (58,2%)	731 (64,7%)	399 (35,3%)	530 (45,7%)	631 (54,3%)
Yuan *et al*., 2015	121 (44,3%)	152 (55,6%)	156 (57,1%)	117 (42,8%)	-	-	-	-
Soto-Quintana *et al.*, 2015	-	-	306 (55,0%)	252 (45,0%)	-	-	172 (62,0%)	104 (38,0%)
X. Wang; Z.G. Huang, 2015	129 (41,7%)	181 (58,3%)	190 (61,3%)	120 (38,7%)	-	-	-	-
Jaramillo-Rangel *et al.,* 2015.	211 (86,8%)	32 (13,2%)	124 (51,5%)	117 (48,5%)	92 (80,9%)	22 (19,1%)	79 (69,9%)	34 (30,1%)
Zhou *et al*.,2015.	226 (53,8%)	194 (46,2%)	211 (50,2%)	209 (49,8%)	-	-	-	-
Wang *et al.*, 2015.	141 (53,8%)	121 (46,2%)	167 (63,7%)	95 (36,3%)	-	-	-	-
Liu *et al*., 2014.	167 (43,7%)	215 (56,3%)	228 (59,7%)	154 (40,3%)	-	-	-	-
Martínez-Ramírez *et al.,* 2013.	57 (695%)	25 (30,4%)	44 (53,6%)	38 (46,%3)	60 (74,0%)	21 (25,9%)	46 (56,7%)	35 (43,2%)
Possuelo *et al*., 2013.	39 (48,1%)	10 (58,8%)	20 (39,2%)	29 (61,7%)	42 (51,9%)	7 (41,2%)	31 (60,8%)	18 (38,3%)
Sohail *et al*., 2013	73 (36,0%)	27 (13,0%)	57 (28,0%)	43 (21,0%)	70 (35,0%)	32 (16,0%)	57 (28,0%)	45 (22,0%)
Tulsyan *et al.,* 2013.	82 (39,6%)	18 (8,7%)	62 (29,9%)	38 (18,3%)	-	-	-	-
Zgheib *et al*., 2013.	183 (81,0%)	43 (19,0%)	115 (50,9%)	111 (49,1%)	78 (79,6%)	20 (20,4%)	51 (52,0%)	47 (48,0%)

GSTT1+: wild genotype; *GSTT1*-: null genotype; *GSTM1*+: wild genotype; *GSTM1*-: null genotype. The symbol - indicates that the variant was not investigated by the corresponding study.

Table 2 - Summary of the main results found by the studies analyzed in relation to the *GSTT1* and *GSTM1* polymorphisms and their correlation with clinicopathological parameters that determine the prognosis of the disease.

Study	Findings
Campos *et al.,*2017	Positive association between *GSTT1* deletion and increased risk of recurrence after initiation of treatment and association between *GSTM1* deletion and histological grade III, worst prognosis.
García-Martínez *et al.,*2017	Positive association between *GSTM1* deletion and breast cancer and inverse borderline association between breast cancer and *GSTT1* deletion.
Kakkoura *et al.,*2017	Association between *GSTT1* null and increased risk of developing breast cancer compared to the wild genotype.
Yuan *et al.,* 2015	Lack of association between *GSTT1* and *GSTM1* polymorphisms and the presence or absence of chemotherapy response for breast cancer.
Soto-Quintana *et al.,* 2015	*GSTM1* null as a protective factor in response to chemotherapy in patients with high plasma levels of glucose.
X. Wang; Z.G. Huang, 2015	Association between null genotype of *GSTM1* and a worst response to chemotherapy compared to wild genotype.
Jaramillo-Rangel *et al.,*2015.	Increased risk of developing breast cancer in patients with *GSTM1* deletion.
Zhou *et al.,*2015.	Lack of association between *GSTT1* and *GSTM1* polymorphisms and overall survival of breast cancer patients.
Wang *et al.,*2015.	Null genotype of *GSTM1* associated with a better response to chemotherapy.
Liu *et al.,*2014.	Absence of association between *GSTT1* and *GSTM1* polymorphisms and the response to chemotherapy for breast cancer.
Martínez-Ramírez *et al.,*2013.	Absence of association between *GSTT1* and *GSTM1* polymorphisms and the development of breast cancer.

Possuelo *et al.,*2013.	Patients with breast cancer had a higher frequency of deletion of the *GSTM1* gene when compared to the control group.
Sohail *et al.,*2013	Simultaneous deletion of *GSTM1* and *GSTT1* associated with a higher risk of developing breast cancer.
Tulsyan *et al.,*2013.	Association between *GSTM1* null-*GSTP1* Ile/Val genotypes and a better response to neoadjuvant chemotherapy.
Zgheib *et al.,*2013.	Absence of association between *GSTT1* and *GSTM1* polymorphisms and the development of breast cancer.

6. ARTIGO 2

RESEARCH ARTICLE

GSTM1 and *GSTT1* polymorphisms are associated with glutathione variations and poor prognosis parameters in breast cancer

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Microabstract

It was included 121 women with invasive ductal breast cancer. *GSTT1* presence was associated with reduced levels of GSH in breast cancer patients with history of chronic stress. There was significance between positive double genotype and an increase in GSH levels in the presence of angiolymphatic emboli, also, significance between the deleted double genotype and a high ki67 value.

Abstract

Glutathione transferase enzymes (GSTs) act on the conjugation of reduced glutathione (γ-L-glutamyl-L-cysteine glycine, GSH) with the products of metabolism and xenobiotics. Polymorphisms in these genes have been associated with susceptibility to develop breast cancer, but few is known regarding its role on disease prognosis. The present study evaluated the relationship of polymorphisms of *GSTM1* and *GSTT1* genes and its relationship with breast cancer susceptibility and prognostic, as well as its impact on plasmatic GSH. A total of 121 women with invasive ductal breast cancer and 151 healthy controls were included. Polymorphisms analyses were performed using the Multiplex PCR technique and GSH levels measured with the Ellman's reagent. *GSTT1* and *GSTM1* polymorphisms did not show any association with breast cancer susceptibility. *GSTT1* present was associated with reduced levels of GSH in breast cancer patients with a history of chronic stress. There was also significance between the positive double genotype and an increase in GSH levels in the presence of angiolymphatic emboli, as well as significance between the deleted double genotype and a high ki67 value (>14%). These results show these gene polymorphisms are associated with specific clinical parameters that determine breast cancer prognosis, and affect the availability of GSH in such patients.

Keywords: breast cancer, gene polymorphism, GST family, xenobiotics, reduced glutathione.

INTRODUCTION

Breast cancer is the most common neoplasia worldwide. This is a heterogeneous and complex disease, resulting from the balance between genetic and environmental factors. Environmental and intrinsic characteristics of the woman influence the development and evolution of this malignant neoplasm (Yerushalmi *et al.*, 2010; Catsburg *et al.*, 2015; Ahmed *et al.*, 2016; Sebastiani *et al.*, 2016). Altogether, these factors contribute to the generation of pro-inflammatory and pro-oxidant mediators, which are responsible for the occurrence of the genomic instability that perpetuates the tumor.

In a pro-oxidant condition, cells present important defenses that act against free radicals and other reactive substances derived from both intrinsic metabolism and exogenous exposition. Among these defenses, there are the glutathione transferase enzymes (GSTs), which act in phase II of xenobiotic metabolization (Hayes *et al.*, 2005). Their action includes the conjugation of reduced glutathione (γ-L-glutamyl-L-cysteine glycine, GSH) with the products of metabolism and xenobiotics to be excreted from the body, by the interaction between the N-terminal residue of GSTs and the thiol group of GSH (Dirr *et al.*, 1994; Oakley, 2011).

