VANESSA JACOB VICTORINO

RELAÇÃO ENTRE A SUPEREXPRESSÃO DO RECEPTOR DO FATOR DE CRESCIMENTO EPITELIAL HUMANO (HER-2) E ESTRESSE OXIDATIVO EM PACIENTES PORTADORAS DO CÂNCER DE MAMA

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina.

Orientador: Prof. Dr. Rubens Cecchini.

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Londrina, 16 de fevereiro de 2012.

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“A ciência serve para nos dar uma idéia

de quão extensa é a nossa ignorância.”

Félicité Robert de Lamennais

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RESUMO

Introdução e objetivos: O câncer de mama é o tumor maligno mais letal em mulheres em todo o mundo, e durante o seu desenvolvimento, cerca de 20% dos pacientes apresentam superexpressão do receptor do fator de crescimento epidérmico humano-2 (HER2/neu, também conhecido como ErbB2). A família HER2 abrange quatro outros membros presentes na membrana celular como dímeros: HER1, HER2, HER3 e HER4. HER2 pode dimerizar com outro HER2 ou com HER1, HER3 ou HER4. A superexpressão desses receptores possuem sinalização prolongada e através de diferentes vias de sinalizações podem mediar diferentes fatores transcricionais que culminarão em diminuição de apoptose, aumento da sobrevivência celular, aumento da migração celular, progressão do tumoral, crescimento e síntese de proteínas, estimula proliferação e progressão do ciclo celular. Assim, a presença da superexpressão de HER2 no tecido mamário canceroso está associado com a malignidade do tumor. Estudos *in vitro* na literatura sugerem que a estimulação de HER2 minimiza o estresse oxidativo reduzindo os danos a estruturas celulares e algumas das vias ativadas por HER2 podem ser mediadas por produtos do estresse oxidativo e espécies reativas de oxigênio. Sabe-se que o estresse oxidativo pode ser modulador, onde o estresse oxidativo moderado pode estimular a proliferação celular enquanto estresse oxidativo alto pode levar a morte celular. Assim, HER2 super-expresso pode desempenhar um papel protetor para o tumor contra danos mediados pelo estresse. Tais constatações são baseadas na relação indireta entre mediadores do estresse oxidativo e HER2 obtidas por estudos *in vitro* e a influência da superexpressão de HER2 em parâmetros oxidante/ antioxidante em humanos permanece desconhecida. Neste trabalho objetivamos investigar o perfil pro/ antioxidante em mulheres portadoras de tumor HER2 positivo. **Pacientes e Métodos:** Este estudo foi aprovado pelo Conselho Nacional de Pesquisa e Ética (CAAE 0009.0.268.000-07) e pacientes assinaram consentimento informado. As mulheres foram divididas em controles saudáveis (52) pareados por idade e mulheres com câncer de mama (52- BC). O grupo BC foi dividido em 30 mulheres negativas para presença de HER2 (HER^-) e 22 mulheres positivas para presença de HER2 (HER^+) determinada por imunohistoquímica (IHC) empregando o método da estreptavidina-biotina ou hibridização fluorescente *in situ* (FISH) para IHC 2+. Critérios de exclusão foram fumantes, consumidores de álcool, usuários de suplementos antioxidantes, gravidez, lactação, o excesso de praticas de exercícios físicos, usuários de terapia de reposição hormonal e com outras doenças crônicas. Caracterizações dos pacientes incluíram idade no momento do diagnóstico, classificação TNM (T = tumor, N = linfonodo, M = metástase), grau histológico do tumor, receptores hormonais (receptores de estrógeno - ER, receptores de progesterona - PR) e tratamento de quimioterapia. O sangue dos participantes foi coletado no Departamento de Patologia Geral, Universidade Estadual de Londrina, PR-Brasil e centrifugado para obtenção de hemácias (RBC) e plasma. Análises bioquímicas foram realizadas em amostras de plasma para avaliar danos cardíacos, renais e hepáticos através da medição de creatina-quinase total (CKT) e fração MB (CKMB), uréia, creatinina, aspartato-aminotransferase (AST), alanina-aminotransferase (ALT), gama -glutamil transpeptidase (GGT) e bilirrubina total. Para avaliação do metabolismo do ferro, ferritina e ferro plasmático foram mensurados. Níveis de ácido úrico também foram avaliados. Para avaliar o estresse oxidativo em amostras de RBC e

plasma, um método baseado em quimiluminescência foi empregado (QL). Os níveis de malondialdeído (MDA) foram determinados através de um novo método padronizado em cromatografia líquida de alta eficiência (HPLC). Níveis plasmáticos de 8-isoprostano F_2 livre foram quantificados utilizando um kit imunoenzimático competitivo após a hidrólise alcalina de ésteres isoprostanos por reação ELISA (do inglês, *Enzime Linked Immuno Assay Sorbent*). Avaliação dos níveis de nitrito como estimativa de óxido nítrico (NO) plasmatico foi estimado empregando o sistema de reação cádmio-cobre-Griess. O conteúdo de proteínas carboniladas foram avaliadas pelo método da dinitrofenilhidrazina (DNPH). Para avaliar o perfil antioxidante, capacidade antioxidante total (TRAP) foi realizada por QL. Nós também determinamos a atividade da ezima superóxido dismutase (SOD) através do método de inibição da auto-oxidação do pirogalol e determinamos a atividade da enzima catalase através do método de avaliação da degração do peróxido de hidrogênio. Os níveis de glutationa reduzida (GSH) foram determinados através do método de reação com 5, 5 '- dithiobis - 2 - ácido nitrobenzóico (DTNB). Todas as análises foram realizadas em triplicatas. Análise estatística foram realizadas utilizando GraphPad Prism versão 5.0 (GraphPad Software, San Diego, CA), Microsoft Office Excel 2007 e OriginLab software 8.0. Os resultados foram expressos como média aritmética e erro padrão das médias (SEM). Diferenças entre os grupos foram avaliados por análise de variância Two-Way (Two-Way ANOVA) para as curvas de peroxidação lipídica, com teste de Bonferroni como pos-hoc, qui-quadrado e teste exato de Fisher para dados clinicopatológicos, e teste *t* de Student não pareado ou Mann-Whitney para outros parâmetros. Todos os dados foram verificados no Software GraphPad para eliminar *outliers* significativos ($p< 0.05$). As diferenças foram consideradas estatisticamente significantes quando $p < 0.05$. **Resultados:** A maioria dos pacientes apresentaram carcinoma ductal infiltrativo, enquanto somente 2.5% de grupo BC e 5% de HER^+ apresentaram carcinoma *in situ*. A maioria do grupo BC, HER^- e HER^+ também mostraram positividade para ER ($p< 0.001$) e PR ($p<0.0001$). Doença avançada (TNM III/ IV) foi prevalente em todos os grupos ($p<0.0004$). Além do mais, a avaliação do grau histológico classificou a maioria dos pacientes como grau 2 ($p< 0.005$). Nenhuma diferença foi observada para tratamento quimioterápico. Os grupos apresentaram níveis considerados normais de uréia (ctr=29.00 ± 1.370 mg/dL; BC= 28.80 ± 1.442 mg/dL; HER^-= 28.55 ± 1.713 mg/dL; HER^+= 29.11 ± 1.873 mg/dL) e alterações nos níveis de creatinina em BC (ctr=0.9190 ± 0.02785 mg/dL; BC= 0.8208 ± 0.0403 mg/dL; p= 0.014) e nenhuma alteração dependente da superexpressão de HER2 (HER^-= 0.7595 ± 0.03296 mg/dL; HER^+= 0.8965 ± 0.06374 mg/dL) foram observados. Danos hepáticos foram maiores no grupo BC, e verificamos que essas alterações tendem a ser dependentes de HER2 baseados nos altos níveis de AST em BC (ctr= 25.39 ± 1.174 U/L; BC= 47.64 ± 6.508 U/L; p= 0.0006) e HER^+ (HER^-= 36.55 ± 4.018 U/L; HER^+= 59.85 ± 10.12 U/L; p= 0.07), altos níveis de ALT em BC (ctr= 32.64 ± 1.427 U/L; BC= 42.49 ± 3.016 U/L; p= 0.007) e HER^+ (HER^-= 38.55 ± 2.950 U/L; HER^+= 47.59 ± 4.522 U/L; p= 0.09), e GGT em BC (ctr= 17.83 ± 2.079 U/L; BC= 94.88 ± 25.40 U/L; $p< 0.0001$) e HER^+ (HER^-= 37.71 ± 6.013 U/L; HER^+= 154.9 ± 40.33 U/L; p= 0.08). Nenhuma alteração foi observada nos níveis de bilirrubina (ctr=0.5477 ± 0.02748 mg/dL; BC= 0.4697 ± 0.04771 mg/dL; HER^-= 0.4437 ± 0.07043 mg/dL; HER^+= 0.5027 ± 0.05014 mg/dL). Os níveis de CKT não foram alterados em BC (ctr=108.2 ± 38.70 U/L; BC= 60.16 ± 6.643 U/L) e tendem a aumentar em HER^+ (HER^-= 51.05 ± 7.186 U/L; HER^+=70.88 ± 9.475 U/L; p=0.09) e nenhuma alteração em CKMB foi observada (ctr=1.300 ± 0.3462 U/L; BC= 2.231 ± 0.5719 U/L; HER^-= 1.762 ± 0.4923 U/L; HER^+= 2.778 ± 0.9237 U/L). BC apresentou aumento significativo nos níveis de ferro (ctr= 81.83 ± 4.791 µg/dL; BC= 115.5 ± 9.168 µg/dL; HER^-= 114.7 ± 14.48 µg/dL; HER^+= 117.1 ± 14.29 µg/dL; p= 0.02) e ferritina (ctr=47.70 ± 2.831 mg/dL; BC= 279.9 ± 72.58 mg/dL; HER^-= 371.5 ± 153.9 mg/dL; HER^+= 154.0 ± 19.29 mg/dL; p= 0.0003) independente da superexpressão de HER2. Para avaliar estresse oxidativo

plasmáticos e em RBC, QL induzida por *tert*-butil foi realizada e aplicamos o teste Two-way ANOVA para avaliar se as curvas são diferentes, teste de Bonferroni para verificar quais pontos da curva são diferentes e teste *t* de Student para analisar a diferença da média das curvas. Perfil de estresse oxidativo em RBC foi significativamente maior no grupo de BC do que no controle ($p< 0.0001$) independentemente do status HER2 e nenhum ponto da curva foi diferente. A integral da área sob a curva (AUC) aumentou somente no grupo BC (ctr= 722,200 ± 58,990 AUC; BC= 1,097,000 ± 228,200 AUC; HER^-= 1,069,000 ± 40,560 AUC; HER^+= 1,086,000 ± 71,130 AUC; $p< 0.05$). Lipídeos hidroperóxidos foram encontrados aumentados para o grupo BC ($p< 0.0005$) e HER^- ($p< 0.0001$). O teste de Bonferroni não mostrou nenhuma diferença nos pontos das curvas e a AUC não apresentou diferença (ctr=1,262,000 ± 135,100 AUC; BC= 1,937,000 ± 308,500 AUC; HER^-= 2,217,000 ± 481,000 AUC; HER^+= 1,338,000 ± 346,800 AUC). Os níveis de MDA foram diminuídos no grupo HER^+ (ctr= 63.99 ± 4.032 nM; BC= 70.60 ± 9.507 nM; HER^-= 73.81 ± 7.997 nM; HER^+= 65.88 ± 21.11 nM; $p= 0.03$). Nenhuma alteração foi observada avaliando níveis de 8-isoprostanos F_2 (ctr= 144.6 ± 0.3083 pg/mL; BC= 144.4 ± 0.2976 pg/mL; HER^-= 144.5 ± 0.4841 pg/mL; HER^+= 144.3 ± 0.3899 pg/mL), conteúdo de proteínas carbonílicas (ctr= 9.087 ± 0.5459 nM; BC= 9.640 ± 0.4461 nM; HER^-= 9.438 ± 0.4649 nM; HER^+= 9.937 ± 0.6302 nM) e nitrito (ctr= 19.33 ± 1.532 µM; BC= 21.77 ± 1.402 µM; HER^-= 21.63 ± 1.795 µM; HER^+= 21.98 ± 2.308 µM). Como os níveis de ácido úrico foram aumentados no grupo BC (ctr=3.578 ± 0.1861 mg/dL; BC= 4.463 ± 0.2336 mg/dL; $p= 0.004$) e HER^+ (HER^-= 4.852 ± 0.3275 mg/dL; HER^+= 4.014 ± 0.1598 mg/dL; $p= 0.03$), a TRAP foi corrigida pela concentração de ácido úrico. Os resultados mostram que a TRAP diminuiu significativamente no grupo BC (ctr= 366.6 ± 17.36 nM Trolox/ mgxdL-¹; BC= 200.7 ± 15.81 nM Trolox/ mgxdL-¹; $p< 0.0001$) independente da superexpressão de HER2 (HER^-= 202.1 ± 20.99 nM Trolox/ mgxdL-¹; HER^+= 198.4 ± 23.29 nM Trolox/ mgxdL-¹). A avaliação da atividade da SOD mostrou aumento somente no grupo HER^+ (ctr=3.744 ± 0.3148 USOD/mL; BC= 4.214 ± 0.4491 USOD/mL; HER^-= 3.234 ± 0.1661 USOD/mL; HER^+= 5.145 ± 0.8173 USOD/mL; $p=0.03$). A atividade da enzima catalase foi diminuída no grupo BC (ctr= 544.5 ± 11.20 Vabs/min/mL; BC= 504.7 ± 8.977 Vabs/min/mL; $p= 0.007$) independentemente da superexpressão de HER2 (HER^-= 503.9 ± 13.12 Vabs/min/mL; HER^+= 505.6 ± 12.40 Vabs/min/mL). Os níveis de GSH foram aumentados em HER^+ (ctr= 16.62 ± 1.813 nM; BC= 14.70 ± 1.428 nM; HER^-= 9.510 ± 1.687 nM; HER^+= 17.24 ± 2.457 nM; $p= 0.04$). **Discussão e conclusão:** No presente estudo relatamos pela primeira vez vários achados patológicos que ocorrem nas mulheres o câncer de mama de acordo com superexpressão de HER2. Avaliação do metabolismo de ferro foi utilizado como indicador de estresse oxidativo e foi aumentado em BC independentemente do status HER2. Sabe-se que altos níveis de ferro livre podem gerar radicais hidroxilas através da reação de Fenton levando a peroxidação lipídica, e observou-se aumento da peroxidação lipídica no plasma do grupo HER^- e em RBC de BC por QL. Além disso, altos níveis de ferro livre podem potencializar o estresse oxidativo e danos hepáticos, como observado no grupo BC e na presença da superexpressão de HER2. Os níveis de ferritina também aumentaram no grupo BC independentemente da superexpressão de HER2, podendo representar uma resposta compensatória para conter a sobrecarga de ferro. Neste trabalho, mostramos um aumento de lipoperoxidação em RBC no grupo BC independentemente da superexpressão de HER2 e aumento no perfil de lipoperoxidação e MDA na ausência de superexpressão de HER2. Assim, HER2 superexpresso parece ser protetor contra a peroxidação lipídica. Nenhuma alteração foi encontrada em 8-isoprostanos F2, nitrito como estimativa de NO e conteúdo de proteínas carbonílicas. TRAP foi diminuída em BC associados com os níveis de ácido úrico plasmático, sugerindo que moléculas de baixo peso molecular foram consumidos como uma defesa contra processos oxidativos, independentemente da superexpressão de HER2. Defesas contra espécies reativas de oxigênio

