Universidade Estadual de Londrina

LAUANA GREICY TONON LEMOS

ESTUDO DOS MECANISMOS OXIDATIVOS ENVOLVIDOS NA RESPOSTA À QUIMIOTERAPIA ASSOCIADA AO TRASTUZUMAB NO CÂNCER DE MAMA

Londrina
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Dissertação de Mestrado apresentado ao Departamento de Patologia Experimental da Universidade Estadual de Londrina.

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Londrina,19 de agosto de 2013.

DEDICO

Aos meus pais que tanto me apoiaram e estiveram presentes com amor, carinho e conselhos durante estes anos.

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“Pouco conhecimento faz com que as pessoas se sintam orgulhosas.
Muito conhecimento, que se sintam humildes.
É assim que as espigas sem grãos erguem desdenhosamente a cabeça para o céu,
enquanto que as cheias as baixam para a terra, sua mãe.”

Leonardo da Vinci

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SUMÁRIO

1. RESUMO EXPANDIDO

Introdução: O câncer de mama é o tipo de neoplasia que mais acomete mulheres em todo o mundo, representando 22% de novos casos a cada ano [1]. Vários fatores contribuem para a agressividade da doença [2] dentre eles a amplificação do proto-oncogene ErbB2/neu, que codifica a proteína ErbB2, também conhecida como HER2 ("*human epidermal growth factor 2*") [3]. Na fisiologia normal, o HER2 é expresso durante as fases do desenvolvimento de glândulas mamárias e sua maturação. Também está envolvido com a sobrevivência de cardiomiócitos, inibindo apoptose e mantendo a função cardíaca [4, 5]. Entretanto, a amplificação/superexpressão é uma alteração genética estabelecida como um fator de mau prognóstico no câncer de mama [6], encontrada em cerca de 20-30% dos casos de tumores de mama [7] e induz sinalização celular desregulada [8], apoptose reduzida [9], sobrevivência celular prolongada [10] e potencial metastático [11]. A dimerização de HER2 regula várias vias celulares que impactam diretamente na homeostase redox [12]. Células cancerosas que superexpressam HER2 geram quantidades significativas de espécies reativas (ERs) que estão relacionadas com a ativação de PI3K-AKT, NF-kB e via p53 [3, 13, 14]. Novas terapias que visam o bloqueio do receptor HER2 vêm sendo desenvolvidas, como a quimioterapia com trastuzumab, um anticorpo monoclonal utilizado com sucesso no tratamento do câncer de mama com amplificação de HER2 [15]. Devido à capacidade de o trastuzumab ligar-se aos receptores HER2 e impedir sua dimerização, não ocorre a formação de ATP intracelular pela via PI3K-AKT, desta forma, a demanda de ATP tende a ser suprida pela produção mitocondrial, o que resulta na geração de espécies reativas de oxigênio (EROs) [16]. Esta abordagem quimioterápica é bastante nova e, portanto, o impacto do tratamento no perfil oxidativo de mulheres com câncer de mama permanece desconhecido. Neste trabalho, investigamos o perfil oxidativo sistêmico em mulheres portadoras de tumores de mama com amplificação de HER2 e em quimioterapia associada ao trastuzumab.