Glutathione transferase mu 1 (*GSTM1*) and glutathione transferase theta 1 (*GSTT1*) genes belong to the mu and theta classes, respectively. The first is located on chromosome 1 and has 3 variants, two of which are functional and one is null by deletion (Widersten *et al.*, 1991). *GSTT1* gene, on the other hand, is located on chromosome 22, presents one functional allele and another null by deletion (Arruda *et al.*, 1998). These null polymorphic variants by deletion are also called deleted or null genotypes, in relation to the wild genotype, which would characterize the presence of the gene and, therefore, the protein expression (Di Pietro *et al.*, 2010).

Polymorphisms in these genes have been studied due to their influence on the susceptibility to diseases such as cancer (Liu, K.; *et al.*, 2014; Sui *et al.*, 2014), including breast (Possuelo *et al.*, 2013; Wang *et al.*, 2015). However, there are still limited studies showing the relationship of these variants with the prognosis of patients separated by, as well as there are no studies correlating the availability of GSH in the presence of these polymorphisms. Thus, this study aimed to evaluate the polymorphisms of *GSTM1* and *GSTT1* in relation to the prognostic parameters of

breast cancer and a possible relationship among the availability of reduced glutathione and the different genotypes.

MATERIALS AND METHODS

Human subjects

This study was approved by the Ethics Committee on Research involving Human subjects under the number CAAE 35524814.4.0000.0107 and the number 5231/CAAE N_171231134.0000.5231 and all volunteer donors signed a free informed consent form prior to biological material collection.

Characterization of the sample

For the case group, 10 mL of peripheral blood were obtained in tubes containing EDTA from 147 patients with operable breast cancer in the initial stage treated at the Cancer Hospital of Francisco Beltrão (Ceonc, Francisco Beltrão - PR) from January 2015 to August 2018. On the day of surgery, the patients were interviewed for obtention of epidemiological and clinicopathological information and also the Self Report Questionnaire (SRQ-20) (Cormanique *et al.*, 2015). Plasma and buffy coat were obtained by centrifugation at 4000 revolutions per minute (rpm) for 5 minutes, with subsequent storage at -20°C until the time of analysis. After confirmation of the diagnosis by biopsy, only those with invasive ductal breast cancer (CDI) were included in the study, resulting in 121 women.

From medical records, the following data were collected: age at diagnosis; menopausal status; body mass index (obtained by calculating the division of the patient's weight by the height squared) which stratifies the patients in low weight (<18.5), eutrophic (18.5 - 24.9) and overweight/obesity (>25). From histological analysis, there were collected the expression of hormone receptors (estrogen and progesterone), HER2 overexpression, Ki67 proliferation index, subtypes, tumor size; tumor histological degree; presence of angiolymphatic emboli; presence of metastases in lymph nodes. And from patient interview: exposure to pesticides, through direct or indirect contact and recurrence., history of chronic stress and family history of breast

cancer, positive for the family history of breast cancer syndrome and/or hereditary ovary according to SFH-7 instrument.

Concerning the control group, 5 mL of heparinized peripheral blood were collected from 151 women who had as inclusion criteria no history of current or previous breast cancer, according to clinical examination and mammography updated for the time of collection, from 2010 to 2011 in a Basic Health Unit of the city of Londrina, Paraná, in partnership with the Laboratory of Studies and Applications of DNA Polymorphism and Immunology (LEAP) of the Londrina State University. These samples were processed and stored as previously described for the patients.

Research design

At first, a case-control study was carried out to evaluate a possible association between the *GSTT1* and *GSTM1* polymorphisms and the susceptibility to breast cancer. A cross-sectional study was then performed searching for a correlation between the studied polymorphisms and the patients' clinicopathological parameters: Age at diagnosis; menopausal status; exposure to pesticides; family history of breast cancer; expression of hormonal receptors (estrogen and progesterone), overexpression of human epidermal growth factor 2 (HER2), Ki67 proliferation index; histological grade; presence of angiolymphatic emboli; presence of metastases in lymph nodes; recurrence and death. Finally, analyses were also performed to evaluate the relationship between glutathione concentration and the polymorphisms studied, in relation to clinicopathological parameters.

DNA extraction

DNA from patients was extracted from peripheral blood of the samples was performed by Salting-out extraction protocol (Miller *et al.*, 1988) and from control women it was extracted by the Mini Spin Plus Extraction Kit (BioPur, Curitiba, Paraná, BR). After extraction, the DNA was quantified by spectrophotometry at 260 nm in the Nanodrop 2000 equipment (NanoDrop Technologies, Wilmington, DE, USA). The purity of the samples was evaluated through the ratio between absorbances obtained in the lengths of 260nm and 280nm.

Genetic polymorphisms analyses

Polymorphism analysis was performed at the Laboratory of Studies and Applications of DNA Polymorphism and Immunology (LEAP) using the PCR technique to verify the presence or absence of polymorphisms in genes *GSTM1* and *GSTT1*, based on the Multiplex PCR protocol (Abdel-Rahman *et al.*, 1996), , modified. The CYP1A1 gene was used as internal control of the reaction. The primers for this gene amplify a non-polymorphic fragment of 312 bp. Fragments of 215 and 480 base pairs (bp) are observed, respectively, in *GSTM1* and *GSTT1* positive individuals. The absence of *GSTM1* (215 bp) or *GSTT1* (480 bp) amplification, in the presence of internal control, indicates the respective null genotypes for each gene or for both. The reaction conditions are shown in table 1, as well as the primer sequences.

The fragments were amplified in PTC-100 thermal cycler (MJ Research, Inc), submitted to electrophoresis (3V/ml) in 10% polyacrylamide gel and colored with silver nitrate. To confirm the size of the amplified products, DNA markers (Ladder) of 100 bp were used, as well as reagent purity controls (negative control) in each amplification reaction. The electrophoretic profile of the polymorphisms *GSTM1* and *GSTT1* is represented in Figure 1.

Glutathione determination

Glutathione measurement were performed in 69 samples, but only 58 results were included. Aliquots of 100 uL of plasma thawed at room temperature in eppendorfs and then were deproteinized with 100 uL of 50% trichloroacetic acid (TCA) and centrifuged for 5 minutes at 4000 rpm. A volume of 50 uL of this supernatant was added to the microplate and mixed with 200 uL of Tris-HCl buffer solution (0.4 Molar; pH 8.9). The yellow compound was detected by spectrophotometry at 405 nm, adapted from Sedlak e Lindsay (1968) and . Data were expressed as nmols/L of GSH.

Statistical Analysis

Data were expressed as means±errors of the means, and the results were compared by Mann-Whitney (non-parametric data) or Student´s t test (parametric data). ANOVA test was also performed when comparing more than 2 groups. The

case-control association was performed by calculating the Odds Ratio (OR) with a 95% confidence interval. Other statistical analyses were performed using SPSS Statistics 25.0 (SPSS inc., Chicago, Illinois, USA) and GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, California, USA). For all data, the level of significance adopted was $p \leq 0.05$.

RESULTS

The mean age at diagnosis was 55.51 ± 12.73 years for patients and control women had a mean value equal to 51.59 ± 11.27 years. Most women were menopausal at the time of diagnosis (71.6%), while 75.5% were overweight, according to the BMI calculation. Most women also reported some contact with pesticides during their lifetime (86.3%). Regarding the chronic history of stress, 59.0% of the women were categorized as negative for this parameter and the history of breast cancer in the family was negative in 91.1% of the cases. Regarding the hormone receptors, 70.8% presented positive estrogen receptor and 52.1% were positive for the progesterone receptor, in relation to HER2, the overexpression of this receptor was observed in 16.2% of the cases and 46.6% of the patients presented moderate cellular proliferation (Ki67). The most common subtype was Luminal B with 37.5%, while the triple negative subtype had a prevalence of 18.3%. Most patients had tumors between 1.5 and 3.0 centimeters (cm) in size (53.5%). Histopathological grade II was present in 50.5% and the absence of angiolymphatic emboli was observed in 62.6% of patients, while lymph node metastasis occurred in 35.4% of patients. The disease relapsed in 5.9% of the cases and 6.6% died during the study period. Some data were not complete in the patients' medical records, for this reason the sample size of the variables is different (Table 2).