incluem enzimas como SOD, que dismuta o radical superóxido em peróxido de hidrogênio e oxigênio, e a enzima catalase, que remove peróxido de hidrogênio dando origem à água e ao oxigênio. Nossos resultados mostram que superexpressão de HER2 aumenta a atividade da SOD, sugerindo que a sinalização por HER2 pode leva à produção do ânion superóxido ou melhora a atividade catalítica da SOD. Estudos têm demonstrado uma diminuição na atividade da catalase em pacientes com doença avançada e importância do peróxido de hidrogênio como mediador positivo em vias PI3K/ Akt e MAPK p38 presente em vias de HER2 superexpresso. Neste trabalho mostramos que a redução da catalase é uma condição presente em BC independentemente do status HER2, mesmo mulheres $HER2^{+}$ exibindo aumento da SOD e, conseqüentemente, peróxido de hidrogênio. Indução de vias mediadas por Jun quinase amino-terminal (JNK), importante via induzida por HER2 super-expresso como um mecanismo de proteção a apoptose, pode induzir a produção de GSH. GSH, na presença de glutationa peroxidase, também pode reduzir o peróxido de hidrogênio à água. Como demonstramos a superexpressão de HER2 impediu a diminuição de GSH provavelmente por um mecanismo de proteção para o excesso de peróxido de hidrogênio. Em conclusão, esta pesquisa fornece novos dados sobre o envolvimento de HER2 superexpresso e estresse oxidativo em mulheres portadoras do câncer de mama. Neste trabalho, informamos que em portadores do câncer de mama, a superexpressão de HER2 atenua o estresse oxidativo, uma vez que diminui a peroxidação lipídica plasmática e formação de MDA, aumentando níveis de SOD e GSH. Assim, esta pesquisa contribui para novas pesquisas com o objetivo de desenvolver novas terapias anti-HER2.

Palavras-chave: Mamas – Câncer. Tumores – Genética – Expressão. Oncologia. Antioxidantes. Células cancerosas.

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ABSTRACT

Introduction and Aims: Breast cancer is the most lethal malignancy in women worldwide, and during its development, about 20% of patients show overexpression of human epidermal growth factor receptor-2 (HER2/neu, also known as ErbB2). HER2 family embraces four members present as dimmers in cell surface: HER1, HER2, HER3 and HER4. HER2 can dimerize with another HER2 or with HER1, HER3 or HER4. Overexpression of these receptors present prolonged signaling activity and through distinct pathways they can mediate several transcriptional factors that decrease apoptosis, augmenting cell survival, increase cell migration, tumor progression, growth, and protein synthesis, stimulates proliferation and cellular cycle progression. Thus, presence of overexpressed HER2 in breast cancer tissue is associated with tumor malignancy. *In vitro* studies in the literature suggest that HER2 stimulation minimize oxidative stress leading to reducing damage to cell structures and some of those HER2 activated pathways can be mediated by products of oxidative stress and reactive oxygen species. It is known that oxidative stress can modulate cellular fate, as mild oxidative stress can stimulates cell proliferation and higher oxidative stress can lead to cell death. Thus, HER2 overexpression may play a protective role for the tumor against damage mediated by stress. Such findings are based on the indirect relationship between mediators of oxidative stress and HER2 obtained by *in vitro* studies and the influence of HER2 overexpression in oxidant/ antioxidant parameters in humans remains unknown. In this report, we aimed to investigate the pro/ antioxidant profile in women bearing HER2 positive tumor. **Subjects and Methods:** This study was approved by Research and Ethics National Council (CAAE 0009.0.268.000-07) and patients provided signed informed consent. Women were divided into 52 healthy age-matched controls and 52 breast cancer women (BC). BC group was divided into 30 negative for HER2 presence (HER^-) and 22 positive for HER2 presence (HER^+) determined by immunohistochemical (IHC) employing streptavidin biotin method or fluorescence in situ hybridization (FISH) for IHC 2+. Excluded criteria were smokers, regular alcohol consumers, antioxidant supplement users, pregnancy, lactation, excessive physical exercises practitioners, hormone replacement therapy users and with another chronic disease. Patients' characterization included age at diagnosis, TNM (T= tumor, N= node, M= metastasis) classification, histological tumor grade, hormonal receptors (estrogen receptors - ER, progesterone receptors - PR) and chemotherapy treatment. Heparinized blood was collected at Department of General Pathology, Londrina State University, PR-Brazil and centrifuged for red blood cells (RBC) and plasma obtainment. Biochemical analysis were performed in plasma samples to assess heart, kidneys and liver damage through measurement total creatine-kinase (CKT) and MB fraction (CKMB), urea, creatinine, aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), gamma-glutamyl-transpeptidase (GGT) and total bilirubin. To assay iron metabolism, ferritin and plasmatic iron were

measured. Levels of uric acid were also evaluated. To evaluate oxidative stress in RBC and plasma samples, a chemiluminescence-based method was employed. Malondialdehyde (MDA) levels was determined improving a new method was adapted by high performance liquid chromatography (HPLC). Free 8-Isoprostane F_2 plasmatic levels were quantified with a competitive immunoenzymatic kit after alcaline hydrolysis of isoprostanes esters by Enzime Linked Immuno Sorbent Assay (ELISA) reaction. Evaluation of nitrite levels as estimative of nitric oxide (NO) concentration in plasma sample was estimated employing cadmium-copper-Griess reaction system. Protein Carbonyls content was evaluated by dinitrophenilhidrazine (DNPH) method. To evaluate antioxidant profile, total radical antioxidant parameter (TRAP) thought chemiluminescence assay was performed. We also determined superoxide dismutase activity through auto-oxidation inhibition of pirogalol method and catalase activity through hydrogen peroxide degradation method. Erythrocytic reduced glutathione (GSH) levels was determined through 5, 5' – dithiobis – 2 – nitrobenzoic acid (DTNB) method. All analysis were conducted in triplicate sets. Statistical analysis were perfomed using GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA), Microsoft Office Excel 2007 and OriginLab 8.0 software. Results were expressed as arithmetic means and standard error of means (SEM). Differences among groups were assessed by two-way analysis of variance (ANOVA) to lipid peroxidation curves, with Bonferroni´s test as pos-hoc, qui-square and Fisher's exact test for clinicapathological data, and Student's unpaired t Test or Mann-Whitney to others parameters. All data were checked in GraphPad Software to eliminate significant outliers ($p< 0.05$). Differences were considered statistically significant when $p< 0.05$. **Results:** Most of patients possessed infiltrating ductal carcinoma, while just 2.5% of BC group and 5% of HER^+ presented *in situ* carcinoma. Most BC, HER^- and HER^+ were also positive to ER ($p< 0.001$) and PR ($p<0.0001$). Advanced disease (TNM III/ IV) was prevalent in all groups ($p<0.0004$). Besides, histological tumor grade analysis classified most patients in grade 2 ($p< 0.005$). No difference was observed for chemotherapy treatments. Groups displayed normal levels of urea (ctr=29.00 ± 1.370 mg/dL; BC= 28.80 ± 1.442 mg/dL; HER^-= 28.55 ± 1.713 mg/dL; HER^+= 29.11 ± 1.873 mg/dL) and alterations in creatinine levels in BC (ctr=0.9190 ± 0.02785 mg/dL; BC= 0.8208 ± 0.0403 mg/dL; p= 0.014) and no alterations for HER2 status (HER^-= 0.7595 ± 0.03296 mg/dL; HER^+= 0.8965 ± 0.06374 mg/dL) was observed. Hepatic damage was higher in BC group, and we found that alterations tend to be HER2 dependent based on higher levels of AST in BC (ctr= 25.39 ± 1.174 U/L; BC= 47.64 ± 6.508 U/L; p= 0.0006) and HER^+ (HER^-= 36.55 ± 4.018 U/L; HER^+= 59.85 ± 10.12 U/L; p= 0.07), higher ALT levels in BC (ctr= 32.64 ± 1.427 U/L; BC= 42.49 ± 3.016 U/L; p= 0.007) and HER^+ (HER^-= 38.55 ± 2.950 U/L; HER^+= 47.59 ± 4.522 U/L; p= 0.09), and GGT levels in BC (ctr= 17.83 ± 2.079 U/L; BC= 94.88 ± 25.40 U/L; $p< 0.0001$) and HER^+ (HER^-= 37.71 ± 6.013 U/L; HER^+= 154.9 ± 40.33 U/L; p= 0.08). No alteration was observed in bilirrubin levels (ctr=0.5477 ± 0.02748 mg/dL; BC= 0.4697 ± 0.04771 mg/dL; HER^-= 0.4437 ± 0.07043 mg/dL; HER^+= 0.5027 ± 0.05014 mg/dL). CKT levels did not differ in BC (ctr=108.2 ± 38.70 U/L; BC= 60.16 ± 6.643 U/L) and tend to increase in HER^+ (HER^-= 51.05 ± 7.186 U/L; HER^+=70.88 ± 9.475 U/L; p=0.09) and no alteration in CKMB levels were found (ctr=1.300 ± 0.3462 U/L; BC= 2.231 ± 0.5719 U/L; HER^-= 1.762 ± 0.4923 U/L; HER^+= 2.778 ± 0.9237 U/L). BC showed significant increased levels of plasmatic iron (ctr= 81.83 ± 4.791 µg/dL; BC= 115.5 ± 9.168 µg/dL; HER^-= 114.7 ± 14.48 µg/dL; HER^+= 117.1 ± 14.29 µg/dL; p=

0.02) and ferritin (ctr=47.70 ± 2.831 mg/dL; BC= 279.9 ± 72.58 mg/dL; HER^-= 371.5 ± 153.9 mg/dL; HER^+= 154.0 ± 19.29 mg/dL; p= 0.0003) independently of HER2 status. To assess plasmatic and RBC oxidative stress, *tert*-butyl induced CL was performed applying Two-way ANOVA statistics to evaluate if curves are different, Bonferroni's test to check which curve's points are different and curve's Student's *t* test to analyze mean curves difference. RBC oxidative stress profile was significantly higher in BC group than in control (p< 0.0001) independently of HER2 status and no points of the curve was different. Integration of area under the curve (AUC) was increased only for BC group (ctr= 722,200 ± 58,990 AUC; BC= 1,097,000 ± 228,200 AUC; HER^-= 1,069,000 ± 40,560 AUC; HER^+= 1,086,000 ± 71,130 AUC; p< 0.05). Significantly higher levels of plasma lipid hydroperoxides in BC (p< 0.0005) and HER^- (p< 0.0001). Bonferroni's test did not show differences in points of the curves and no difference in AUC (ctr=1,262,000 ± 135,100 AUC; BC= 1,937,000 ± 308,500 AUC; HER^- = 2,217,000 ± 481,000 AUC; HER^+= 1,338,000 ± 346,800 AUC) was observed. MDA levels was decreased in HER^+ women (ctr= 63.99 ± 4.032 nM; BC= 70.60 ± 9.507 nM; HER^-= 73.81 ± 7.997 nM; HER^+= 65.88 ± 21.11 nM; p= 0.03). No alterations in pro-oxidative products were observed assessing levels of 8-isoprostanes F_2 (ctr= 144.6 ± 0.3083 pg/mL; BC= 144.4 ± 0.2976 pg/mL; HER^-= 144.5 ± 0.4841 pg/mL; HER^+= 144.3 ± 0.3899 pg/mL), protein carbonyls content (ctr= 9.087 ± 0.5459 nM; BC= 9.640 ± 0.4461 nM; HER^-= 9.438 ± 0.4649 nM; HER^+= 9.937 ± 0.6302 nM) and nitrite (ctr= 19.33 ± 1.532 μM; BC= 21.77 ± 1.402 μM; HER^-= 21.63 ± 1.795 μM; HER^+= 21.98 ± 2.308 μM). As uric acid levels were altered in BC (ctr=3.578 ± 0.1861 mg/dL; BC= 4.463 ± 0.2336 mg/dL; p= 0.004) and HER^+ (HER^-= 4.852 ± 0.3275 mg/dL; HER^+= 4.014 ± 0.1598 mg/dL; p= 0.03), antioxidant profile assessed by TRAP levels was corrected by uric acid levels. Results showed significant TRAP decrease in BC group (ctr= 366.6 ± 17.36 nM Trolox/ mgxdL-¹; BC= 200.7 ± 15.81 nM Trolox/ mgxdL^{-1}; p< 0.0001) independently of HER2 status (HER^-= 202.1 ± 20.99 nM Trolox/ mgxdL^{-1}; HER^+= 198.4 ± 23.29 nM Trolox/ mgxdL-¹). SOD evaluation showed significant augment in HER^+ group (ctr=3.744 ± 0.3148 USOD/mL; BC= 4.214 ± 0.4491 USOD/mL; HER^-= 3.234 ± 0.1661 USOD/mL; HER^+= 5.145 ± 0.8173 USOD/mL; p=0.03). Catalase activity was lowered in BC (ctr= 544.5 ± 11.20 Vabs/min/mL; BC= 504.7 ± 8.977 Vabs/min/mL; p= 0.007) independently of HER2 status (HER^-= 503.9 ± 13.12 Vabs/min/mL; HER^+= 505.6 ± 12.40 Vabs/min/mL). GSH levels displayed significant augment in HER^+ (ctr= 16.62 ± 1.813 nM; BC= 14.70 ± 1.428 nM; HER^-= 9.510 ± 1.687 nM; HER^+= 17.24 ± 2.457 nM; p= 0.04). **Discussion and conclusion:** In the present study we reported for the first time several pathological findings occurring in breast cancer women accordingly to HER2 overexpression. Iron metabolism evaluation employed as indicator of oxidative stress was increased in BC independently of HER2 status. It is well known that high levels of free iron can generate hydroxyl radicals through Fenton's reaction leading to lipid peroxidation, and we observed increased lipid peroxidation in plasma of HER^- group and RBC of BC by CL. Additionally, high levels of free iron potentiate oxidative stress and enhance hepatic damage, as noted in BC group dependent of HER2 presence. Ferritin levels are also increased in BC independently of HER2 overexpression presence and it could represent a compensatory response to contain iron overload. Here, we showed an increased lipid hydroperoxides formation in RBC in BC independently of HER2 status and increase in profile of pre-formed lipid hydroperoxides and MDA levels in the absence of HER2 overexpression. Thus, HER2