Estratégias: Para avaliar a capacidade do medicamento em modular o equilíbrio redox, realizamos inicialmente experimentos *in vitro* a partir de linhagens celulares de carcinoma da mama humano HER2 positivas (HCC1954). As células foram plaqueadas em placas de 6 poços ($2x10^6$) e incubadas com o meio de cultura contendo trastuzumab (50µg/mL), sendo o volume final de cada poço 1mL. Após 24 horas de incubação, a cultura foi tripsinizada. O conteúdo total dos poços (células + sobrenadante) foi coletado e congelado a -86^oC para a análise *in vitro* do perfil oxidativo estimado pelos níveis de Óxido Nítrico (NO) plasmáticos e tióis totais [17]. A verificação *in vitro* do perfil redox modulado pelo quimioterápico, permitiu a investigação em pacientes que são portadoras de câncer de mama HER2 amplificado e que encontravam-se em tratamento com o trastuzumab. Neste estudo, um total de 57 mulheres diagnosticadas com carcinoma de mama ductal infiltrativo com amplificação de HER2 foram inscritas para compor dois grupos: o grupo de mulheres portadoras de tumores HER2 amplificados e sem tratamento quimioterápico (grupo CA, n=24), e o grupo de mulheres portadoras de tumores HER2 amplificados e que se submeteram à quimioterapia baseada em trastuzumab (CT plus TZ, n=33). O tratamento com trastuzumab consiste em infusão intravenosa de $6mg/m^2$ com intervalos de 21 dias com o esquema prévio ACT (4 ciclos de doxorubicina $60mg/m^2$ e ciclofosfamida $600mg/m^2$, seguido por paclitaxel $175mg/m^2$ durante 4 semanas e com intervalos de 21 dias). As amostras foram coletadas em média no 8º ciclo para assegurar que a concentração de trastuzumab no plasma humano atingisse o estado de equilíbrio [18]. Um grupo controle (CTR) composto por 119 voluntárias saudáveis foi pareado por etnia, idade e índice de massa corporal (IMC) com o grupo CA e CT plus TZ. Foi adotado o para o grupo controle, critérios de exclusão, tais como: hábito de fumar, uso de medicamentos hormonais, uso de complexos vitamínicos, doenças crônicas e histórico de câncer. Além disso, as voluntárias encontravam-se em jejum de 4 horas para que as coletas fossem feitas. Este estudo foi aprovado pelo Conselho Nacional de Pesquisa e Ética (CAAE 0009.0.268.000-07) e todas as participantes assinaram um termo de consentimento. As amostras de sangue das pacientes foram coletadas no Instituto do Câncer de Londrina (ICL) e as amostras das doadoras do grupo

CTR foram coletadas no Departamento de Patologia Geral da Universidade Estadual de Londrina. Todas as amostras foram centrifugadas para obtenção de plasma que fora armazenado a -86°C (Ultra Indrel Congelador) para análise posterior. Para experimentos a fim de avaliar o estresse oxidativo causado pela quimioterapia, avaliamos parâmetros pró-oxidantes plasmáticos por meio da determinação de malondialdeído (MDA) por técnica de HPLC, quantificação de produtos avançados da oxidação de proteínas (AOPP) por espectrofotometria, determinação dos níveis de nitrito como estimativa das concentrações plasmática de óxido nítrico (NO) e a valiação da lipoperoxidação plasmática e tecidual por quimiluminescência de alta sensibilidade induzida por *tert*-butil hidroperóxido. O perfil antioxidante foi demonstrado a partir da avaliação da capacidade antioxidante total plasmática por quimiluminescência de alta sensibilidade (TRAP) corrigida por Tiol (TRAP/Tiol) e ácido úrico (TRAP/URCA). Essas correções foram necessárias para avaliarmos se outros antioxidantes, além de tiol e ácido úrico eram também mobilizados durante o tratamento com trastuzumab, pois estes são os principais antioxidantes presentes no plasma humano, sendo que tiol representa 800-1000µmol/L e ácido úrico, 150-400µmol/L [19]. Foi também feita análise da quantidade de tiol total plasmática, pois estes compostos são sinalizadores plasmáticos do balanço redox em resposta a estresse oxidativo e dano celular Análises bioquímicas para avaliação de danos teciduais foram feitas a partir da dosagem de marcadores presentes no plasma como aspartato-aminotransferase (AST), alanina-aminotransferase (ALT), gama-glutamil transpeptidase (GGT), proteína-C-reativa (PCR), fração MB da creatina-quinase (CKMB), lactato desidrogenase (LDH) e ácido úrico. As análises foram realizadas empregando o Systema Automatizado Dimension RxL® (Dade-Behring - Siemens Corp.). Os resultados foram expressos como média e erro padrão da média (SEM). Os parâmetros foram comparados pelo teste de Mann-Whitney (dados não-paramétricos) ou teste *t* de Student (dados paramétricos). Correlação de Spearman foram também realizados. Todas as análises estatísticas foram realizadas utilizando GraphPad Prism versão 5.0 (GraphPad Software, San Diego, EUA). Um valor de $P<0,05$ foi adotado como significativo e os valores representados em gráficos.