In this study, there were observed that 73.6% of the patients had the genotype present for *GSTT1*, and 26.4% presented with null genotype in homozygosis. Among the female controls, 78.1% were present and 21.9% were absent for this gene. Regarding *GSTM1*, 49.6% of the patients had presence for this gene, while in 50.4% the null genotype was obtained. In relation to the control group, 51% presented wild genotype, while 49%, null genotype, as shown in table 3. The double deletion was present in 12.4% of patients and 13.2% of control women. The case-control association

study revealed no significant association of risk or protection between polymorphisms and the development of breast cancer (Table 3).

When correlated with patients' clinicopathological conditions, age at diagnosis, menopausal status, exposure to pesticides, family history of breast cancer, expression of hormone receptors (estrogen and progesterone), HER2 overexpression, Ki67 proliferation index, tumor histological grade, presence of angiolymphatic emboli, presence of metastases in lymph nodes, recurrence and death, significant results were not obtained for both polymorphisms (Table 4).

A possible relationship between null and present genotypes for *GSTT1* and *GSTM1* was evaluated in relation to glutathione concentrations, but no significance was found (p>0,999), as shown in Figure 2. The mean concentration values in nmols/L of GSH were 20.37±5.82 for the double deleted genotype, 19.75±3.47 for null *GSTT1*, 17.22±1.35 for present *GSTT1*, 18.82±1,96 for absent *GSTM1* and 16.59±1.66 for present *GSTM1*.

In addition, the genotypes *GSTT1* null and present, *GSTM1* null and present, double positive and double deleted were related to clinicopathological parameters (age at diagnosis; menopausal status; body mass index; exposure to pesticides; history of chronic stress; family history of breast cancer; expression of estrogen and progesterone hormone receptors, HER2 overexpression, Ki67 proliferation index, subtypes, tumor size; tumor histological degree; presence of angiolymphatic emboli and presence of metastases in lymph nodes) in relation to GSH concentrations. Significance was obtained between the presence of *GSTT1* and a reduced value of GSH concentration in patients with a history of chronic stress (p=0.055, unpaired test and p=0.016, Mann whitney test). There was also significance between the positive double genotype and an increase in GSH in the presence of angiolymphatic emboli (p=0.02, unpaired test and p=0.025, Mann whitney test), as well as significance between the deleted double genotype and a high value of ki67 (p=0.057), as shown in Figure 3. In the other parameters, no significant results were found.

DISCUSSION

Our study showed the existence of association among different GST polymorphisms with clinical parameters that are determinant of prognosis in breast

cancer. Further, we found that these specific polymorphisms can affect systemic GSH availability.

Breast carcinogenesis is a multifactorial event, and may be accompanied by several stimuli related to the generation of genomic instability. Currently it is known that besides the accumulation of cellular mutations that mark the beginning of a neoplasm, other processes have been listed as the maintenance of cellular proliferation, inhibition of growth suppressors, resistance to apoptosis, increase in angiogenesis and capacity to perform metastasis (Hanahan e Weinberg, 2000). It is known that the role of external environmental factors, generically called xenobiotics, impacts the development and course of cancer. The metabolization of these compounds, performed in part by enzymes of the GST family, ensures the elimination of substances that could be harmful to the body (Townsend, D. M. e Tew, K. D., 2003).

According to a study that evaluated the percentage of *GSTT1* and *GSTM1* polymorphisms in the Brazilian population, it was demonstrated that the null profile for *GSTT1* is present in 18.5% of Caucasian individuals and 19% of black individuals in the country, while the homozygous deletion of *GSTM1* was found in 55% and 33% of these groups, respectively (Arruda *et al.*, 1998). In the present study, 26.4% of the patients and 21.9% of the control women presented with null genotype in homozygosis for *GSTT1*. Regarding the *GSTM1* polymorphism, 50.4% of the patients and 49% of the participants in the control group presented null genotype. The genotypic frequencies found are consistent with the literature, considering that the studied population is mostly self-declared Caucasian.

Polymorphisms have been studied searching for new markers for susceptibility to cancer development, thus, studies have shown an association between *GSTT1* and *GSTM1* polymorphisms with uterine leiomyoma (Mostafavi *et al.*, 2016), prostate carcinoma (Malik *et al.*, 2016) and hepatocellular carcinoma (Sui *et al.*, 2014), among other diseases, but there are still discrepancies in the world literature. This is because, although the absence of these enzymes may predispose the individual to a reduction in the efficiency of the process of detoxification of products of cellular metabolism, when evaluating the response to chemotherapy treatment, this relationship may be of protection, because the therapy compounds would not be excreted so easily and could act for longer against tumor cells (Soto-Quintana *et al.*, 2015; Wang *et al.*, 2015).

Some studies show null profile of *GSTM1* can be associated with a higher risk for the development of this disease(Possuelo *et al.*, 2013), similarly, another study

showed a higher risk of recurrence in patients with the null genotype for *GSTT1* (Campos *et al.*, 2017). The present study, however, revealed no significant association of risk or protection between polymorphisms and the development of breast cancer (Table 3). When the genotypes were correlated with the clinicopathological data of the patients, no significant results were obtained for both polymorphisms (Table 4).

The subtypes that have expression of estrogen and/or progesterone hormone receptors are the most common in breast cancer (about 80% of cases), corresponding to luminal A and B subtypes (Thomas e Gustafsson, 2011; Thomas e Gustafsson, 2015). On the other hand, the triple negative subtype is found in approximately 10% to 20% of cases (Perou, 2011; O'toole *et al.*, 2013). In this study, the luminal subtypes corresponded to 68.1% of the cases, while the triple negative was found in 18.5%. Although the prevalence found for the triple negative subtype in this study is consistent with the limits already established in the literature, it is important to highlight the high prevalence found, which corresponds to a worse prognosis profile, given the absence of specific therapy for this subtype, as well as the high rate of cell proliferation and aggressiveness (Goldhirsch *et al.*, 2011a; Keam *et al.*, 2011; Doepker *et al.*, 2018).

Glutathione is a thiol existing in eukaryotic cells in the reduced (GSH) and oxidized form (N Kaplowitz *et al.*, 1985). The first is the most commonly found in the body (Wu *et al.*, 2004) and integrates the process of xenobiotics metabolization facilitating the excretion of these compounds by the conjugation to these products through the GSTs enzymes (Oakley, 2011). Thus, in order to assess the influence of the polymorphisms of *GSTT1* and *GSTM1* on the availability of GSH, the null and present genotypes for these genes were analyzed in relation to the plasma glutathione levels, but no significance was found, as shown in Figure 2. Also, levels were very similar to that showed in both control and advanced breast cancer group for a recent research(Panis *et al.*, 2012).