overexpression seems to be a protective pathway against lipid peroxidation. No alteration was found in others lipid peroxidation products as 8-isoprostanes F_2 and no differences were found for nitrite as estimative of NO and carbonyl content regarding BC and HER2 status. TRAP was decreased in BC associated with plasmatic uric acid levels, suggesting that low molecular weight molecules have been consumed as a defense against oxidative processes, independently of HER2 overexpression. Defenses against reactive oxygen species includes enzymes like SOD, which dismutate superoxide radical forming hydrogen peroxide and oxygen, and catalase, which removes hydrogen peroxide giving rise to water and oxygen. Our results show that HER2 overexpression increases SOD activity indicating that HER2 signaling leads to enhancement in superoxide anion or improves SOD catalytic activities. Studies have demonstrated a decrease in catalase activity in AD patients and the importance of hydrogen peroxide as positively mediators in PI3K/ Akt and p38 MAPK pathways present in $HER2^+$ tumor cells. In this report, we show that catalase reduction is a condition present in BC independently of HER2 status, even though $HER2^+$ women displaying increase in superoxide dismutase and, consequently, hydrogen peroxide. Induction of pathways mediated by Jun amino-terminal kinase (JNK), important pathway induced by HER family as a mechanism of apoptosis protection, can induce GSH production. GSH, in the presence of glutathione peroxidase, can also reduce hydrogen peroxide in water. As we demonstrated here HER2 overexpression prevented GSH decreases probably by a protective mechanism for hydrogen peroxide excess. In conclusion, this research provides new data about the involvement of HER2 overexpression and oxidative stress in bearing breast cancer women accordingly to HER2 status. Here, we reported that in breast cancer host, HER2 overexpression attenuates oxidative stress as it prevents plasmatic lipid peroxidation and MDA formation, increases SOD and GSH. Thus, this research contributes to further researches aiming to develop new anti-HER2 therapy.

Keywords: Breast – Cancer. Tumors – Genetics – Expression. Oncology. Antioxidants. Cancer cells.

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LISTA DE ABREVIATURAS E SIGLAS

AKT	proteína quinase B
ALT	alanina-aminotransferase
aPKC	proteína quinase C atípica
ASK1	Regulador quinase de apoptose 1
AST	aspartato-aminotransferase
BAD	membro pró-apoptótico da família Bcl-2, do inglês "*Bcl-2 antagonist of cell death*"
CAT	catalase
CD1	ciclina D1
CDC42	proteína controladora de divisão celular 42
CDK2	ciclina dependente de quinase 2
CDK6	ciclina dependente de quinase 6
CKMB	fração MB da creatina quinase
CKT	creatina quinase total
EGF	fator de crescimento epitelial
EGFR	receptores do fator de crescimento epitelial
ER	receptores de estrógeno
ERNs	espécies reativas do nitrogênio
EROs	espécies reativas do oxigênio
FKHR	fator de transcrição *forkhead*
GGT	gama-glutamil-transpeptidase
GPx	glutationa peroxidase
GPX	glutationa peroxidase
Grb2/ Sos	proteína 2 ligada a receptor de fator de crescimento/ filho de *Sevenless*
GSH	glutationa
GSK3	glicogênio sintase quinase 3
HER1	receptores do fator de crescimento epitelial 1
HER2/ ErBb2	receptores do fator de crescimento epitelial 2
HER3	receptores do fator de crescimento epitelial 3
HER4	receptores do fator de crescimento epitelial 4
MAPK	proteína quinase ativadora de mitógenos
MDA	malondialdeído

MDM2	do inglês, *"murine double minute 2"*
MEK-ERK	proteína quinase ativadora de mitógenos/ quinase regulada por sinais extracelulares
MMP2	metaloproteinases 2
NF-κB	fator nuclear-{kappa}B
NO	óxido nítrico
PAR	proteína de participação defeituosa
PI3K	fosfatidil inositol-3-quinase
PKC	proteína quinase C
PLCγ1	fosfolipase *Cγ1*
PR	receptores de progesterona
QL	quimiluminescência
SOD	superóxido dismutase
TNM	T, tumor; N, do inglês "node", linfonodo; e M, metástase
TRAP	capacidade antioxidante total plasmática
TSC1 e TSC2	complexo de esclerose tuberosa 1 e 2
UICC	União Internacional Contra o Câncer

SUMÁRIO

1 INTRODUÇÃO

1.1 CÂNCER DE MAMA: ASPECTOS GERAIS

O câncer de mama consiste na neoplasia maligna que mais mata mulheres em todo o mundo, sendo a mais incidente tanto em países desenvolvidos como em desenvolvimento (WHO, 2011). Estimativas para o ano de 2012 válidas para o ano de 2013 apontam ocorrência de aproximadamente 518.510 novos casos de cânceres no Brasil, onde 52.860 representam o câncer de mama para a população feminina, com risco estimado de 52 casos para cada 100 mil mulheres (INCA, 2011). O desenvolvimento do carcinoma de mama é multifatorial, onde o ambiente e susceptibilidades genéticas podem desempenhar um importante papel (JOHNSON-THOMPSON; GUTHRIE, 2000).

Clinicamente, os tumores são caracterizados segundo a classificação TNM (T, tumor; N, do inglês "node", linfonodo; e M, metástase). O estadiamento do câncer de mama, proposto pela União Internacional Contra o Câncer (UICC, 2004), considera o tamanho do tumor, presença/ ausência de metástases em linfonodos e presença/ ausência de metástases à distância. A classificação clínica e o estadiamento do câncer de mama pelo sistema TNM encontram-se exemplificados nas tabelas 1 e 2, respectivamente (BARROS et al., 2001; BRASIL, 2004; UICC, 2004).

O estadiamento de tumores segundo o TNM permite a caracterização do tumor, porém é falha para indicação terapêutica (VIEIRA et al., 2008). Perou et al. (2000) caracterizaram a variação do padrão de expressão genética em peças tumorais de mama, utilizando a técnica de DNA microarray. Os padrões encontrados forneceram um retrato molecular distinto de cada tumor, cujos padrões refletiram uma relação entre os tumores e conexões entre um gene específico e tumores específicos. Segundo essa classificação os tumores seriam identificados em quatro diferentes grupos: luminal A e B, os quais expressam tumores positivos para receptores de estrógeno (ER) e progesterona (PR), triplo negativo (também dito "*basal-like*") e receptores do fator de crescimento epitelial humano (HER2, do inglês:"*human epidermal growth factor 2*", também conhecidos como ErbB2) super-expresso (SIOSHANSI et al., 2011).

Tabela 1 – Classificação clínica do câncer de mama pelo sistema TNM.

T - Tumor	
Tx	O tumor primário não pode ser avaliado
T0	Sem evidência de tumor primário
Tis	Carcinoma *in situ*: carcinoma intraductal ou carcinoma lobular *in situ* ou doença de Paget da papila sem tumor
T1	Tumor com 2cm ou menos em sua maior dimensão
	T1a- tumor com 0,5cm ou menos em sua maior dimensão
	T1b- tumor com mais de 0,5cm e até 1cm em sua maior dimensão
	T1c- tumor com mais de 1cm e até 2cm em sua maior dimensão
T2	Tumor com mais de 2cm e até 5cm em sua maior dimensão
T3	Tumor com mais de 5cm em sua maior dimensão
T4	Tumor de qualquer tamanho, com extensão direta à parede torácica ou à pele
	T4a- extensão para parede torácica
	T4b- edema ou ulceração da pele da mama ou nódulos cutâneos satélites, confinados a mesma mama
	T4c- T4a e T4b associados
	T4d- carcinoma inflamatório
N- Linfonodo	
Nx	Os linfonodos regionais não podem ser avaliados
N0	Ausência de metástases nos linfonodos regionais
N1	Metástases em linfonodos auxiliares homolaterais móveis
N2	Metástases nos linfonodos axilares homolaterais fixos uns aos outros ou a outras estruturas
N3	Metástases nos linfonodos da cadeia mamária interna homolateral
M- Metástase	
Mx	Presença de metástases à distância não pode ser avaliada
M0	Ausência de metástases à distância
M1	Metástases à distância

Fonte: adaptado de Barros et al. (2001) e UICC (2004).

Assim, novos alvos terapêuticos estão sendo formulados visando o subtipo molecular do tumor. A superexpressão de HER2 tem sido um importante alvo de terapias, mesmo em tumores que possuem concomitantemente receptores para estrógeno e progesterona, devido aos fortes sinais reguladores de sobrevivência celular do tumor que essa superexpressão causa (DOGAN et al., 2011; HYNES; LANE, 2005). Contudo, terapias anti-HER permanecem inacessíveis a muitas mulheres e, quando acessível, algumas ainda podem se tornar resistentes a terapia (NAHTA et al., 2006).

Tabela 2 – Estadiamento do câncer de mama em função das diversas combinações possíveis pelo sistema TNM.

Estádio 0 (Carcinoma *in situ*)	Tis	N0	M0
Estádio I (Invasão local)	T1	N0	M0
Estádio IIa (Tumor primário limitado ou invasão linfática regional mínima)	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Estádio IIb (Tumor primário limitado ou invasão linfática regional mínima)	T2	N1	M0
	T3	N0	M0
Estádio IIIa (Tumor local extenso ou invasão linfática regional extensa)	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1, N2	M0
Estádio IIIb (Tumor local extenso ou invasão linfática regional extensa)	T4	Qualquer N	M0
Estádio IIIc (Tumor local extenso ou invasão linfática regional extensa)	Qualquer T	N3	M0
Estádio IV (Tumor localmente avançado ou presença de metástases à distância)	Qualquer T	Qualquer N	M1

Fonte: adaptado de Barros et al. (2001) e UICC (2004).

1.2 RECEPTORES HER2 E O CÂNCER DE MAMA

Durante o desenvolvimento do câncer de mama, 20% das pacientes apresentam positividade para a amplificação ou super expressão de HER2. A superexpressão de HER2 pode ser decorrente de amplificação genética ou desregulação transcricional (GUTIERREZ; SCHIFF, 2011).

Receptores HER2 estão inclusos na subclasse I de receptores tirosina quinase, a qual abrange quatro membros: receptores do fator de crescimento epitelial 1 (HER1), HER2, receptores do fator de crescimento epitelial 3 (HER3) e receptores do fator de crescimento epitelial 4 (HER4). Através de diferentes vias de sinalização ligante-dependente, os receptores da família HER medeiam diversos fatores de transcrição que atuam no controle de apoptose, migração, crescimento, adesão e diferenciação celular. Em processos

fisiológicos, estão relacionados com a embriogênese, organogênese, regeneração tecidual e cicatrização de feridas (YARDEN; SLIWKOWSKI, 2001).

Na membrana celular, o receptor HER2 é encontrado na forma de heterodímero ou homodímero. Os dímeros HER2 possuem atividade sinalizadora prolongada e atenuam a evasão da sinalização (YARDEN, 2001) (Figura 1).

Os receptores da família HER possuem uma região extracelular composta por quatro domínios: I – IV. A conformação extracelular de HER2 é fixa e se assemelha a um estado "ligante- ativado", onde os domínios de interação II-IV estão ausentes e o loop de dimerização no domínio II é exposto. Esse estado permite que HER2 seja o parceiro de dimerização preferencial por outros integrantes da família HER e faz com que a interação com um ligante não seja possível, pois o local de ligação encontra-se oculto e inacessível a ligações (HYNES; LANE, 2005).

Vias de sinalização celular ativadas por ligantes ao heterodímero HER2/ HER1, atuam diretamente em vias fosfolipase Cγ1 (PLCγ1), através do resíduo de tirosina-fosforilada 1248 do receptor HER2. Esta via tem relação indireta com a via proteína quinase C (PKC) e sinaliza oscilação da concentração de cálcio intracelular e reorganização dos filamentos de actina, formando projeções do citoesqueleto celular. Dessa forma, a ativação da via PLC está diretamente relacionada com o aumento da migração e progressão tumoral (DITTMAR et al., 2002).