Resultados: O tratamento com trastuzumab *in vitro* de células HCC1954 causou redução dos níveis de NO e aumento dos níveis de tiol, quando comparado com as células não tratadas, mostrando, dessa forma, que o tratamento modula o perfil redox de linhagens HER2 positivas. Para avaliação do perfil oxidativo durante o tratamento com trastuzumab de portadoras de carcinoma de mama ductal infiltrativo HER2-amplificado, identificamos a média de idade das doadoras, sendo semelhante em todos os grupos avaliados (47,77 anos para CTR, 49,5 anos para o grupo CA e 48,12 anos para CT plus TZ). Dentre os parâmetros pró-oxidantes avaliados, obtivemos níveis de MDA menores no grupo CT plus TZ quando comparado com os grupos CA e CTR. Os níveis de AOPP encontraram-se aumentados no grupo CT plus TZ quando comparado com o grupo CA. Neste caso, a quimioterapia restabelece os padrões de AOPP com relação ao grupo CTR. Níveis de NO plasmáticos encontraram-se elevados no grupo CT plus TZ quando comparado ao CA e CTR. Análise das curvas de peroxidação lipídica mostrou que o grupo CT plus TZ apresentou maior perfil de peroxidação lipídica quando comparado com o grupo CTR e com o grupo CA. O perfil antioxidante plasmático foi verificado do TRAP. Observamos a partir deste teste que o grupo CA apresentou capacidade antioxidante reduzida, enquanto que o grupo CT plus TZ, níveis mais elevados com relação ao grupo CA, mostrando que o quimioterápico restabelece os padrões antioxidantes das pacientes em tratamento. Quando os valores de TRAP foram corrigidos por níveis de ácido úrico (URCA), ainda observamos uma redução da capacidade antioxidante no grupo CA que encontra-se aumentada no grupo CT plus TZ com relação a CA. O valor de TRAP também foi corrigido por níveis de tiol. Deste modo, níveis de TRAP/TIOL apresentaram-se mais elevados no grupo CT plus TZ quando comparado ao grupo CA e ao CTR. A quantidade de ácido úrico analisado separadamente, não varia entre qualquer um dos grupos analisados. O conteúdo tiol foi significativamente maior em pacientes do grupo CA quando comparado ao CTR. CT plus TZ exibiu quantidades diminuídas de tiol com relação a CA, mas encontra-se aumentado com relação ao CTR. O perfil bioquímico permitiu a determinação de marcadores de danos hepático e cardíaco. Para avaliarmos o

dano hepático optamos por marcadores como AST, ALT e GGT. As três enzimas apresentaram-se aumentadas no grupo CA quando comparado ao CTR e ao CT plus TZ, mostrando que o quimioterápico tenta restabelecer os padrões mais baixos dessas enzimas. Quanto aos marcadores cardíacos, a proteína C reativa apresentou-se elevada no grupo CA quando comparado ao CTR e ao CT plus TZ. O tratamento parece restabelecer estes níveis, pois em CT plus TZ, a quantidade de proteína C reativa encontra-se diminuída em relação a CA. Níveis de CKMB e LDH apresentam-se aumentados durante o tratamento, pois CT plus TZ reflete num aumento com relação ao grupo CA e ao grupo CTR. Analisando a correlação entre AOPP e CKMB por teste de Spearman, observamos que há uma correlação positiva de 0,0091. Este dado reflete numa estreita relação entre níveis de AOPP e CKMB, ou seja, altos níveis de AOPP estão relacionados à, também, elevados níveis de CKMB.