In addition, the genotypes *GSTT1* null and present, *GSTM1* null and present, double positive and double deleted were related to the clinicopathological parameters in relation to the concentrations of GSH. Among the external factors that can affect women increasing the risk for the development of breast cancer there is the history of chronic emotional stress (Kornblith *et al.*, 2001). In a Brazilian study conducted with 34 women with this neoplasm, it was demonstrated that this parameter was associated with a cohort with worse prognosis, characterized by the presence of a majority of HER2 overexpression and obesity (Cormanique *et al.*, 2015). In the present study,

significance was obtained between the presence of *GSTT1* and a reduced value of GSH concentration in patients with a history of chronic stress. There was also significance between the positive double genotype and an increase in GSH in the presence of angiolymphatic emboli. The presence of emboli is related to an endothelial response due to the inflammatory process present in the tumor microenvironment (Hasebe *et al.*, 2008; Samad e Ruf, 2013). This pathology is characterized by a prothrombotic and proinflammatory state, and the formation of emboli is related to tumor growth, formation of new vessels and invasion of new sites, in addition, this process is favored in the presence of obesity (Rubio-Jurado *et al.*, 2018). Additionally, high levels of Ki-67 are associated with a more aggressive clinical nature of this pathology (Keam *et al.*, 2011). In the present study, there was significance between the double deleted genotype and a high value of ki67 (>14%) with increased GSH. This result shows a higher mobilization of this parameter in the context of enzymatic absence of T1 and M1 genes combined with a status of high cellular proliferation.

Its important to highlight some limitations like the fact that we did not analyze variants in the genes that regulate the detoxification phase I, as well as the rest of the GST family. Disagreements found in the literature about polymorphisms of the GSTs family can be attributed to the different ethnic groups analyzed.

CONCLUSION

According to the case-control study, the *GSTT1* and *GSTM1* polymorphisms were not candidates for susceptibility markers for breast cancer. Additionally, they were not shown as prognostic markers, because the correlation analyses indicated no significance between these polymorphisms and the patients' clinicopathological parameters. However, significance was found between some of the parameters analyzed in relation to the concentration of GSH in some of the genotypes studied, showing that this peptide behaves differently in relation to the presence or absence of these genes. In general, this study is expected to contribute to the development of additional studies, because we need to establish new markers for this neoplasm, given its considerable importance in clinical practice.

Clinical Practice Points

GST polymorphisms have been studied searching for biomarkers for the susceptibility to the development of cancer. Studies have shown an association between null variants of *GSTT1* and *GSTM1* with uterine leiomyoma, prostate carcinoma, among other neoplasms. However, in relation to breast cancer, some authors show that the absence of these enzymes may be related to a higher risk of developing this disease, while it may be a factor of better response to chemotherapy. In our study, the *GSTT1* and *GSTM1* polymorphisms were not shown as biomarkers for breast cancer and were not shown as prognostic factors either. However, our research revealed a different behavior of GSH concentrations in relation to some genotypes in the presence of certain clinicopathological parameters. This finding may indicate that this peptide is mobilized more or less according to the presence or absence of these enzymes in cases of some worse prognostic factors, like history of chronic stress, presence of angiolymphatic emboli and higher levels of Ki67.

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TABLES

Table 1 - Sequences of primers and their cycling used in the amplification reaction of *GSTT1*, *GSTM1* and *CYP1A1* genes.

Genes	Sequences	Reaction conditions
GSTM1	5'GAACTCCCTGAAAAGCTAAAGC3' 5'GTTGGGCTAAATATACGGTGG3'	94°C for 5 min, 30 cicles (94°C for 1 min, 59°C for 1 min, 72°C for 1 min) 72°C for 5 min
CYP1A1:	5'GAACTGCCACTTCAGCTGTCT3'	
GSTT1:	5'CAGCTGCATTTGGAAGTGCTC3' 5'TTCCTTACTGGTCCTCACATCTC3' 5'TCACCGGATCATGGCCAGCA3'	

Min: minutes.

Table 2 - Clinicopathological characterization of patients diagnosed with invasive ductal breast cancer.

Clinicopathological features		**Patients n(%)**
Age (years) (n=120)	<40	13 (10.8%)
	40-60	64 (53.4%)
	>60	43 (35.8%)
Menopause (n=116)	Negative	33 (28.4%
	Positive	83 (71.6%)
Body mass index (n=110)	<18.5	1 (0.9%)
	18.5 - 24.9	26 (23.6%)
	>24.9	83 (75.5%)
Pesticides (n=102)	No	14 (13.7%)
	Yes	88 (86.3%)
Stress history (n=110)	No	65 (59.0%)
	Yes	45 (41.0%)
Family history of breast cancer (n=101)	No	92 (91.1%)
	Yes	9 (8.9%)
Estrogen receptor (n=120)	Negative	35 (29.2%)
	Positive	85 (70.8%)
Progesterone receptor (n=119)	Negative	57 (47.9%)
	Positive	62 (52.1%)
HER (n=119)	Negative	99 (83.2%)
	Positive	20 (16.2%)
Ki67 (n=118)	Low	42(35.6%)
	Intermediate	55 (46.6%)
	High	21 (17.8%)
Subtypes (n=120)	Luminal A	37 (30.8%)
	Luminal B	45 (37.5%)
	HER2	16 (13.4%)
	Triple negative	22 (18.3%)
Tumor size (n=101)	<1.5	13 (12.9%)
	1.5-3.0	54 (53.5%)
	>3.0	34 (33.6%)
Histopathological grade (n=103)	I	30 (29.1%)
	II	52 (50.5%)
	III	21 (20.4%)
Angiolymphatic emboli (n=107)	Absence	67 (62.6%)
	Presence	40 (37.4%)
Limph node metastasis (n=96)	Absence	62 (64.6%)
	Presence	34 (35.4%)
Recurrence (n=119)	No	112 (94.1%)
	Yes	7 (5,9%)

HER2: human epidermal growth factor receptor 2. Low: <14%; Moderate: between 14% and 50%; High: >50%.

Table 3 - Association between *GSTT1* and *GSTM1* polymorphisms and breast cancer.

Plymorphism	**Controls n(%)**	**Breast cancer n(%)**	***OR*[a]**	**IC 95%**	***p* value**
GSTT1					
Presence	118 (78.1%)	89 (73.6%)		-	-
Deletion	33 (21.9%)	32 (26.4%)	1.29	0.79 – 1.85	0.39
GSTM1					
Presence	77 (51.0%)	60 (49.6%)		-	-
Deletion	74 (49.0%)	61 (50.4%)	1.03	0.81 – 1.31	0.91

*Odds Ratio (OR), confidence interval (IC 95%) and p values.

Table 4 - Association of *GSTT1* and *GSTM1* polymorphisms with prognostic parameters of patients with breast cancer.

Clinicopathological features		Pacients n (%)	*GSTT1* *p value*	*GSTM1* *p value*
Age (years) (n=120)	<40	13 (10.8%)		
	40-60	64 (53.4%)	0.37	0.42
	>60	43 (35.8%)		
Menopause (n=116)	Negative	33 (28.4%		
	Positive	83 (71.6%)	0.70	0.93
Pesticides (n=102)	No	14 (13.7%)		
	Yes	88 (86.3%)	0.30	0.22
Family history of breast cancer (n=101)	No	92 (91.1%)		
	Yes	9 (8.9%)	0.18	0.06
Estrogen receptor (n=120)	Negative	35 (29.2%)	0.88	0.93
	Positive	85 (70.8%)		
Progesterone receptor (n=119)	Negative	57 (47.9%)	0.89	0.77
	Positive	62 (52.1%)		
HER (n=119)	Negative	99 (83.2%)	0.46	0.55
	Positive	20 (16.2%)		
Ki67 (n=118)	Low	42(35.6%)		
	Intermediate	55 (46.6%)	0.28	0.34
	High	21 (17.8%)		
Histopathological grade (n=103)	I	30 (29.1%)		
	II	52 (50.5%)	0.95	0.33
	III	21 (20.4%)		
Angiolymphatic emboli (n=107)	Absence	67 (62.6%)	0.57	0.61
	Presence	40 (37.4%)		
Limph node metastasis (n=96)	Absence	62 (64.6%)	0.17	0.32
	Presence	34 (35.4%)		
Recurrence (n=119)	No	112 (94.1%)		
	Yes	7 (5,9%)	0.54	0.26

RE: estrogen receptor; RP: progesterone receptor; HER2: human epidermal growth factor receptor 2. Low: <14%; Moderate: between 14% and 50%; High: >50%.