A ativação do heterodímero HER2/ HER1 pode, ainda, favorecer a progressão tumoral e hiperproliferação. A superexpressão de HER2 nessa via ativa e amplifica a sinalização ras através de complexos proteína 2 ligada a receptor de fator de crescimento/ filho de Sevenless (Grb2/ Sos), mediando indiretamente os fatores nucleares ciclina D1 (CD1), ciclina dependente de quinas K6 (CDK6), ciclina E e $P27^{KIP1}$ (JANES et al., 1994).

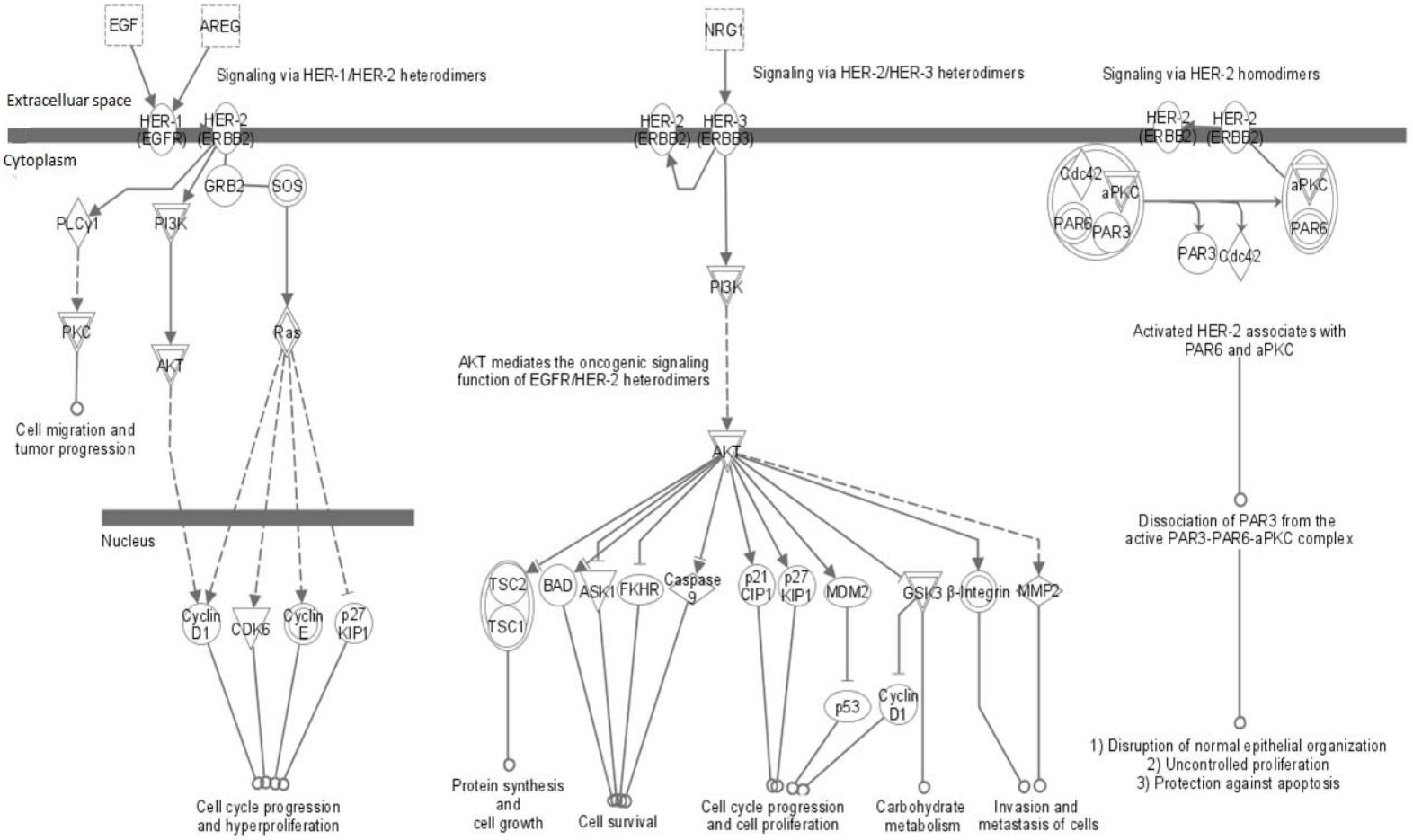
Uma vez que o receptor HER2 se apresenta na forma de homodímero na membrana celular não há ligante conhecido (YARDEN; SLIWKOWSKI, 2001). Quando em homodímero, o HER2 age sobre o complexo de proteína de participação defeituosa (PAR), dissociando o PAR-3 do complexo ativo PAR3-PAR6- proteína quinase C atípica (aPKC)-proteína controladora de divisão celular 42 (CDC42) e associando PAR6-aPKC. A dissociação do complexo PAR rompe a organização normal do epitélio, levando a proliferação descontrolada e proteção a apoptose (ARANDA et al., 2006). É bem descrito na literatura a sinalização intracelular que vias mediadas por HER2 incluem também ras-proteína quinase ativadora de mitógenos (MAPK), MAPK quinase S6 independente e

fosfolipase C-gama. Porém as conseqüências biológicas da ativação dessas vias não são completamente conhecidas (KUREBAYASHI, 2001). Recentemente, Tanizaki e colaboradores (2011) publicaram que a superexpressão de HER2 pode ativar também vias de sinalizações de proteína quinase ativadora de mitógenos/ quinase regulada por sinais extracelulares (MEK-ERK) como um mecanismo a fim de inibir a apoptose.

O dímero HER2 é o parceiro de dimerização preferencial de todos dímeros da família HER (GRAUS-PORTA et al., 1997). Zhou e Agazie (2011) demonstraram que superexpressão de HER2 sozinha aumenta a cascata de sinalização basal por ele estimulada. Contudo, quando estimulado por fator de crescimento epitelial (EGF), mesmo em baixas concentrações, ocorre aumento da intensidade da sinalização. Foi também demonstrado por esse grupo de pesquisa que quando inibida a estimulação de receptores do fator de crescimento epitelial (EGFR), observa-se a supressão da via EGFR- ERK1/ 2 – proteína quinase B (AKT) na superexpressão de HER2. Assim, considera-se que o EGFR é necessário para a sustentação do sinal e transformação celular mediados pela superexpressão de HER2.

A sinalização via heterodímero HER2/ HER3 é conhecida principalmente pela ativação de fosfatidil inositol-3-quinase (PI3K), agindo indiretamente sob a via AKT, a qual é responsável por mediar sinalizações oncogênicas diversas. É conhecido que a estimulação inicial de metástase e invasão do tumor é mediado por ativação de HER3 através de vias PI3K (SMIRNOVA et al., 2011).

Figura 1 – Vias de sinalização mediadas por membros da família HER (fonte: IPA Ingenuity Systems).



Dentre as sinalizações mediadas por HER2/ HER3-PI3K-AKT, encontra-se a fosforilação do complexo de esclerose tuberosa 1 e 2 (TSC1 e TSC2) e $p27^{KIP1}$. Como resultado da ativação dessa via, ocorre aumento da atividade de ciclina dependente de quinase 2 (CDK2), de síntese de DNA e da fase S do ciclo celular. Dessa forma, este processo acarreta em síntese de proteínas e crescimento celular (DAN et al., 2002). O heterodímero HER2/ HER3 pode também mediar vias AKT fosforilando diretamente a caspase-9 e membro pró-apoptótico da família Bcl-2 (BAD) ou regular negativamente regulador quinase de apoptose (ASK1) (KIM et al., 2001) e fator de transcrição *forkhead* (FKHR) (TANG et al., 1999), estimulando a sobrevivência celular tumoral.

A proliferação e progressão do ciclo celular podem ser estimuladas por HER2/HER3 pela ativação direta da AKT sobre $p21^{CIP1}$ (ZHOU et al., 2001) e proteína MDM2 (do inglês, *murine double minute 2*) (HIGASHIYAMA et al., 1997). A AKT pode se associar com $p21^{CIP1}$ e fosforilar o resíduo treonina 145 no núcleo da célula. Essa fosforilação irá mediar um sinal para a localização citoplasmática de $p21^{CIP1}$, promovendo crescimento celular (ZHOU et al., 2001). A oncoproteína MDM2 já é descrita como super-expressa em alguns tumores, possuindo função inibitória de p53 (HIGASHIYAMA et al., 1997).

A proliferação e progressão do ciclo celular pode, também, ser favorecida através da inibição de glicogênio sintase quinase 3 (GSK3), inibindo a CD1. A ativação HER2/ HER3, é capaz, ainda, de favorecer o metabolismo de carboidratos através da inibição de GSK3. Além disso, mecanismos de invasão e metástase celular são mediados pela ativação de β-integrina e inibição de metaloproteinases 2 (MMP2) (MANOUKIAN; WOODGETT, 2002).

A forma heterodímera HER2/ HER4 é receptora para um grupo de proteínas denominadas neuregulinas e está associado a processos de quimiotaxias, proliferação e diferenciação celular (YARDEN; SLIWKOWSKI, 2001).

Assim, fica clara a relação entre a superexpressão de HER2 com o mau prognóstico de mulheres portadoras do câncer de mama que apresentam tumores positivos para a superexpressão de HER2 e não possuem tratamento alvo para este receptor.

1.3 Participação do Estresse Oxidativo como Regulador em Vias de Sinalização HER

Espécies reativas são definidas como moléculas orgânicas, inorgânicas e átomos os quais possuem um ou mais elétrons não pareados, com capacidade de existência

independente. Quando o elétron desemparelhado encontra-se centrado nos átomos de oxigênio ou nitrogênio são denominados espécies reativas do oxigênio (EROs) ou espécies reativas do nitrogênio (ERNs), respectivamente. A instabilidade das espécies reativas é atribuída à sua configuração, a qual conduz a uma meia-vida curta e confere alta reatividade (HALLIWELL, 1989). Em contrapartida, o organismo dispõe de um arsenal de moléculas de defesas antioxidantes que agem como *scavengers* de espécies reativas (HALLIWELL; GUTTERIDGE, 2007).

O sistema de defesa antioxidante é dividido em enzimático e não enzimático. O sistema enzimático inclui enzimas como a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX). O sistema não enzimático inclui compostos sintetizados pelo organismo, como a glutationa (GSH), ácido úrico, bilirrubina e hormônios sexuais, e por compostos ingeridos pela dieta, como flavonóides, ácido ascórbico e α-tocoferol. (NIJVELDT et al., 2001; SCHNEIDER; OLIVEIRA, 2004). De acordo com Halliwell e Gutteridge (2007), a defesa antioxidante pode freqüentemente ser induzida pela exposição do organismo a espécies reativas e a moléculas sinalizadoras celulares, como as citocinas. Assim, estresse oxidativo é caracterizado pelo desequilíbrio entre moléculas oxidantes e antioxidantes, em favor das oxidantes, resultando em danos celulares.

A iniciação de tumores pode ser mediada por espécies reativas (ROBBINS et al., 2010). Porém, sabe-se que células em repouso permanecem em um ambiente geralmente reduzido, com altas concentrações de glutationa reduzida e outros antioxidantes. Em níveis moderados de estresse oxidativo, a célula é estimulada a proliferar, envolvendo um aumento de cálcio intracelular e aumento de fosforilação de proteínas. Quando os níveis de estresse oxidativo são considerados mais altos, danos oxidativos a estruturas celulares se instalam. Caso esses danos não sejam reparados e os danos oxidativos não cessados, a célula é estimulada a entrar em apoptose ou necrose (HALLIWELL, 2007). Assim, torna-se claro o papel regulador do estresse oxidativo na sobrevivência celular.

Atualmente, trabalhos na literatura vêem demonstrando que muitos produtos da ação de espécies reativas possuem papel regulador no câncer. Mannello e colaboradores (2007) mensuraram níveis de malondialdeído (MDA) e 8- epímero de prostaglandina $F_{2\alpha}$, dois produtos de lipoperoxidação, em fluido aspirado mamário de mulheres saudáveis e com câncer de mama. Foi encontrado, por este grupo de pesquisa, que níveis de 8- epímero de prostaglandina $F_{2\alpha}$ foram maiores em mulheres saudáveis do que em mulheres com câncer de mama e nenhuma diferença nos níveis de MDA. Os resultados desse grupo sugerem um papel fisiológico para 8- epímero de prostaglandina $F_{2\alpha}$ em glândulas mamárias normais. Gago-

Dominguez et al. (2007) também propõem a lipoperoxidação como um mecanismo protetor no câncer de mama, onde produtos da lipoperoxidação podem participar de cascatas de sinalizações mediando o controle de proliferação celular, indução de diferenciação e apoptose.

Há ainda trabalhos que mostram a participação de espécies reativas, como o peróxido de hidrogênio, como modulador de vias PI3K/ AKT e p38 MAPK (ANGELONI et al., 2010), importante via sinalizada por ativação de receptores HER2 como já discutido anteriormente. Trabalhos *in vitro* também demonstram que antraquinonas podem ser capazes de induzir apoptose em células que super-expressam HER2 através do aumento da expressão de caspase 9 e p53 através de mediação de espécies reativas (CHANG et al., 2011).

Seo e colaboradores (2011) mostraram que antioxidantes como a genisteína e quercetína são capazes de induzir a via de apoptose extrínseca através de super-regulação de p53 e inibindo a sinalização por fator nuclear-{kappa}B (NF-κB). Outros trabalhos *in vitro*, como os de Shin-Kang et al. (2011) demonstram que antioxidantes como tocotrienóis reduzem a ativação de MAPK-ERK e suprimem a ativação de AKT e levando a baixa-regulação de GSK3, outra importante via a qual quando o HER2 é super-expresso, possui sinalização ativada. Kuo e colaboradores (2011) também demonstraram que um alcalóide (berbearina) pode suprimir o crescimento de células tumorais que super-expressam HER2 através de modulação da via de sinalização HER2/ PI3K/ AKT. Este composto é capaz de interferir na expressão de CD1 e ciclina E e induzir apoptose através da indução de vias mitocondriais/ caspases.