Discussão: Evidências indicam que níveis moderados de estresse oxidativo podem exercer efeitos moduladores na maquinaria celular, enquanto níveis elevados acabam por danificar estruturas celulares [20, 21]. Neste contexto, avaliamos o perfil oxidativo das mulheres diagnosticadas com câncer de mama infiltrativo ductal HER2 amplificado que se submeteram à quimioterapia associada ao trastuzumab. Inicialmente realizamos testes *in vitro* com células positivas para HER2 (HCC1954) com a finalidade de observar se este quimioterápico era mesmo capaz de alterar o balanço redox celular. Nossos dados mostraram que o trastuzumab altera o estado redox *in vitro* reduzindo os níveis de NO e aumentando o conteúdo de tiol dessas células. Em seguida, avaliamos o impacto da administração de trastuzumab sobre a oxidação lipídica e proteica de pacientes portadoras de câncer de mama HER2 positivo em tratamento com trastuzumab. O tratamento induziu um estado pró-oxidante demonstrado pelos níveis elevados de peroxidação de lipídios e formação aumentada de produtos avançados de oxidação das proteínas. A peroxidação lipídica resulta na formação de vários metabólitos de baixo peso molecular, que reage com estruturas celulares como proteínas e DNA [22]. No presente estudo observaram-se níveis elevados de peroxidação lipídica e NO no plasma de pacientes submetidos a tratamento com trastuzumab. Este estado pró-

oxidante, indica que uma fonte de peroxidação lipídica induzida pelo tratamento com trastuzumab pode ser pela conversão de NO plasmático elevado em peroxinitrito [23]. Além disso, verificou-se redução dos níveis de MDA em pacientes tratadas com trastuzumab, indicando que esta droga afeta o equilíbrio da peroxidação lipídica no plasma. MDA é um produto secundário dos processos de lipoperoxidação, representando um papel protetor no câncer de mama, já que estimula a lise de células cancerosas [20]. De acordo com nossos achados, os baixos níveis de MDA durante o tratamento sugerem que o processo de lipoperoxidação encontre-se em uma outra etapa, e isto poderia ser confirmado por outras técnicas de identificação de produtos de lipoperoxidação, mas que ainda não estão disponíveis em nosso laboratório. Essa hipótese tem como base os níveis elevados de peroxidação lipídica durante o tratamento, mostrados também em nossos resultados. O fenômeno de oxidação lipídica e proteica afeta diretamente as defesas antioxidantes [24], assim, investigamos o estado antioxidante sistêmico da quimioterapia associada ao trastuzumab. Os nossos resultados indicam que a capacidade antioxidante restaurada por administração de trastuzumab é independente dos níveis de tiol e ácido úrico, sugerindo que outros antioxidantes podem também ser mobilizados durante o tratamento com trastuzumab. Apesar do aumento da sobrevida após tratamento à base de trastuzumab [7], alguns efeitos colaterais como toxicidade hepática e cardíaca são descritos. Nossos dados mostraram que o tratamento diminui os níveis de AST, ALT e GGT chegando a valores muito próximos aos do grupo controle. Desta forma, observamos que para padrões de dano hepático, o trastuzumab não apresenta toxicidade para estas pacientes. O mesmo não se pode inferir sobre o dano cardíaco, o risco de cardiotoxicidade mediada pelo trastuzumab representa 4% quando é administrado sem associação a outros quimioterápicos e esta porcentagem pode chegar a 27% quando administrado em combinação com antraciclinas e ciclofosfamida [25]. Cerca de 75% das pacientes HER2 submetidas ao protocolo de trastuzumab apresentaram níveis de LDH e CKMB superiores aos valores de referência e estes achados sugerem a ocorrência de dano cardíaco [16]. Uma correlação positiva entre CKMB e os produtos avançados de oxidação de proteínas (AOPP) foi encontrada no plasma, sugerindo que a

ocorrência da oxidação de proteínas pode estar relacionada com alterações na permeabilidade de cardiomiócitos durante tratamento com trastuzumab. O bloqueio de HER2 torna-os incapazes de lidar com o acúmulo de espécies reativas, favorecendo a oxidação de estruturas celulares como proteínas [12]. Em conclusão, nossos dados pioneiros que permitiram avaliar o perfil redox de pacientes submetidas ao protocolo de quimioterapia associada ao trastuzumab, revelam que o tratamento modula o estado redox sistêmico das portadoras de câncer de mama com amplificação de HER2. Também, evidencia uma interação entre a oxidação proteica e dano cardíaco. Estes dados servem como base para o desenvolvimento de terapias menos tóxicas como também atentam sobre a importância de examinar as pacientes avaliando dano tecidual. O monitoramento faz-se necessário durante todo o tratamento dessas pacientes visto que ainda não se conhecem os efeitos a longo prazo desta modulação por trastuzumab.

2. LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanina-aminotransferase
AST	Aspartato-aminotransferase
ATP	Adenosina Trifosfato
AOPP	Produtos Avançados da Oxidação de Proteínas
CKMB	Fração MB da Creatina-quinase
DNA	Ácido Desoxirribonucleico
DOX	Doxorrubicina
ERs	Espécies Reativas
EROs	Espécies Reativas de Oxigênio
GGT	Gama-Glutamil Transpeptidase
HCC1954	Células do Carcinoma Mamário Humano
HER2+ HER2	Fator de Crescimento Epidermal Humano 2
HPLC	Cromatografia Líquida de Alta Performance
IMC	Índice de Massa Corpórea
INCA	Instituto Nacional do Câncer
LDH	Lactato Desidrogenase
MDA	MALONDIALDEÍDO
NADPH	Nicotinamida Adenosina Difosfato Reduzida
NF-κB	Fator Nuclear de Transcrição κB
NO	Óxido Nítrico
PCR	Proteína C Reativa
PI3K-AKT	Phosphoinositol 3 Kinase
TRAP	Capacidade Antioxidante Total Plasmática
URCA	Ácido Úrico

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4. ANEXOS

4.1 – Carta de aceite do projeto com parecer do Comitê de Ética da Universidade Estadual de Londrina e folha de rosto do cadastro no Sistema Nacional de Ética em Pesquisa

Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

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

Parecer CEP/UEL:	037/2012
CAAE:	00762512.2.0000.5231
Processo:	38617/2012
Pesquisador(a):	Carolina Panis
Unidade/Órgão:	CCB – Departamento de Ciências Patológicas

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"**Estudo dos Mecanismos Oxidativos Envolvidos na Resposta à Quimioterapia com Trastuzumab no Câncer de Mama**"

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá ser encaminhado ao CEP/UEL relatório final da pesquisa, conforme prevê a Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares.

Londrina, 11 de junho de 2012.

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

4.2 Termo de Consentimento Livre e Esclarecido aplicado às doadoras participantes deste estudo

Londrina, ___ de _____, 2012

UNIVERSIDADE

ESTADUAL DE LONDRINA

TERMO DE CONSENTIMENTO LIVRE E

ESCLARECIDO A – Informações sobre a pesquisa:

Você está sendo convidada a participar, como voluntária, da pesquisa intitulada **"**Estudo dos mecanismos oxidativos envolvidos na resposta à quimioterapia associada ao trastuzumab no câncer de mama**",** que tem por objetivo avaliar de que forma pacientes portadoras de câncer de mama respondem à quimioterapia quando tratadas com a droga trastuzumab.

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 – Procedimentos do Estudo:

Os procedimentos da pesquisa envolvem a obtenção de uma amostra de 10mL de sangue de voluntárias, obedecendo os critérios de exclusão pré-estabelecidos. A coleta será feita no Departamento de Ciências Patológicas da Universidade Estadual de Londrina e/ou no Instituto de Câncer de Londrina antes e após a administração do quimioterápico. Será analisado o estresse oxidativo a partir do plasma sanguíneo.

C – Confidencialidade da Pesquisa

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados não serão divulgados de forma a possibilitar sua identificação. A participação no estudo não acarretará custos para você e não haverá nenhuma compensação financeira adicional. Você receberá uma cópia deste termo onde consta o telefone e o endereço do coordenador do projeto de pesquisa, podendo tirar suas dúvidas sobre o projeto e sua participação, agora ou a qualquer momento. 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, tel 3371-4521.Você poderá entrar em contato com o Comitê de Ética em

Pesquisa com seres humanos da Universidade Estadual de Londrina pelo telefone (43) 3371-2490.

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

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecido pelo(s) pesquisador(es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste serviço.