SUPPLEMENTARY TABLES

Supplementary tables included all the results of the analyses about the genotypes of *GSTT1* and *GSTM1* null and present, double positive and double deleted related to clinicopathological parameters, also in relation to GSH concentrations. The tables show means and *p* values.

Supplementary table 1 – Correlations of *GSTT1* positive genotype and clinicopathological parameters in breast cancer patients.

Clinicopathological features		**Mean ± error**	***p* value**
Age (years) (n=45)	<50 (n=14)	19.2±2.3	
	>50 (n=31)	16.2±1.6	0.31
Menopause (n=43)	Negative (n=11)	20.0±2.8	
	Positive (n=32)	16.2±1.6	0.25
Body mass index (n=42)	18.5 - 24.9 (n=12)	14.2±2.6	
	>24.9 (n=30)	18.6±1.6	0.16
Pesticides (n=34)	No (n=3)	14.5±2.4	
	Yes (n=31)	18.8±1.8	0.48
Stress history (n=38)	No (n=22)	20.3±2.0	
	Yes (n=16)	14.1±2.2	0.055
Family history of breast cancer (n=33)	No (n=30)	18.6±1.8	
	Yes (n=3)	14.4±8.5	0.50
Estrogen receptor (n=45)	Negative (n=16)	17.5±2.5	
	Positive (n=29)	17.0±1.6	0.84
Progesterone receptor (n=44)	Negative (n=23)	17.9±2.0	
	Positive (n=21)	16.6±1.8	0.65
HER (n=44)	Negative (n=39)	16.8±1.4	
	Positive (n=5)	20.7±3.9	0.38
Ki67 (n=42)	<14% (n=14)	15.9±1.3	
	>14% (n=28)	20.2±2.8	0.12
Subtypes (n=45)	Luminal A (n=12)	19.6±3.1	
	Luminal B (n=18)	15.2±1.3	0.57
	HER2 (n=4)	19.8±4.9	
	Triple negative (n=11)	16.7±3.3	
Tumor size (n=36)	<2cm (n=17)	17.3±2.5	
	>2cm (n=19)	17.0±2.0	0.93
Histopathological grade (n=38)	I (n=12)	18.8±3.2	
	II (n=18)	16.3±1.9	
	III (n=8)	15.3±3.6	0.68
Angiolymphatic emboli (n=38)	Absence (n=20)	16.7±2.2	
	Presence (n=18)	16.6±1.6	0.98

Limph node metastasis (n=35)	Absence (n=21)	18.4±2.4	
	Presence (n=14)	17.5±2.0	0.78

Supplementary table 2 – Correlations of *GSTT1* negative genotype and clinicopathological parameters in breast cancer patients.

Clinicopathological features		**Mean ± error**	***p* value**
Age (years) (n=31)	<50 (n=8)	22.5±4.9	
	>50 (n=23)	17.5±2.0	0.35
Menopause (n=30)	Negative (n=6)	20.2±4.7	
	Positive (n=24)	18.4±2.3	0.72
Body mass index (n=29)	18.5 - 24.9 (n=8)	22.6±5.6	
	>24.9 (n=21)	17.9±1.9	0.31
Stress history (n=27)	No (n=14)	22.5±3.2	
	Yes (n=13)	15.6±2.8	0.12
Family history of breast cancer (n=23)	No (n=21)	21.6±2.6	
	Yes (n=2)	10.7±1.5	0.23
Estrogen receptor (n=31)	Negative (n=10)	19.3±3.3	
	Positive (n=21)	18.5±2.4	0.85
Progesterone receptor (n=31)	Negative (n=15)	20.3±3.2	
	Positive (n=16)	17.4±2.2	0.47
HER (n=31)	Negative (n=27)	19.2±2.1	
	Positive (n=4)	15.7±3.7	0.55
Ki67 (n=31)	<14% (n=8)	18.2±3.6	
	>14% (n=23)	19.0±2.3	0.85
Subtypes (n=31)	Luminal A (n=10)	16.3±3.1	
	Luminal B (n=12)	19.9±3.5	0.82
	HER2 (n=3)	17.8±4.2	
	Triple negative (n=6)	21.29±5.1	
Tumor size (n=23)	<2cm (n=11)	19.4±3.9	
	>2cm (n=12)	16.9±2.3	0.58
Histopathological grade (n=23)	I (n=8)	17.8±3.7	
	II (n=11)	19.5±3.5	
	III (n=4)	15.7±4.4	0.82
Angiolymphatic emboli (n=27)	Absence (n=18)	19.1±2.5	

Limph node metastasis (n=23)	Presence (n=9)	15.8±2.6	0.82
	Absence (n=18)	21.2±2.8	
	Presence (n=5)	18.7±4.4	0.68

Supplementary table 3 – Correlations of *GSTM1* positive genotype and clinicopathological parameters in breast cancer patients.

Clinicopathological features		**Mean ± error**	***p* value**
Age (years) (n=31)	<50 (n=11)	19.1±2.6	
	>50 (n=20)	14.6±1.6	0.14
Menopause (n=31)	Negative (n=7)	17.7±2.6	
	Positive (n=24)	15.3±1.7	0.50
Body mass index (n=24)	18.5 - 24.9 (n=8)	13.2±2.1	
	>24.9 (n=16)	18.4±2.5	0.19
Pesticides (n=24)	No (n=2)	15.8±3.5	
	Yes (n=22)	16.3±1.7	0.92
Stress history (n=23)	No (n=15)	17.5±2.0	
	Yes (n=8)	16.5±4.1	0.80
Family history of breast cancer (n=22)	No (n=20)	16.2±1.5	
	Yes (n=2)	15.4±14.7	0.90
Estrogen receptor (n=27)	Negative (n=9)	17.2±3.7	
	Positive (n=18)	16.2±1.7	0.78
Progesterone receptor (n=26)	Negative (n=14)	17.7±2.4	
	Positive (n=12)	15.6±2.4	0.55
HER (n=26)	Negative (n=21)	15.9±1.9	
	Positive (n=5)	20.2±3.4	0.33
Ki67 (n=29)	<14% (n=11)	19.8±3.4	
	>14% (n=18)	14.4±1.2	0.65
Subtypes (n=26)	Luminal A (n=6)	19.3±4.7	
	Luminal B (n=10)	14.6±1.3	0.56
	HER2 (n=3)	20.5±5.8	
	Triple negative (n=7)	15.4±4.2	
Tumor size (n=23)	<2cm (n=9)	13.5±1.1	
	>2cm (n=14)	18.9±2.9	0.19
Histopathological grade (n=25)	I (n=8)	19.7±4.3	
	II (n=11)	15.2±1.3	
	III (n=6)	14.3±4.0	0.45
Angiolymphatic emboli (n=23)	Absence (n=11)	14.0±2.7	

	Presence (n=12)	16.8±1.6	0.39
Limph node metastasis (n=22)	Absence (n=11)	15.9±3.1	
	Presence (n=11)	16.5±1.8	0.86

Supplementary table 4 – Correlations of *GSTM1* negative genotype and clinicopathological parameters in breast cancer patients.