Assim, pode-se concluir que espécies reativas e antioxidantes medeiam importantes vias de sinalização celular que são super ativadas em células com amplificação/ superexpressão de HER2. Essas vias nas células tumorais agem em favor do tumor, gerando características mais agressivas. Assim, recentes trabalhos visam colaborar com o entendimento do funcionamento das vias celulares e pouco se sabe sobre as conseqüências da ativação dessas vias no hospedeiro.

2 OBJETIVOS

Avaliar a influência da expressão da proteína HER2 em parâmetros oxidantes/ antioxidantes em mulheres com câncer de mama.

2.1 OBJETIVOS ESPECÍFICOS

- Caracterizar os níveis de expressão protéica de HER2 em pacientes portadoras de carcinoma ductal mamário infiltrativo ou *in situ*;
- Avaliar o perfil de dano sistêmico sobre o tecido cardíaco, hepático e renal;
- Caracterizar o metabolismo do ferro e perfil pró-oxidativo;
- Avaliar o perfil antioxidante.

2.2 ESTRATÉGIAS

Para avaliar a expressão tecidual de HER2, amostras de tumores serão identificadas por imunohistoquímica pelo método da avidina-peroxidase. A caracterização do dano tecidual específico será feita através da medida dos níveis plasmáticos creatina quinase total (CKT) e da fração MB (CKMB) e, creatinina, aspartato-aminotransferase (AST), alanina-aminotransferase (ALT), gama-glutamil-transpeptidase (GGT), bilirrubina total. O metabolismo do ferro será acessado através dos níveis de ferro plasmático e ferritina.

A avaliação do perfil pró-oxidativo será realizada através de quimiluminescência induzida por *tert*-butil (QL), níveis de malondialdeído (MDA), 8-isoprostanos F_2, nitrito como estimativa de óxido nítrico (NO) e proteínas carboniladas. O perfil antioxidante será mensurado através da atividade das enzimas SOD e CAT, níveis de GSH, ácido úrico e capacidade antioxidante total (TRAP).

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ANEXO

ANEXO A

Normas Técnicas para Publicação na Revista Científica *"Breast Cancer Research and Treatment"*

Types of papers:

The journal publishes articles dealing with original laboratory investigations and articles dealing with clinical studies. It also hosts invited review articles, pro and con discussions of controversial subjects, meeting reports, editorials, and letters.

The length of submitted papers should not exceed 3500 words.

Tumor Marker Studies .

Manuscript describing the results of tumor marker studies should include the essential elements of "Reporting recommendations for tumor marker prognostic studies (REMARK)" (McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM (2006). Reporting recommendations for tumor marker prognostic studies (REMARK). Breast Cancer Res Treat 100(2):229-235).

Failure to do so will result in the manuscript being returned to the author without peer review, as outlined by the editors of Breast Cancer Research and Treatment : Hayes DF, Ethier S, Lippman ME (2006) New guidelines for reporting of tumor marker studies in breast cancer research and treatment: REMARK. Breast Cancer Res Treat 100(2):237-238). J Clin Oncol. 2005 23:9067-9072.

Reporting recommendations for tumor marker prognostic studies (REMARK)

New guidelines for reporting of tumor marker studies in breast cancer research and treatment: REMARK

Cost Effective Analyses

The Editors are interested in scholarly cost-effectiveness studies. However, any such study will require a documented statement that either the study was not supported by a pharmaceutical company, or if so, that the sponsor had no input into design or analysis of the cost effective analysis, nor did they have final review. Each author should be asked to document his or her role in the study. As with any manuscript, potential authors are encouraged to decline if they feel that their role is insufficient to merit their inclusion.

For more details, authors are referred to Lippman et al (2009) Cost effective analyes. Breast Cancer Res Treat 115:221-222.

Cost effective analyses

Cell Line Studies

In general, Breast Cancer Research and Treatment will not accept for publication papers in which all of the data shown in the paper were obtained using a single cell line. Indeed, for most studies, experiments involving the use of multiple cell lines (more than 2) is highly recommended. In rare exceptions 'single cell line' papers will be considered but only when the use of a single line was necessitated by the scope of the other experiments, such as those involving screens of thousands of shRNAs or compounds. When submitting a paper in which all reported data were obtained with a single cell line, the authors must justifiy why one cell line was used, or the paper will be returned without review.

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Authors should submit their manuscripts online. Electronic submission substantially reduces the editorial processing and reviewing times and shortens overall publication times. Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

Title page

The title page should include:

o The name(s) of the author(s)

o A concise and informative title

o The affiliation(s) and address(es) of the author(s)
o The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 300 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Manuscripts should be submitted in Word.

o Use a normal, plain font (e.g., 10-point Times Roman) for text.
o Use italics for emphasis.
o Use the automatic page numbering function to number the pages.
o Do not use field functions.
o Use tab stops or other commands for indents, not the space bar.
o Use the table function, not spreadsheets, to make tables.
o Use the equation editor or MathType for equations.
o Note: If you use Word 2007, do not create the equations with the default equation editor but use the Microsoft equation editor or MathType instead.
o Save your file in doc format. Do not submit docx files.

Manuscripts with mathematical content can also be submitted in LaTeX.

o LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter. A table of Abbreviations can be included following the Abstract.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.
Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:
1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
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o Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8
Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

o Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

o Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

o Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

o Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

o Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

o www.issn.org/2-22661-LTWA-online.php

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

o EndNote style (zip, 2 kB)

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

o All tables are to be numbered using Arabic numerals.

o Tables should always be cited in text in consecutive numerical order.

o For each table, please supply a table caption (title) explaining the components of the table.

o Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
o Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

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o Supply all figures electronically.
o Indicate what graphics program was used to create the artwork.
o For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
o Vector graphics containing fonts must have the fonts embedded in the files.
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Line Art

o Definition: Black and white graphic with no shading.
o Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
o All lines should be at least 0.1 mm (0.3 pt) wide.
o Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
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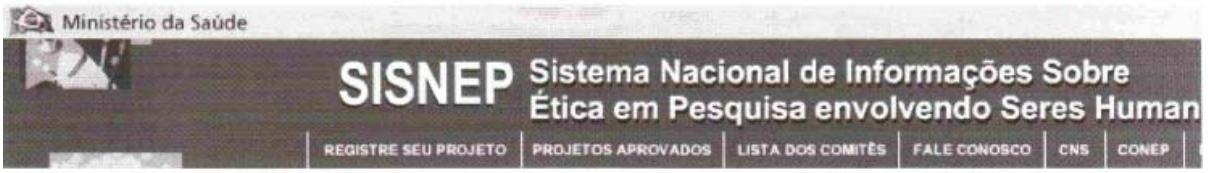
ANEXO B

Aprovação do Comitê de Ética em Pesquisa

CAAE 0009.0.268.000-07



Projetos Aprovados - PR no ano de 2007

Busca de Projeto de Pesquisa por palavra chave: Filtrar

Total de Projetos encontrados: 8

CAAE	Título do Projeto	Instituição Sediadora
0009.0.268.000-07	ESTUDO DA ANEMIA HEMOLÍTICA E DOS MECANISMOS OXIDATIVOS PRÉ-HEMOLÍTICOS EM PACIENTES PORTADORES DE CANCER DE MAMA SUBMETIDOS A QUIMIOTERAPIA PELO PACLITAXEL.	Universidade Estadual de Londrina e Hospital Universitár Regional do Norte do Paraná - UEL
0301.0.093.000-06	Prevalência da anemia ferropriva em crianças de 6 a 24 meses em centros municipais de educação infantil de Cascavel, Paraná.	Universidade Estadual de Maringá
0073.0.093.000-07	Ocorrência de anemia em pacientes atendidos no laboratório Labomar na cidade de Luiziania-PR	Universidade Estadual de Maringá
0103.0.093.000-07	Prevalência de Anemia em Pacientes Atendidos pelo LEPAC-UEM	Universidade Estadual de Maringá
0175.0.208.000-07	Orientações Nutricionais para Pacientes Portadores de Anemia Falciforme	Hospital de Clínicas - Universidade Federal do Paran - UFPR
0086.0.078.000-07	Trajetoria de um individuo no enfrentamento da anemia aplastica.	Universidade Norte do Paraná - UNOPAR
0189.0.093.000-07	Estudo da Freqüência de Anemia Ferropriva em crianças (0-12 anos) atendidas pelo LEPAC	Universidade Estadual de Maringá
0201.0.093.000-07	Distribuição espacial da anemia:prevalência em escolares ingressantes nas escolas públicas de Maringá-PR, no ano de 2007	Universidade Estadual de Maringá

Andamento do projeto - CAAE - 0009.0.268.000-07

Título do Projeto de Pesquisa

ESTUDO DA ANEMIA HEMOLÍTICA E DOS MECANISMOS OXIDATIVOS PRÉ-HEMOLÍTICOS EM PACIENTES PORTADORES DE CANCER DE MAMA SUBMETIDOS A QUIMIOTERAPIA PELO PACLITAXEL.

Situação	Data Inicial no CEP	Data Final no CEP	Data Inicial na CONEP	Data Final na CONEP
Aprovado no CEP	06/02/2007 18:25:02	20/03/2007 09:46:07		

Descrição	Data	Documento	Nº do Doc	Origem
2 - Recebimento de Protocolo pelo CEP (Check-List)	06/02/2007 18:25:02	Folha de Rosto	0009.0.268.000-07	CEP
1 - Envio da Folha de Rosto pela Internet	01/02/2007 11:18:31	Folha de Rosto	FR121928	Pesquisador
3 - Protocolo Aprovado no CEP	20/03/2007 09:46:07	Folha de Rosto	008/07	CEP

ANEXO C

Termo de Consentimento Livre e Esclarecido

A – <u>Informações sobre a pesquisa:</u>

Você está sendo convidada a participar, como voluntária, da pesquisa intitulada **"**ESTUDO DA ANEMIA HEMOLÍTICA E DOS MECANISMOS OXIDATIVOS PRÉ-HEMOLÍTICOS EM PACIENTES PORTADORES DE CANCER DE MAMA SUBMETIDOS À QUIMIOTERAPIA**",** que tem por objetivo avaliar os níveis de lesão pré-hemolítica e o estresse oxidativo no sangue de pacientes antes e após a sessão de quimioterapia.
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.

B – <u>Procedimentos do Estudo:</u>

Os procedimentos da pesquisa envolvem a obtenção de 20mL de sangue periférico antes e após cada sessão de quimioterapia. Serão analisados o estresse oxidativo (lipidio hidroperoxido de eritrócitos e plasma, capacidade antioxidante do plasma, as enzimas superóxido dismutase e catalase e sistema GSH de hemácias e adicionalmente, os parâmetros hematológicos de rotina. Nossa espectativa é de que este estudo possa motivar pesquisas posteriores clínicas e experimentais empregando antioxidantes no protocolo de tratamento com este e outros quimioterápicos que induzem hemólise e anemia.

C – <u>Confidencialidade da Pesquisa</u>

As informações obtidas natravés desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados não serão divulgados de forma a possibilitar sua identificação.

.

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O coordenador do projeto é o Prof. Dr Rubens Cecchini, que pode ser encontrado no endereço: Rod. Celso Garcia cid, 445, Departamento de ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970.

D – <u>Consentimento livre esclarecido e informado:</u>

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ANEXO D

Artigo: Oxidative Stress Profile of Breast Cancer Patients is Correlated with HER-2/neu Protein Overexpression

Este é um trabalho realizado no Laboratório de Patofisiologia dos Radicais Livres da Universidade Estadual de Londrina, formado pelo artigo científico: **Oxidative stress profile of breast cancer patients is correlated with HER-2/neu protein overexpression.** V. J. Victorino, C. Panis, F. C. Campos, A. C. A. Herrera, A. N. Colado-Simão, A. L. Cecchini, R. Cecchini.

As formatações do artigo seguem as normas da revista *Breast Cancer Research and Treatment* (Anexo 1).

Oxidative stress profile of breast cancer patients is related with HER-2/neu protein overexpression

Run-title: Oxidative stress in HER-2/neu breast cancer patients

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Abstract: During breast cancer development, about 20% of patients show overexpression of human epidermal growth factor receptor-2 (HER2) which is associated with tumor malignancy. The influence of HER2 overexpression in oxidant/ antioxidant parameters in humans remains unknown. We investigated the pro/ antioxidant profile in women bearing HER2 positive tumor. Women were divided into 52 healthy age-matched controls and 52 breast cancer women (BC), divided into 30 negative for HER2 presence (HER^-) and 22 positive for HER2 overexpression (HER^+) determined by immunohistochemical or fluorescence *in situ* hybridization (FISH). We measured plasmatic iron metabolism and tissue-specific damage, systemic oxidative profile was evaluated through high sensitive chemiluminescence, malondialdehyde (MDA) levels, free 8-isoprostanes F2 levels, protein carbonyl content and estimate nitric oxide (NO) levels through nitrite levels. Antioxidant profile was assessing through measurement of total radical antioxidant parameter (TRAP), superoxide dismutase (SOD), catalase activity and glutathione (GSH) levels determination. We found iron metabolism evaluation increased in BC independently of HER2 overexpression. High levels of plasmatic iron potentiate oxidative stress and enhance hepatic damage, as noted in BC group probably dependent of HER2 presence. Ferritin levels are increased in BC and it could represent a compensatory response to contain iron overload. We found increased hepatic and cardiac damage in BC more pronounced for HER2 presence. Increased red blood cells lipid peroxidation in BC and plasmatic lipid peroxidation in BC and HER^- and decreased MDA levels in HER^+ were found suggesting that HER2 overexpression protects against plasmatic lipid peroxidation. No alteration was found for 8-isoprostanes F_2, nitrite and carbonyl content levels. TRAP was decreased in BC, suggesting that antioxidants have been consumed as a defense against oxidative processes. HER2 overexpression increased SOD activity indicating that HER2 signaling leads to enhancement in superoxide anion or improves SOD catalytic activities and catalase activity was decreased in BC. We also demonstrated that HER2 overexpression prevented GSH decreases probably by a protective mechanism for hydrogen peroxide excess. Taken together, we show that HER2 overexpression attenuates oxidative stress as it prevents plasmatic lipid peroxidation and MDA formation, increases SOD and GSH, contributing to further researches aiming to develop new anti-HER2 therapy.