_____ _____

Assinatura do doador Assinatura do pesquisador responsável

4.3 Normas para publicação de artigo científico na revista Breast Cancer Reseach and Treatment

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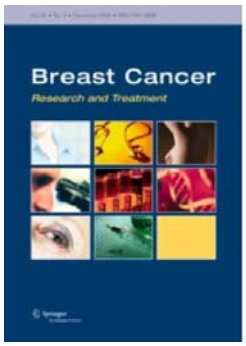

Breast Cancer Research and Treatment

Editor-in-Chief: Marc Lippman

ISSN: 0167-6806 (print version)
ISSN: 1573-7217 (electronic version)

Journal no. 10549

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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

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

Cost effective analyses

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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.

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- Book chapter

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genomics, 3rd edn. Wiley, New York, pp 230-257

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

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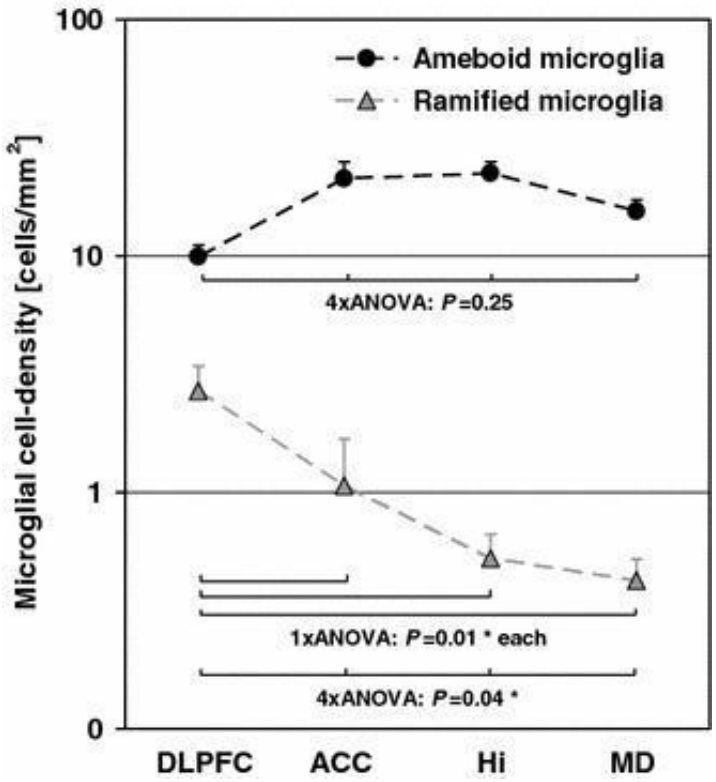

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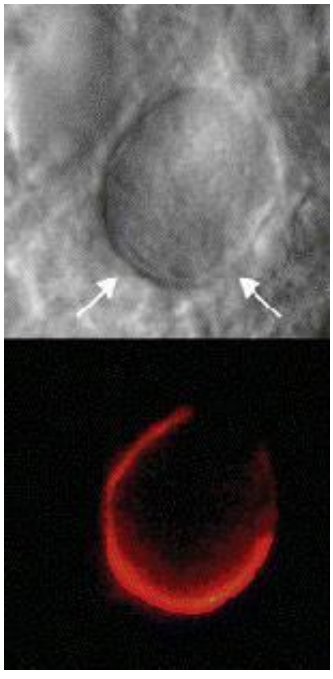