Clinicopathological features		**Mean ± error**	***p* value**
Age (years) (n=31)	<50 (n=8)	22.5±4.9	
	>50 (n=23)	17.5±2.0	0.27
Menopause (n=29)	Negative (n=6)	20.2±4.7	
	Positive (n=23)	18.6±2.3	0.76
Body mass index (n=29)	18.5 - 24.9 (n=8)	22.6±5.6	
	>24.9 (n=21)	17.9±1.9	0.31
Pesticides (n=23)	No (n=1)	11.8±0.0	
	Yes (n=22)	21.0±2.6	0.46
Stress history (n=27)	No (n=14)	22.5±3.2	
	Yes (n=13)	15.6±2.8	0.12
Family history of breast cancer (n=23)	No (n=21)	21.6±2.6	
	Yes (n=2)	10.7±1.5	0.23
Estrogen receptor (n=31)	Negative (n=10)	19.3±3.3	
	Positive (n=21)	18.5±2.4	0.85
Progesterone receptor (n=31)	Negative (n=15)	20.3±3.2	
	Positive (n=16)	17.4±2.2	0.47
HER (n=31)	Negative (n=27)	19.2±2.1	
	Positive (n=4)	15.7±3.7	0.55
Ki67 (n=31)	<14% (n=8)	18.2±3.6	
	>14% (n=23)	19.0±2.3	0.85
Subtypes (n=31)	Luminal A (n=10)	16.3±3.1	
	Luminal B (n=12)	19.9±3.5	0.82
	HER2 (n=3)	17.8±4.2	

	Triple negative (n=6)	21.2±5.1	
Tumor size (n=23)	<2cm (n=11)	19.4±3.9	
	>2cm (n=12)	16.9±2.3	0.58
	I (n=8)	17.8±3.7	
Histopathological grade (n=23)	II (n=11)	19.5±3.5	
	III (n=4)	15.7±4.4	0.82
Angiolymphatic emboli (n=27)	Absence (n=18)	19.1±2.5	
	Presence (n=9)	15.8±2.6	0.42
Limph node metastasis (n=23)	Absence (n=18)	21.2±2.8	
	Presence (n=5)	18.7±4.4	0.68

Supplementary table 5 – Correlations of double null genotype and clinicopathological parameters in breast cancer patients.

Clinicopathological features		**Mean ± error**	***p* value**
Age (years) (n=7)	<50 (n=3)	23.1±11.7	
	>50 (n=4)	18.3±6.7	0.72
Menopause (n=24)	Negative (n=1)	10.5±0.0	
	Positive (n=23)	20.2±2.6	0.46
Body mass index (n=7)	18.5 - 24.9 (n=4)	26.5±9.3	
	>24.9 (n=3)	10.8±0.8	0.21
Stress history (n=7)	No (n=4)	17.3±7.0	
	Yes (n=3)	24.4±11.1	0.59
Family history of breast cancer (n=6)	No (n=5)	24.5±7.4	
	Yes (n=1)	9.2±0.0	0.44
Estrogen receptor (n=7)	Negative (n=1)	9.2±0.0	
	Positive (n=6)	22.2±6.5	0.48
Progesterone receptor (n=7)	Negative (n=3)	22.3±12.1	
	Positive (n=3)	18.9±6.5	0.79
HER (n=7)	Negative (n=5)	23.7±7.7	
	Positive (n=2)	11.8±2.6	0.57
Ki67 (n=7)	<14% (n=4)	10.7±0.6	
	>14% (n=3)	33.1±9.6	0.039
Subtypes (n=7)	Luminal A (n=4)	10.7±0.6	
	Luminal B (n=2)	42.5±4.1	0.56
	HER2 (n=1)	14.5±0.0	
	Triple negative (n=0)	0	
Tumor size (n=5)	<2cm (n=1)	9.2±0.0	
	>2cm (n=4)	19.0±6.4	0.54
Histopathological grade (n=4)	I (n=2)	13.3±1.1	
	II (n=1)	38.3±0.0	
	III (n=1)	11.4±0.0	0.07
Angiolymphatic emboli (n=6)	Absence (n=5)	16.2±5.5	
	Presence (n=1)	14.5±0.0	0.90

Supplementary table 6 – Correlations of double positive genotype and clinicopathological parameters in breast cancer patients.

Clinicopathological features		**Mean ± error**	***p* value**
Age (years) (n=31)	<50 (n=9)	17.6±2.3	
	>50 (n=12)	14.5±2.7	0.42
Menopause (n=21)	Negative (n=6)	18.2±3.0	
	Positive (n=15)	14.9±2.2	0.42
Body mass index (n=24)	18.5 - 24.9 (n=6)	11.7±2.4	
	>24.9 (n=13)	17.7±2.6	0.17
Pesticides (n=17)	No (n=2)	15.8±3.5	
	Yes (n=15)	16.7±2.5	0.89
Stress history (n=16)	No (n=14)	16.2±2.2	
	Yes (n=2)	15.4±14.7	0.90
Family history of breast cancer (n=22)	No (n=20)	16.2±1.5	
	Yes (n=2)	15.4±14.7	0.91
Estrogen receptor (n=21)	Negative (n=7)	13.8±3.4	
	Positive (n=14)	16.9±2.2	0.44
Progesterone receptor (n=20)	Negative (n=11)	15.8±2.4	
	Positive (n=9)	16.3±3.2	0.91
HER (n=20)	Negative (n=17)	15.1±2.0	
	Positive (n=3)	21.5±5.9	0.24
Ki67 (n=20)	<14% (n=10)	18.1±3.2	
	>14% (n=10)	14.0±2.0	0.63
Subtypes (n=21)	Luminal A (n=6)	19.3±4.7	
	Luminal B (n=8)	15.1±1.6	0.41
	HER2 (n=2)	20.1±10.0	
	Triple negative (n=5)	11.2±3.0	
Tumor size (n=18)	<2cm (n=7)	12.8±0.6	
	>2cm (n=11)	17.9±3.3	0.24
Histopathological grade (n=19)	I (n=6)	18.3±4.6	
	II (n=8)	14.6±1.7	
	III (n=5)	14.1±4.9	0.69
Angiolymphatic emboli (n=17)	Absence (n=7)	10.0±1.6	
	Presence (n=10)	17.1±2.0	0.023

Limph node metastasis (n=18)	Absence (n=9)	15.5±3.8	
	Presence (n=9)	16.8±2.2	0.76

FIGURES

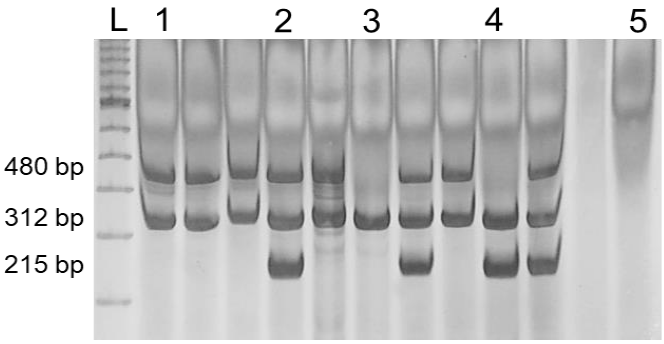

Figure 1 - Electrophoretic profile for the polymorphisms of genes *GSTM1* and *GSTT1*. L - fragment marker of 100 bp (Ladder); 1 - *GSTT1* present; 2 - *GSTT1* and *GSTM1* present; 3 - *GSTT1* and *GSTM1* absent; 4 - *GSTM1* present; 5 - Negative control (DNA free) for both alleles.