Keywords: HER2 – oxidative stress – pro-oxidative profile –antioxidant profile.

Introduction

Breast cancer is the most lethal malignancy in women worldwide, presenting higher incidence in both developed and developing countries [1]. During breast cancer evolution, about 20% of patients show amplification or overexpression of human epidermal growth factor receptor-2 (HER2/neu, also known as ErbB2) [2]. Overexpression of HER2 can be due to genetic amplification or transcriptional deregulation. HER2 are included in tyrosine-kinase subclasse I of receptors, which embraces four members: HER1, HER2, HER3 and HER4 [3].

HER2 is present as dimmers in cell surface, as homodimer with another HER2 or heterodimer with HER1, HER3 or HER4. Overexpression of these receptors present prolonged signaling activity and through distinct pathways they can mediate several transcriptional factors that decrease apoptosis [4] augmenting cell survival [5,6], increase cell migration, favor tumor progression [7] and growing [8], induce protein synthesis [9], stimulates proliferation and cellular cycle activation [8,10]. In this context, it is apparent that overexpressiong of HER2 in breast cancer is associated with tumor malignancy.

In vitro studies in the literature suggest that HER2 stimulation minimize oxidative stress [11], leading to reduced damage to cell structures. In addition, HER2 activated pathways can be mediated by products of oxidative stress [12] and reactive oxygen species [13-17]. Moreover, it is also known that oxidative stress can modulate cellular fate, stimulating cellular proliferation or death in mild or high concentrations, respectively [18]. Thus, overexpression of those receptors in breast cancer may play a protective role for the tumor favoring its growing and metastasis. Such findings are based on the indirect relationship between mediators of oxidative stress and HER2 obtained by *in vitro* studies. Although HER2 is overexpressed in several tumors types and this receptor has been widely studied and employed as a target of therapeutic research [19], the influence of HER2 overexpressed in oxidant/ antioxidant parameters in humans remains unknown. In this report, we investigated the oxidative damages and the antioxidant profile in women bearing HER2 positive tumor. To reach these goals, we characterized HER2 protein levels breast tumor tissue, measured iron metabolism, tissue-specific damage and systemic oxidative and also antioxidant profile.

Subjects and Methods

This study was approved by Research and Ethics National Council (CAAE 0009.0.268.000-07) and patients provided signed informed consent.

Patient Selection and Study Design

A prospective study of 104 volunteer's was undertaken between January 2009 and January 2011. Women were divided into 52 healthy age-matched controls and 52 breast cancer women (BC). BC group was divided into 30 negative for HER2 overexpression (HER^-) and 22 positive for HER2 protein overexpression (HER^+) determined by immunohistochemical (IHC) employing streptavidin biotin method or fluorescence in situ hybridization (FISH) for IHC 2+. Excluded subjects from this study were smokers, regular alcohol consumers, antioxidant supplement users, pregnancy, lactation, excessive physical exercises practitioners, hormone replacement therapy users and with another chronic disease. Patients' characterization included age at diagnosis, TNM (T= tumor, N= node, M= metastasis) classification, histological tumor grade, hormonal receptors (estrogen receptors - ER, progesterone receptors - PR) and chemotherapy treatment. Heparinized blood of all participants was collected at Department of General Pathology, Londrina State University, PR-Brazil. Blood was centrifuged for red blood cells (RBC) and plasma obtainment. All analysis employing RBC were performed at the collect day and plasma aliquots were stored in -86°C (Indrel Ultra Freezer) to further analysis.

Biochemical Analysis

Biochemical analysis were performed in plasma samples to assess heart, kidneys and liver damage through measurement of total creatine-kinase (CKT) and MB fraction (CKMB), urea, creatinine, aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), gamma-glutamyl-transpeptidase (GGT) and total bilirubin. To assay iron metabolism, ferritin and plasmatic iron were measured. Levels of uric acid were also evaluated. Analyses were automatically performed in Dimension RxL® (Siemens Corp., Illinois - USA).

Evaluation of Pro-oxidative Profile

Oxidative stress - High sensitive chemiluminescence (CL)

To evaluate oxidative stress in RBC and plasma samples, a chemiluminescence-based method was employed [20]. RBC was diluted 1200x in 10mM monobasic phosphate buffer, at 37°C and reaction started with addition of 10µL of 3mM tert-butyl hydroperoxide. Plasmatic chemiluminescence reaction was initiated by the addiction of 10µL of 3mM tert-butyl hydroperoxide in 125µL of plasma and 865µL of 30mM disodium phosphate-KCl 120mM buffer, pH 7.4, 37°C. Readings were performed in Glomax luminometer (TD 20/20 Turner Designers) for 40 minutes, one reading/ second. Data were treated and analyzed in Origin 8.0 Software and results were expressed in relative light's units (RLU).

Malondialdehyde (MDA) levels

High performance liquid chromatography (HPLC) determinations were made in equipment HPLC-20AT Shimadzu equipped with a LC20AT pump and SPDM20A UV – diode array absorbance detector employing a C18 reverse phase column. To determine MDA concentration, a standard curve was performed as Karatas (2002) [21]. For preparation of standard solution of MDA, 10mL of 0.1M HCl were added in 10mL of 1,1,3,3-tetraetoxipropane (TEP) and this solution was maintained for 5 minutes in boiling water, following ice bath to complete synthesis of MDA. To improve chromatograms purity and to preserve the conservation of HPLC column, a new method was adapted [21, 22]. A 160µL of plasma samples or standard solution reacted with 100µL of 0.5M percloric acid. Samples were centrifuged for 5 minutes, 5000 x *g*, 4°C. A 180µL of supernatant was recovered to react with 100µL of thiobarbituric acid for 30 minutes, 95°C. Reaction was stopped by ice bath and 100µL of 1M NaH_2PO_4, pH 7.0, were added to stabilize sample pH. Further, samples were centrifuged for 10 minutes, 5000 x *g* at 4°C. Mobile phase was constituted of 65% 50mM KH_2PO_4 buffer, pH 7.0, and 35% methanol HPLC grade. Readings were executed at 535nm during 12 minutes with isocratic flow of 0.8mL/minute and results were expressed in nM of MDA.

Free 8-isoprostanes F_2 levels in plasma

8-Isoprostane F_2 plasmatic levels were quantified with a competitive immunoenzymatic kit (Cayman Chemical, USA), after alcaline hydrolysis of isoprostanes esters. Supernatants were added to the microplate Enzime Linked Immuno Sorbent Assay (ELISA) reaction. All sample concentrations were determined when compared to recombinant standard curve in pg/mL.

Evaluation of nitrite levels

Nitric oxide (NO) concentration in plasma sample was estimated by measuring nitrite as previous described [23]. About 60µL of plasma samples were deproteinized by adding 50µL of 75mM $ZnSO_4$ solution (Merck). Samples were mixed, centrifuged at 9500 x *g* for 2 minutes, 25°C and deproteinized with 55mM NaOH (Merck). Samples were centrifuged at 9500 x *g* for 5 minutes, 25°C and supernatants recovered and diluted 5:1 in 45 g/L glycine, pH 9.7 (Merck). Cadmium granules (Fluka) were activated in 5mM $CuSO_4$ in 15g/L glycine-NaOH buffer, pH 9.7 (Merck) during 5 minutes. Activated granules were added to samples and incubated for 10 minutes. Griess reagent (Sigma) was added to supernatants. A calibration curve was prepared by dilution of $NaNO_2$ (Merck) in distilled sterile water. The absorbance was performed at 550 nm using a standard microplate reader (Multiskan EX, LabSystems, Minnesota USA). Results were expressed in µM of nitrite.

Protein Carbonyls

To evaluate protein carbonyls levels, 100µL of plasma sample reacted with 1mL of dinitrophenilhidrazine 10mM and HCl 2.5M were employed to precipitate proteins. After 1 hour, precipitation with 1.25mL of trichloroacetic acid (TCA) 20% was performed following a 20 minutes ice bath and 15 minutes of 1500 x *g* centrifugation. A 1.25mL of TCA 10% was added to pellet. After 20 minutes of ice bath, samples were centrifuged for 15 minutes, 1500 x *g*. Pellets were rinsed with ethanol and diluted in 6M guanidine [24]. Readings were performed in spectrophotometer (UV- Shimadzu 355-390nm) and results were expressed in nM mL^{-1} mg^{-1} total proteins based on molar extinction coefficient of 22 M^{-1} cm^{-1}.

Evaluation of Antioxidant Profile

Total Radical Antioxidant Parameter (TRAP) Thought Chemiluminescence Assay

2, 2'azo-bis, 2 amidinopropane (ABAP), a potent free radical generator decomposes itself and emits photons in this process. The action of ABAP is neutralized as long as antioxidants are capable of inhibiting its function. Initially ABAP emission (900µL of glicine buffer 0.1 M pH 8.6, 50µL of luminol and 50µL of ABAP) was measured as a pro-emission standard. An antioxidant standard solution (Trolox - 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 25µM) was added in order to neutralize ABAP autoxidation (830µL of Glicine buffer 0.1 M pH 8.6, 70µL of Trolox, 50µL of luminol and 50µL ABAP). Subsequently, plasma samples diluted 1:50 (830µL of Glicine buffer 0.1M pH 8.6, 70µL of sample, 50µL of luminol and 50µL of ABAP) were used. Readings were performed in a Glomax luminometer (Turner Designs TD 20/20) during 30 minutes, 5 readings/ second.Time which sample's antioxidants can inhibit ABAP action was compared to Trolox. Results were expressed as nM sample equivalents of Trolox [25].

Superoxide Dismutase (SOD) activity determination

RBC were hemolysed in distillated water in a proportion of 1:20. Runs of 5µL, 10µL and 20µL of samples were measured. To each run, destilated water, 1M TRIS (tris – hydroxymethyl – aminomethane) buffer and pirogalol (1.2 mg/mL) were added. The auto-oxidation inhibition of pirogalol was measured at 420 nm in spectrophotometer (Shimadzu UV-1650 PC) during 6 minutes kinetic. The results were expressed as SOD unities/mL accordingly to recommendations for SOD activity over pirogalol oxidation [26].

Catalase activity determination

RBC were diluted in distillated water in a proportion of 1:80. Then, 10µL of sample were incubated in a system containing 1M TRIS buffer and 200 mM hydrogen peroxide. Kinetic of absorbance disappearance was monitored in spectrophometer at 240 nm (Shimadzu UV-1650 PC). The results were expressed in absorbance values/minute/mL [27].

Erythrocytic reduced glutathione (GSH) levels

RBC were hemolysed at a ratio of 1:10 in distillated water and then, 1.25 mL of EDTA (Ethylenediamine tetraacetic acid) and 250 µL of 50% TCA was added. After 15 minutes, samples were centrifuged at 2400 x *g* for 15 minutes. Next, 1 mL of supernatant was added to 2 mL of 0.4M TRIS buffer, pH 8.9. Finally, 50µL of DTNB (5, 5' – dithiobis – 2 – nitrobenzoic acid) was added to react with GSH. A standard curve was performed in order to determine GSH concentration in samples. The absorbance was read at 412 nm and results were expressed in nM [28].

Statistical Analysis

All analysis were conducted in triplicate sets. Statistical analysis were perfomed using GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA), Microsoft Office Excel 2007 and OriginLab 8.0 software. Results were expressed as arithmetic means and standard error of means (SEM). Differences among groups were assessed by two-way analysis of variance (ANOVA) to lipid peroxidation curves, with Bonferroni´s test as pos-hoc, qui-square and Fisher's exact test for clinicapathological data, Mann-Whitney and Student's unpaired t Test to others parameters. Data were checked in GraphPad Software to eliminate significant outliers ($p< 0.05$). Differences were considered statistically significant when $p< 0.05$. * marked differences between control and BC group; # marked differences between HER^- and HER^+ group; $^{\Delta}$ marked p value between 0.1 and 0.05.

Results

Clinicopathological data are summarized in Table 1. Most of patients possessed infiltrative ductal carcinoma, while just 2.5% of BC group and 5% of HER^+ presented *in situ* carcinoma. Most BC, HER^- and HER^+ were also positive to ER ($p< 0.001$) and PR ($p<0.0001$). Advanced disease (TNM III/ IV) was prevalent in all groups ($p<0.0004$). Besides, histological tumor grade analysis classified most patients in grade 2 ($p< 0.005$). No difference was observed for chemotherapy treatments.

As shown in Table 2, groups displayed normal levels of urea (ctr=29.00 ± 1.370 mg/dL; BC= 28.80 ± 1.442 mg/dL; HER^-= 28.55 ± 1.713 mg/dL; HER^+= 29.11 ± 1.873 mg/dL) and alterations in creatinine levels in BC (ctr=0.9190 ± 0.02785 mg/dL; BC= 0.8208

± 0.0403 mg/dL; p= 0.014) and no alterations for HER2 status (HER^-= 0.7595 ± 0.03296 mg/dL; HER^+= 0.8965 ± 0.06374 mg/dL) was observed.

Hepatic damage was higher in BC group, and alterations tend to be HER2 dependent based on higher levels of AST in BC (ctr= 25.39 ± 1.174 U/L; BC= 47.64 ± 6.508 U/L; p= 0.0006) with tendency for HER^+ (HER^-= 36.55 ± 4.018 U/L; HER^+= 59.85 ± 10.12 U/L; p= 0.07), higher ALT levels in BC (ctr= 32.64 ± 1.427 U/L; BC= 42.49 ± 3.016 U/L; p= 0.007) with tendency for HER^+ (HER^-= 38.55 ± 2.950 U/L; HER^+= 47.59 ± 4.522 U/L; p= 0.09), and GGT levels in BC (ctr= 17.83 ± 2.079 U/L; BC= 94.88 ± 25.40 U/L; p< 0.0001) with tendency for HER^+ (HER^-= 37.71 ± 6.013 U/L; HER^+= 154.9 ± 40.33 U/L; p= 0.08). No alteration was observed in bilirrubin levels (ctr=0.5477 ± 0.02748 mg/dL; BC= 0.4697 ± 0.04771 mg/dL; HER^-= 0.4437 ± 0.07043 mg/dL; HER^+= 0.5027 ± 0.05014 mg/dL) (Table 2).

CKT levels in BC did not differ (ctr=108.2 ± 38.70 U/L; BC= 60.16 ± 6.643 U/L) and tend to increase in HER^+ (HER^-= 51.05 ± 7.186 U/L; HER^+=70.88 ± 9.475 U/L; p=0.09) and no alteration in CKMB levels were found (ctr=1.300 ± 0.3462 U/L; BC= 2.231 ± 0.5719 U/L; HER^-= 1.762 ± 0.4923 U/L; HER^+= 2.778 ± 0.9237 U/L) (Table 2).

BC showed significant increased levels of plasmatic iron (ctr= 81.83 ± 4.791 µg/dL; BC= 115.5 ± 9.168 µg/dL; HER^-= 114.7 ± 14.48 µg/dL; HER^+= 117.1 ± 14.29 µg/dL; p= 0.02) and ferritin (ctr=47.70 ± 2.831 mg/dL; BC= 279.9 ± 72.58 mg/dL; HER^-= 371.5 ± 153.9 mg/dL; HER^+= 154.0 ± 19.29 mg/dL; p= 0.0003) independently of HER2 status (Table 2).

To assess plasmatic and RBC oxidative stress, *tert*-butyl induced CL was performed applying Two-way ANOVA statistics to evaluate if curves are different, Bonferroni's test to check which curve's points are different and curve's Student's *t* test to analyze mean curves difference. RBC oxidative stress profile was significantly higher in BC group than in control (p< 0.0001) independently of HER2 status and no points of the curve was different (Figure 1a, b). Integration of area under the curve (AUC) was increased only for BC group (ctr= 722,200 ± 58,990 AUC; BC= 1,097,000 ± 228,200 AUC; HER^-= 1,069,000 ± 40,560 AUC; HER^+= 1,086,000 ± 71,130 AUC; p< 0.05). Statistical analyses of RBC lipid peroxidation evaluation are presented in Figure 1b.

Figure 1c shows significantly higher levels of plasma lipid hydroperoxides in BC (p< 0.0005) and HER^- (p< 0.0001). Bonferroni's test did not show differences in points of the curves and no difference in AUC (ctr=1,262,000 ± 135,100 AUC; BC= 1,937,000 ± 308,500 AUC; HER^-= 2,217,000 ± 481,000 AUC; HER^+= 1,338,000 ± 346,800 AUC) was observed

(Figure 1c, d). Statistical analyses of plasma lipid hydroperoxide evaluation are presented in Figure 1d.

MDA levels were decreased only in HER^+ group (ctr= 63.99 ± 4.032 nM; BC= 70.60 ± 9.507 nM; HER^-= 73.81 ± 7.997 nM; HER^+= 65.88 ± 21.11 nM, p= 0.03. Figure 2a) and no alterations were found in levels of 8-isoprostanes F_2 (ctr= 144.6 ± 0.3083 pg/mL; BC= 144.4 ± 0.2976 pg/mL; HER^-= 144.5 ± 0.4841 pg/mL; HER^+= 144.3 ± 0.3899 pg/mL. Figure 2b), protein carbonyls content (ctr= 9.087 ± 0.5459 nM; BC= 9.640 ± 0.4461 nM; HER^-= 9.438 ± 0.4649 nM; HER^+= 9.937 ± 0.6302 nM. Figure 2c) and nitrite (ctr= 19.33 ± 1.532 μM; BC= 21.77 ± 1.402 μM; HER^-= 21.63 ± 1.795 μM; HER^+= 21.98 ± 2.308 μM. Figure 2d).

As uric acid levels were altered in BC (ctr=3.578 ± 0.1861 mg/dL; BC= 4.463 ± 0.2336 mg/dL; p= 0.004) and HER^+ (HER^-= 4.852 ± 0.3275 mg/dL; HER^+= 4.014 ± 0.1598 mg/dL; p= 0.03), antioxidant profile assessed by TRAP levels was corrected by uric acid levels. Results showed significant TRAP decrease in BC group (ctr= 366.6 ± 17.36 nM Trolox/ $mgxdL^{-1}$; BC= 200.7 ± 15.81 nM Trolox/ $mgxdL^{-1}$; p< 0.0001) independently of HER2 status (HER^-= 202.1 ± 20.99 nM Trolox/ $mgxdL^{-1}$; HER^+= 198.4 ± 23.29 nM Trolox/ $mgxdL^{-1}$) (Figure 3a).

SOD evaluation showed significant augment in HER^+ group (ctr=3.744 ± 0.3148 USOD/mL; BC= 4.214 ± 0.4491 USOD/mL; HER^-= 3.234 ± 0.1661 USOD/mL; HER^+= 5.145 ± 0.8173 USOD/mL; p=0.03) (Figure 3b). Catalase activity was lowered in BC (ctr= 544.5 ± 11.20 Vabs/min/mL; BC= 504.7 ± 8.977 Vabs/min/mL; p= 0.007) independently of HER2 status (HER^-= 503.9 ± 13.12 Vabs/min/mL; HER^+= 505.6 ± 12.40 Vabs/min/mL) (Figure 4c). GSH levels displayed tendency to decrease in BC (ctr= 16.62 ± 1.813 nM; BC= 14.70 ± 1.428 nM; p= 0.08) and augment in HER^+ (HER^-= 9.510 ± 1.687 nM; HER^+= 17.24 ± 2.457 nM; p= 0.04) (Figure 4d).

Discussion

In the present study we reported several pathological findings occurring in breast cancer women accordingly to HER2 overexpression. Relevant findings includes increased hepatic damage in BC with strong tendency of being HER2 dependently, increased RBC lipid peroxidation in BC, increased plasmatic lipid peroxidation in BC and decreased lipid peroxidation and MDA levels in HER^+, augmented plasmatic iron and ferritin in BC women. We also detected changes in antioxidant profile, as decreased TRAP and catalase activity in BC independently of HER2 overexpression, increased SOD and GSH for HER2 presence.

HER2 positive tumors are associated with increased proliferation rates, high histological and nuclear grade and aneuploidy [2]. Although HER2 overexpression leads to aggressive phenotype, in this report most of patients were included in advanced stages of the disease and histological grade 2 of tumor independently of HER2 status.

One point of interest of our study was to characterize HER2 overexpression and its relation with iron metabolism and specific tissue damage. Iron metabolism evaluation employed as indicator of oxidative stress was increased in BC independently of HER2 status. High levels of plasmatic iron can generate hydroxyl radicals through Fenton's reaction [29] leading to lipid peroxidation [30], as we observed in plasma of HER^- and RBC of BC. Additionally, high levels of plasmatic iron potentiate oxidative stress and enhance hepatic damage [31], as noted in BC probably dependent of HER2 presence. Iron overload increase oxidative stress and sustain mitogen-activated protein kinase activation (MAPK) [32], an important activated pathway by HER2 signaling [3], playing an important role in breast cancer development. Ferritin levels are also increased in BC, independently of disease staging and it is also associated with hepatic damage [33]. We demonstrated that elevation in ferritin levels is independent of HER2 overexpression and it could represent a compensatory response to contain iron overload.

Another important finding of our study was the characterization of the differential oxidative profile according to HER2 overexpression. Lipid peroxidation involves initiation, propagation, and termination [34]. Here, initiation is characterized by the ascendant part of the curve and depends on the antioxidant content of the sample. High antioxidant content is able to retard the initiation process and decreased AUC represents reduced lipid hydroperoxides [35]. Here, we showed increased lipid peroxidation in RBC of BC independently of HER2 status and increase in pre-formed lipid hydroperoxides in the absence of HER2 overexpression. As Gago-Dominguez and colleagues hypothesize [36], lipid peroxidation can represent a protective mechanism against cancer. Thus, HER2 overexpression seems to protect against lipid peroxidation. Non-enzymatic lipid peroxidation can result in several small metabolites, as MDA, isoprostanes and hydroperoxides [34]. We show that HER2 overexpression decrease MDA and no alteration was found in 8-isoprostanes F_2 levels.

Although we have previously demonstrated increased nitrite as estimative of NO and carbonyl content in advanced breast cancer [37], this report did not show differences in these parameters regarding BC and HER2 status.

TRAP was decreased in BC, suggesting that low molecular weight molecules have been consumed as a defense against oxidative processes, independently of HER2 overexpression. Enhancement of TRAP consumption associated with plasmatic uric acid levels has been investigated in previous studies with breast cancer patients when advanced disease patients were immediately treated with doxorubicin [37]. Although those findings indicate that low molecular antioxidant defenses are impaired in BC, no previous study investigated the meaning of HER2 overexpression regarding this parameter.

Defenses against reactive oxygen species includes enzymes like SOD, which dismutate superoxide radical forming hydrogen peroxide and oxygen, and catalase, which removes hydrogen peroxide giving rise to water and oxygen [38]. Our results show that HER2 overexpression increases SOD activity indicating that HER2 signaling leads to enhancement in superoxide anion or improves SOD catalytic activities. Previous works have demonstrated a decrease in catalase activity in advanced disease patients [37] and the importance of hydrogen peroxide as positively mediators in PI3K/ Akt and p38 MAPK pathways present in $HER2^+$ tumor cells [13]. Here we show that catalase reduction is a condition present in BC independently of HER2 status, even though HER^+ displaying increases in SOD.

Induction of pathways mediated by Jun amino-terminal kinase (JNK), important pathway induced by HER family as a mechanism of apoptosis protection [3], can induce GSH production [39]. GSH, in the presence of glutathione peroxidase, can reduce hydrogen peroxide in water [38]. As we demonstrated here, HER2 overexpression prevented GSH decreases and tend to increase GGT probably by a protective mechanism for hydrogen peroxide excess. In agreement with our results, Dogan and colleagues [40] showed that blocked of HER2 decreases SOD, catalase and GSH leading to increased oxidative stress.

In conclusion, this research provides new data about the involvement of HER2 overexpression and oxidative stress in bearing breast cancer women accordingly to HER2 status. Here, we reported that in breast cancer host, HER2 overexpression attenuates oxidative stress as it prevents plasmatic lipid peroxidation and MDA formation, increases SOD and GSH. Thus, this research contributes to further researches aiming to develop new anti-HER2 therapy.

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Conflict of Interest

The authors state no conflict of interest.

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TABLE OF ABBREVIATIONS

HER2 - human epidermal growth factor receptor-2;

BC - breast cancer women;

HER^- - negative for HER2 presence;

HER^+ - positive for HER2 presence;

IHC - immunohistochemical;

FISH - fluorescence in situ hybridization;

TNM - T= tumor, N= node, M= metastasis;

ER - estrogen receptors;

PR - progesterone receptors;

RBC - red blood cells;

CKT - total creatine-kinase;

CKMB - MB fraction of creatine-kinase;

AST - aspartate-aminotransferase;

ALT - alanine-aminotransferase;

GGT - gamma-glutamyl-transpeptidase;

CL - chemiluminescence;

RLU - relative light's units;

MDA - Malondialdehyde;

HPLC - High performance liquid chromatography;

TEP - 1,1,3,3 – tetraetoxipropane;

ELISA - Enzime Linked Immuno Sorbent Assay;

NO - Nitric oxide;

TCA - trichloroacetic acid;

TRAP - Total Radical Antioxidant Parameter;

ABAP - 2, 2'azo-bis, 2 amidinopropane;

Trolox - 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid;

SOD - superoxide dismutase;

Tris - hydroxymethyl – aminomethane;

EDTA - Ethylenediamine tetraacetic acid;

DTNB - 5, 5' – dithiobis – 2 – nitrobenzoic acid;

MAPK - mitogen-activated protein kinase activation;

JNK - Jun amino-terminal kinase.

TABLES AND FIGURES

Table 1: Clinicopathological data of breast cancer patients (BC), breast cancer women negative for HER2 (HER^-) and positive for HER2 overexpression (HER^+).

		BC	***HER^-***	***HER^+***
Histologycal type	*In situ*	2.5%	0%	5%
	Infiltrative ductal carcinoma	97.5% *	100% *	95% *
Hormone receptors	ER+	81.5% *	90% *	73% *
	ER-	18.5%	10%	27%
	PR+	82% *	87% *	77% *
	PR-	18%	13%	23%
TNM staging	I/ II	19.5%	25%	14%
	III/ IV	80.5% *	75% *	86% *
Histological Grade	1	14%	10%	18%
	2	62% *	70% *	55% *
	3	24%	20%	27%
Chemotherapic regimen	No	26%	25%	27%
	Paclitaxel	40%	35%	46%
	Doxorrubicin	34%	40%	27%

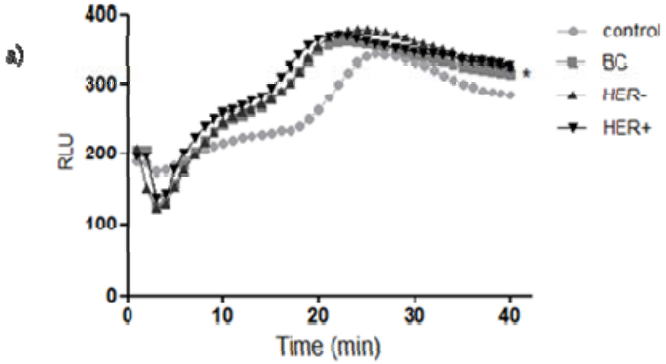
Legend: ER= estrogen receptor, PR= progesterone receptor, TNM= tumor, node, metastasis classification. * indicates statistical significance ($p < 0.05$).