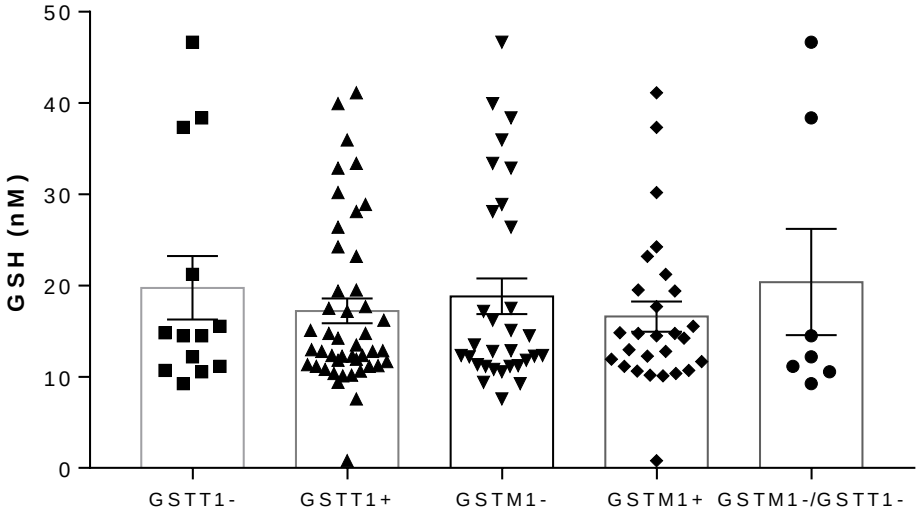

Figure 2 - GSH concentration among the different polymorphisms.
GSTT1-: null genotype; *GSTT1*+: wild genotype; *GSTM1*-: null genotype; *GSTM1*+: wild genotype; *GSTM1-/ GSTM1-:* double deletion.

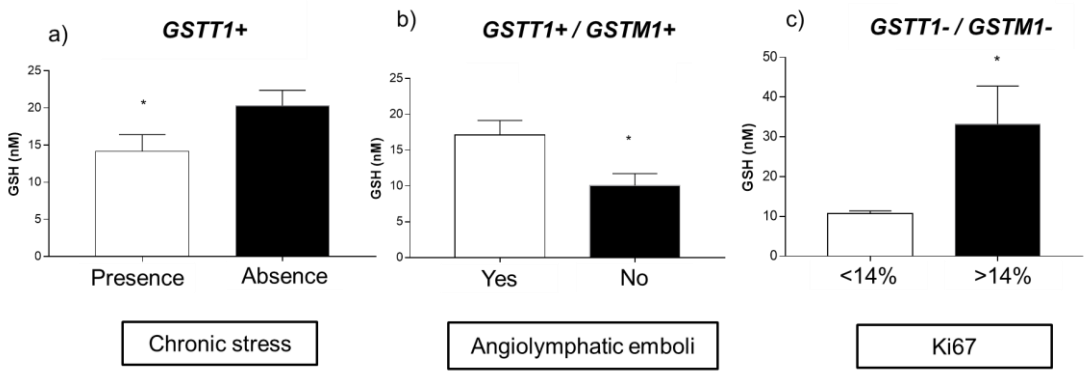

Figure 3 - Significant relations between different genotypes and clinicopathological parameters in pacients with determined GSH levels.

a) Significant association between *GSTT1+* and decrease GSH in chronic psychological stress.

b) Significant association between *GSTT1+/GSTM1+* and increase GSH in presence of angiolymphatic emboli.

c) Significant association between *GSTT1-/GSTM1-* and increase GSH in ki67>14%.

7. ANEXOS

Anexo A

PARECER CONSUBSTANCIADO DO CEP DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Mapeamento do câncer de mama familial no sudoeste do Paraná e estudo de associação de risco coma exposição ocupacional à agrotóxicos.

Pesquisador: CAROLINA PANIS

Área Temática: Versão: 1 CAAE: 35524814.4.0000.0107

Instituição Proponente: UNIVERSIDADE ESTADUAL DO OESTE DO PARANA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 810.501

Data da Relatoria: 25/09/2014

Apresentação do Projeto: Neste estudo pretende-se avaliar todas as mulheres diagnosticadas com câncer de mama, atendidas no Hospital de Câncer de Francisco Beltrão (Ceonc), em um período de 48 meses. A partir da análise de anotações em prontuários serão selecionadas para investigação dos genes de interesse aquelas mulheres com história de câncer de mama familial com ou sem exposição ocupacional à agrotóxicos. Atende aos requisitos teóricos, metodológicos e éticos. Objetivo da Pesquisa: Mapear os casos de câncer de mama familial na região Sudoeste do Paraná e identificar possível associação a exposição ocupacional à agrotóxicos. Avaliação dos Riscos e Benefícios: Não há riscos diretos aos sujeitos, uma vez que serão estudados materiais coletados durante cirurgias oncológicas.

Comentários e Considerações sobre a Pesquisa: Relevante para a área de oncologia.

Considerações sobre os Termos de apresentação obrigatória: Todos apresentados.

Recomendações: Não há recomendações.

Conclusões ou Pendências e Lista de Inadequações: Não há pendências.

Situação do Parecer: Aprovado

Necessita Apreciação da CONEP: Não

Considerações Finais a critério do CEP: Aprovado. O projeto não necessita adequações.

CASCAVEL, 29 de Setembro de 2014

Assinado por: João Fernando Christofoletti (Coordenador)

Endereço: UNIVERSITARIA Bairro: UNIVERSITARIO CEP: 85.819-110 UF: PR Município: CASCAVEL Telefone: (45)3220-3272 E-mail: cep.prppg@unioeste.br

Anexo B

Universidade Estadual de Londrina

PARANÁ

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	189/2013
CAAE:	17123113.4.0000.5231
Data da Relatoria:	30/09/2013
Pesquisador(a):	Maria Angelica Ehara Watanabe
Unidade/Órgão:	Programa de PG em Patologia Experimental

Prezado(a) Senhor(a)

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.

Londrina, 30 de setembro de 2013.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Anexo C

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - TCLE

Título do Projeto: Mapeamento do câncer de mama familial no Sudoeste do Paraná e estudo de associação de risco com a exposição ocupacional à agrotóxicos.

Pesquisador responsável: Profa Dra CAROLINA PANIS – Telefones (43)99165316 e (46) 30571079

Convidamos você a participar de nossa pesquisa que tem o objetivo de identificar os casos de câncer de mama em mulheres que tem história da doença na família, que moram na região Sudoeste do Paraná. Para isso será realizada a coleta de um tubo de sangue (10 mL) e um tubo de saliva (1 mL) para fazer os exames necessários para identificar porque alguns tumores de mama levam à doenças tão agressivas. Durante a execução do projeto também vamos precisar de uma parte do tecido tumoral que o médico irá remover durante a sua cirurgia ou que foi coletado para o diagnóstico da doença (na biópsia). Também precisaremos consultar o prontuário médico, para saber informações sobre sua saúde e sua ocupação de trabalho. Para algum questionamento, dúvida ou relato de algum acontecimento os pesquisadores poderão ser contatados a qualquer momento, pelos telefones (43)99165316 e (46) 30553026. Estamos disponíveis para esclarecer quaisquer dúvidas, a qualquer momento. Desta forma, você está contribuindo para a identificação de fatores que levam à alta incidência de cânceres agressivos na nossa região. Este termo será entregue em duas vias, sendo que uma ficará com você. Você não pagará nem receberá para participar do estudo. Seus dados serão mantidos em sigilo, ou seja, ninguém além dos pesquisadores terá acesso ao material ou informações coletadas. Estes dados serão utilizados somente para fins científicos. Você poderá cancelar sua participação a qualquer momento. Se necessitar de maiores informações, o telefone do comitê de ética é 3220-3272 e da pesquisadora responsável é 46 30553026. A coleta de material será feita dentro do Ceonc, portanto qualquer imprevisto será resolvido imediatamente no local. Ao término do projeto, se a pesquisa identificar que a sua doença se classifica como câncer familial, você será chamado ao Ceonc para receber esclarecimentos sobre como proceder no acompanhamento da doença nos próximos anos.