Table 2: Plasmatic biochemical parameters as indicative of specific tissue damage.and iron metabolism evaluated in health women (control), breast cancer patients (BC), breast cancer women negative for HER2 (HER$^-$) and positive for HER2 overexpression (HER$^+$).

		Control	BC	HER-	HER+
Kidney markers	Urea (mg/dL)	29.00 ± 1.370	28.80 ± 1.442	28.55 ± 1.713	29.11 ± 1.873
	Creatinine (mg/dL)	0.9190 ± 0.02785	0.8208 ± 0.0403 *	0.7595 ± 0.03296	0.8965 ± 0.06374
Hepatic markers	Bilirrubin (mg/dL)	0.5477 ± 0.0275	0.4697 ± 0.0477	0.4437 ± 0.07043	0.5027 ± 0.05014
	AST (U/L)	25.39 ± 1.174	47.64 ± 6.508 *	36.55 ± 4.018	59.85 ± 10.12 $^{\Delta}$
	ALT (U/L)	32.64 ± 1.427	42.49 ± 3.016 *	38.55 ± 2.950	47.59 ± 4.522 $^{\Delta}$
	GGT (U/L)	17.83 ± 2.079	94.88 ± 25.40 *	37.71 ± 6.013	154.9 ± 40.33 $^{\Delta}$
Cardiac markers	CK T (U/L)	108.2 ± 38.70	60.16 ± 6.643	51.05 ± 7.186	70.88 ± 9.475 $^{\Delta}$
	CKMB (U/L)	1.300 ± 0.3462	2.231 ± 0.5719	1.762 ± 0.4923	2.778 ± 0.9237
Iron metabolism	Plasmatic iron (μg/dL)	81.83 ± 4.791	115.5 ± 9.168 *	114.7 ± 14.48	117.1 ± 14.29
	Ferritin (mg/dL)	47.70 ± 2.831	279.9 ± 72.58 *	371.5 ± 153.9	154.0 ± 19.29

Legend: AST= aspartate-aminotransferase, ALT= alanine-aminotransferase, GGT= gamma-glutamyl-transpeptidase, CKMB= MB fraction of creatine-kinase, CKT= total creatine-kinase. Individual distributions of values and means were evaluated by Student's unpaired t-test or Mann-Whitney test. * indicates a statistically significant difference between control and BC, # indicates a statistically significant difference between HER- and HER+. $P < 0.05$ was adopted as significant. $^{\Delta}$ indicates p value between 0.1 and 0.05.

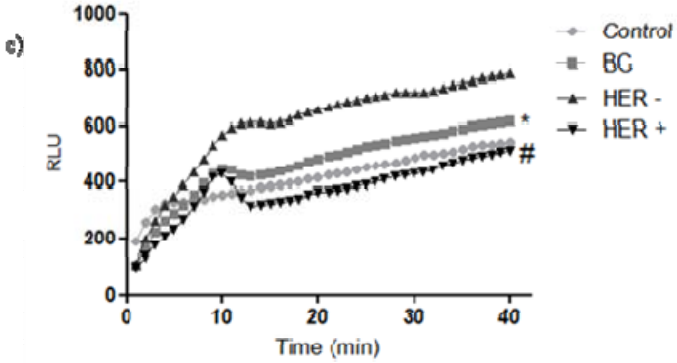
a)



b)

Statistical Analysis	CTRX BC	HER- X HER+
Two-way ANOVA	P < 0.0001	No significance
Bonferroni's Test	No points	No points
Curves Student's t Test	P = 0.0508	No significance
AUC unpaired Student's t Test	No significance	No significance

c)



d)

Statistical Analysis	CTRX BC	HER- X HER+
Two-way ANOVA	P = 0.0015	P < 0.0001
Bonferroni's Test	No points	No points
Curves Student's t Test	P = 0.0460	P < 0.0001
AUC unpaired Student's t Test	No significance	No significance

Figure 1: Oxidative stress evaluated by high-sensitivity chemiluminescence in breast cancer patients (BC), breast cancer women negative for HER2 (HER-) and positive for HER2 over expression (HER+). (a) Profile of red blood cells (RBC) lipid peroxidation, (b) statistical significance of RBC lipid peroxidation curves, (c) profile of plasmatic lipid peroxidation and (d) statistical significance of plasmatic lipid peroxidation curves are shown. RLU= relative light unities, AUC= area under the curve * indicates a statistically significant difference between control and BRCA, # indicates a statistically significant difference between HER- and HER+ P< 0.05 was adopted as significant

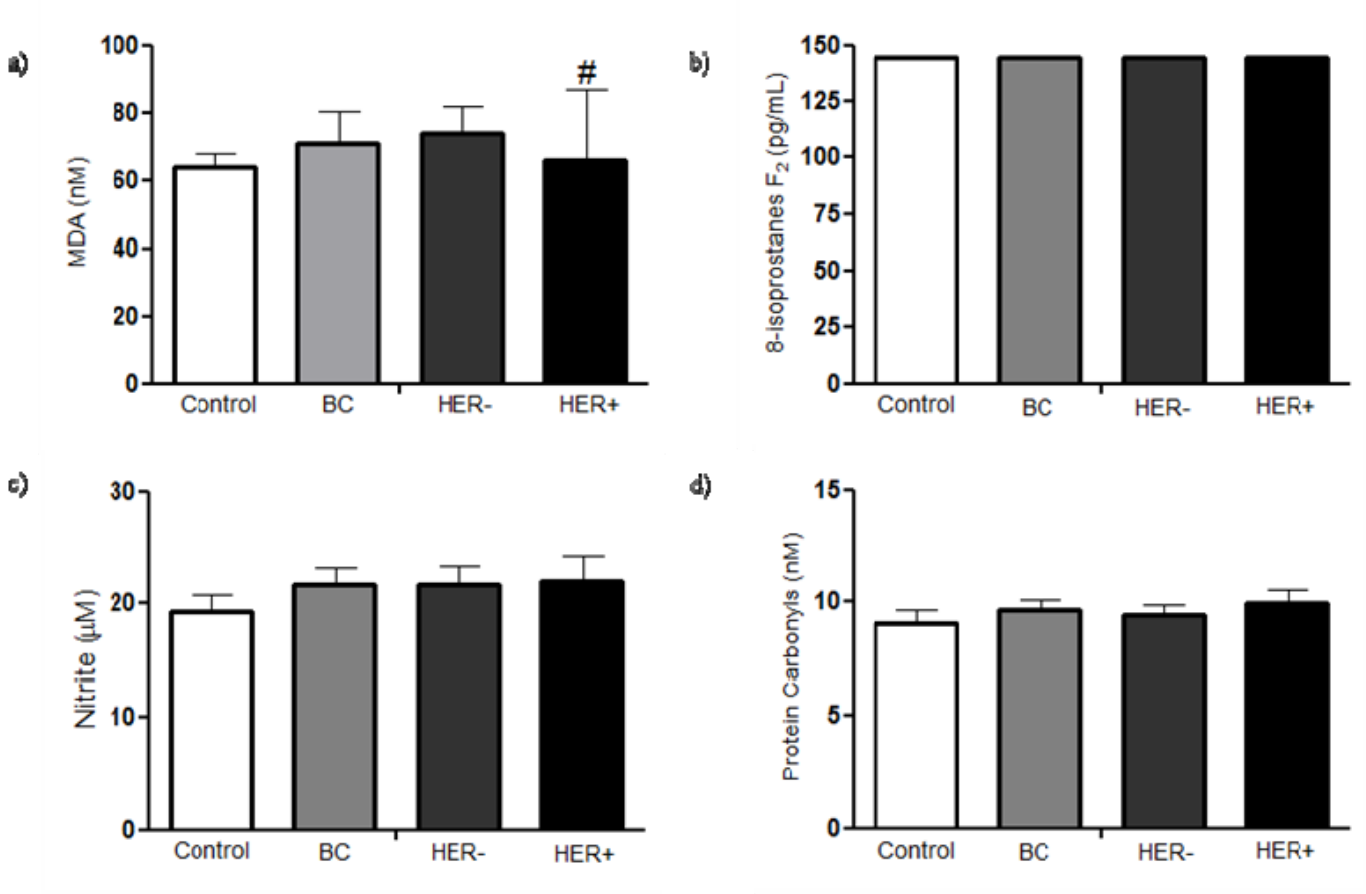


Figure 2: Pro-oxidative profile of breast cancer patients (BC), breast cancer women negative for HER2 (HER-) and positive for HER2 overexpression (HER+). (a) malondialdehyde (MDA) levels measured by HPLC, (b) 8-isoprostanes F_2 levels, (c) nitrite as estimative of nitric oxide (NO), and (d) protein carbonyls levels. Individual distributions of values and means were evaluated by Student's unpaired t-test or Mann-Whitney test. # indicates a statistically significant difference between HER- and HER+ $P < 0.05$ was adopted as significant.

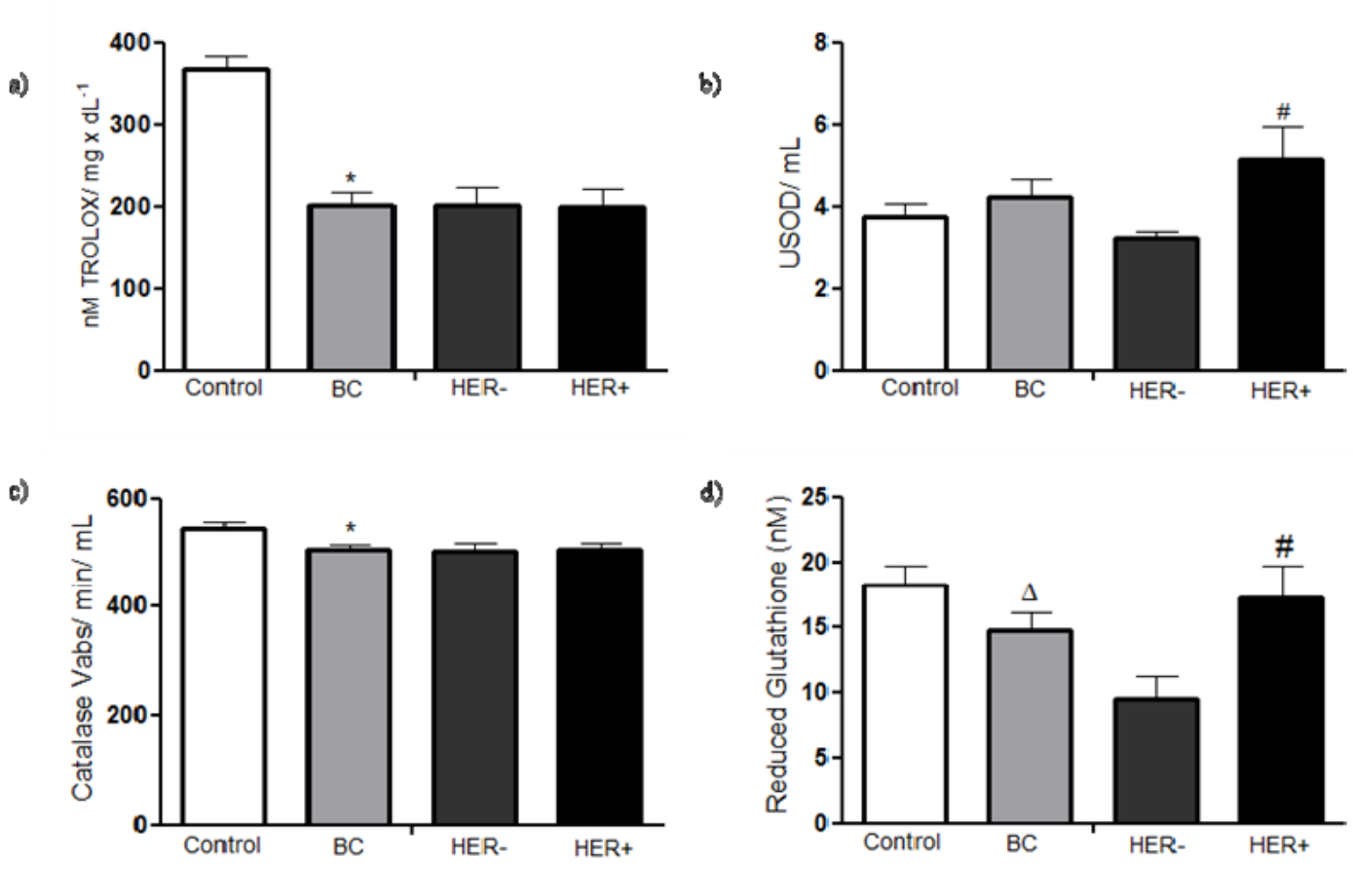


Figure 3. Antioxidant parameters in breast cancer patients (BC), breast cancer women negative for HER2 (HER^-) and positive for HER2 overexpression (HER^+). (a) Antioxidant profile determined by total radical antioxidant parameter (TRAP) corrected by uric acid levels, (b) superoxide dismutase activity, (c) catalase activity and (d) reduced glutathione levels. Individual distributions of values and means were evaluated by Student's unpaired t-test * indicates a statistically significant difference between control and BRCA, # indicates a statistically significant difference between HER- and HER+ $P < 0.05$ was adopted as significant Δ indicates p value between 0.1 and 0.05.