Declaro estar ciente do exposto e desejo participar do projeto. Nome do sujeito de pesquisa ou responsável:

Assinatura:

CPF:

Eu, _____, declaro que forneci todas as informações do projeto ao participante e/ou responsável. Data:

Anexo D

UNIVERSIDADE ESTADUAL DE LONDRINA
TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Informações sobre a pesquisa:

Você está sendo convidada a participar, como **voluntária saudável**, da pesquisa intitulada **"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer"**, que tem por objetivo analisar determinadas moléculas que podem influenciar na imunidade da paciente. Você será esclarecida sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

Procedimentos do Estudo:

Os procedimentos da pesquisa envolvem a obtenção de 5mL de sangue periférico para análise das células e moléculas do sistema imunológico.

Confidencialidade da Pesquisa

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados não serão divulgados de forma a possibilitar sua identificação. A amostra de sangue e tecido obtidos, serão utilizados para obtenção de DNA, RNA e plasma para a realização deste projeto. A participação no estudo não acarretará custos para você e não haverá compensação financeira adicional. A coordenadora do projeto é a Profª. Drª Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel / Fax: (43) 3371-5629, como também procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 3371 – 2490.

Pesquisador Responsável _____

RG:_____

Consentimento livre esclarecido e informado:

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecida pelo(s) pesquisador(es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente como doadora saudável desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante a execução deste projeto.

Londrina, ____ de _____, 20 ___.

Assinatura do doador (ou responsável): _____

Anexo E

Questionário

Número de identificação
Nome paciente
Prontuário
Data DIAGNOSTICO
Topografia do câncer
Idade ao diagnóstico
Menopausa (sim ou não)
Finalidade da QT (paliativa, adjuvante, neo)
Histórico familial (pais ou irmãos com ca)
Grau histopatológico do tumor
Dados de Imunohistoquímica
Classificação TNM
Invasão Linfonodal
Invasão vascular
Fez cirurgia (qual)
Esquema de drogas da quimio
Fez radioterapia?
Tamanho do tumor
Comorbidades
Doença recorrente ou primária?
Sítios de metástase
Histórico de estresse emocional crônico?
Histórico médico
Toma café ou chimarrão?
Índice de massa corporal (peso e altura)
Trabalha Com agrotóxicos
Sobre a história de ca familial:

1. Algum parente teve ca de ovário?
   ( ) sim ( ) não
2. Alguém teve ca de mama bilateral?
   ( ) sim ( ) não
3. Algum homem teve ca de mama?
   ( ) sim ( ) não
4. Alguém teve ca de ovário E de mama?
   ( ) sim ( ) não
5. Alguma mulher da família teve ca de mama antes dos 50 anos?
   ( ) sim ( ) não

Anexo F

SRQ (SELF – REPORT QUESTIONNAIRE) – QUESTIONÁRIO DE AUTO RELATO

DADOS PESSOAIS

NOME
ORIENTAÇOES PARA REALIZAÇÃO DO TESTE

RESPONDA AS SEGUINTES PERGUNTAS A RESPEITO DA SUA SAÚDE NA ÉPOCA DA DESCOBERTA DA SUA DOENÇA

1.Tinha dores de cabeça freqüentes?	( ) SIM[1]	( ) NÃO [0]
2. Tinha falta de apetite?	( ) SIM [1]	( ) NÃO [0]
3. Dormia mal?	( ) SIM [1]	( ) NÃO [0]
4. Assustava-se com facilidade?	( ) SIM [1]	( ) NÃO [0]
5. Tinha tremores de mão?	( ) SIM [1]	( ) NÃO [0]
6. Sentia-se nervoso (a), tenso (a) ou preocupado (a)?	( ) SIM [1]	( ) NÃO [0]
7. Tinha má digestão?	( ) SIM [1]	( ) NÃO [0]
8. Tinha dificuldade para pensar com clareza?	( ) SIM [1]	( ) NÃO [0]
9. Tinha se sentido triste ultimamente?	( ) SIM [1]	( ) NÃO [0]
10. Tinha chorado mais do que de costume?	( ) SIM [1]	( ) NÃO [0]
11. Encontrou dificuldades para realizar com satisfação suas atividades diárias?	( ) SIM [1]	( ) NÃO [0]
12. Tinha dificuldades para tomar decisões?	( ) SIM [1]	( ) NÃO [0]
13. Tinha dificuldades no serviço (seu trabalho é penoso, causa sofrimento)?	( ) SIM [1]	( ) NÃO [0]
14.Senti-se incapaz de desempenhar um papel útil em sua vida?	( ) SIM [1]	( ) NÃO [0]
15. Tinha perdido o interesse pelas coisas?	( ) SIM [1]	( ) NÃO [0]
16. Sentia-se uma pessoas inútil, sem préstimo?	( ) SIM [1]	( ) NÃO [0]
17 Tinha tido ideias de acabar com a vida	( ) SIM [1]	( ) NÃO [0]
18. Sentiu-se cansado (a) o tempo todo?	( ) SIM [1]	( ) NÃO [0]
19. Tinha sensações desagradáveis no estômago?	( ) SIM [1]	( ) NÃO [0]
20. Cansava-se com facilidade?	( ) SIM [1]	( ) NÃO [0]

TOTAL:

NOME RESPONSÁVEL PELA APLICAÇÃO DO TESTE
DATA

Anexo G

CLINICAL BREAST CANCER

AUTHOR INFORMATION PACK

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ISSN: 1526-8209

DESCRIPTION

Clinical Breast Cancer is a peer-reviewed bimonthly journal that publishes original articles describing various aspects of clinical and translational research of **breast cancer**. *Clinical Breast Cancer* is devoted to articles on **detection**, **diagnosis**, **prevention**, and **treatment** of breast cancer. The main emphasis is on recent scientific developments in all areas related to breast cancer. Specific areas of interest include clinical research reports from various therapeutic modalities, cancer genetics, drug sensitivity and resistance, novel imaging, tumor genomics, biomarkers, and chemoprevention strategies.

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GUIDE FOR AUTHORS

INTRODUCTION

Clinical Breast Cancer is a peer-reviewed bimonthly journal that publishes original articles describing various aspects of clinical and translational research of breast cancer. Clinical Breast Cancer is devoted to articles on detection, diagnosis, prevention, and treatment of breast cancer. The main emphasis is on recent scientific developments in all areas related to breast cancer. Specific areas of interest include clinical research and mechanistic approaches; drug sensitivity and resistance; gene and antisense therapy; pathology, markers, and prognostic indicators; chemoprevention strategies; multimodality therapy; and integration of various approaches.

Types of paper

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Anexo H

Clinical Breast Cancer

GSTM1 and GSTT1 polymorphisms are associated with glutathione variations and poor prognosis parameters in breast cancer.

--Manuscript Draft--

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Abstract:	Glutathione transferase enzymes (GSTs) act on the conjugation of reduced glutathione (γ-L-glutamyl-L-cysteine glycine, GSH) with the products of metabolism and xenobiotics. Polymorphisms in these genes have been associated with susceptibility to develop breast cancer, but few is known regarding its role on disease prognosis. The present study evaluated the relationship of polymorphisms of GSTM1 and GSTT1 genes and its relationship with breast cancer susceptibility and prognostic, as well as its impact on plasmatic GSH. A total of 121 women with invasive ductal breast cancer and 151 healthy controls were included. Polymorphisms analyses were performed using the Multiplex PCR technique and GSH levels measured with the Ellman's reagent. GSTT1 and GSTM1 polymorphisms did not show any association with breast cancer susceptibility. GSTT1 presence was associated with reduced levels of GSH in breast cancer patients with a history of chronic stress. There was also significance between the positive double genotype and an increase in GSH levels in the presence of angiolymphatic emboli, as well as significance between the deleted double genotype and a high ki67 value (>14%). These results show these gene polymorphisms are associated with specific clinical parameters that determine breast cancer prognosis, and affect the availability of GSH in such patients.
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