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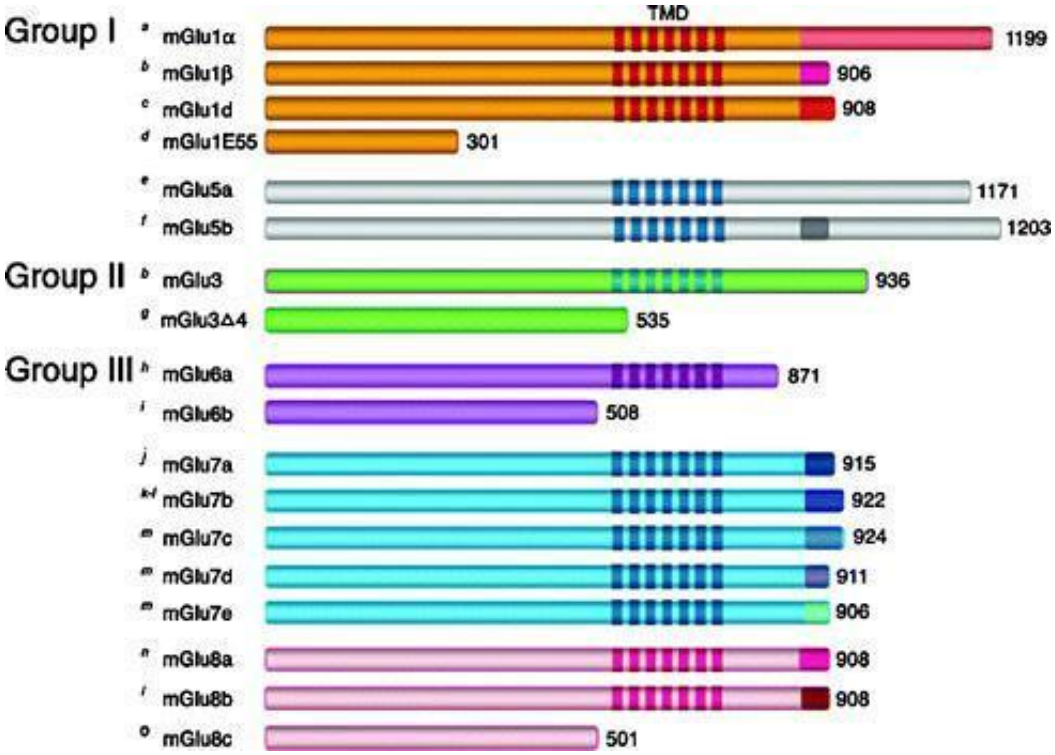

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4.4 Artigo: *"Impact of trastuzumab-based chemotherapy on redox homeostasis in women with HER2-positive breast cancer"*

Breast Cancer Research and Treatment

Impact of trastuzumab-based chemotherapy on redox homeostasis in women with HER2-positive breast cancer

--Manuscript Draft--

Manuscript Number:	
Full Title:	Impact of trastuzumab-based chemotherapy on redox homeostasis in women with HER2-positive breast cancer
Article Type:	Preclinical study
Keywords:	breast cancer; trastuzumab; HER2; oxidative stress
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Abstract:	Trastuzumab is a targeted therapeutic agent against breast tumors with amplification of the human epithelial growth factor receptor 2 (HER2). Patients with HER2-amplified tumors exhibit altered redox homeostasis; however, the impact of trastuzumab-based chemotherapy on redox processes is still unknown. In this study, we determined the oxidative systemic profile of women with HER2-amplified tumors, who were undergoing trastuzumab-based chemotherapy (CT plus TZ), and compared these with the profiles of healthy controls (CTR) and untreated patients with HER2-amplified breast cancer (CA). The oxidative profile of plasma was assessed by evaluating lipid peroxidation and total antioxidant capacity of plasma (TRAP) and determining the levels of advanced oxidation protein products (AOPPs), nitric oxide (NO), and total thiol. Biochemical markers of cardiac and hepatic damage were also assessed. Our findings showed that AOPP levels were significantly higher in the CT + TZ cohort than the CA group. NO levels were higher in the CT + TZ group than in either CA or CTR groups. Furthermore, CT + TZ treatment augmented TRAP and reduced total thiol in comparison to the CA group. AOPP and the MB fraction of creatine-kinase (CKMB) levels were found to be positively correlated in CT + TZ patients. These results suggest that trastuzumab-associated chemotherapy can modulate the redox status of patients with HER2-positive breast cancer. Furthermore, the findings indicate that trastuzumab-induced toxicity may be mediated in part via induction of protein oxidation.

Potential reviewers

Daniel Martins-deSouza, dm533@cam.ac.uk

Dennis C Sgroi, dsgroi@partners.org

TITTLE PAGE

Preclinical Study

Impact of trastuzumab-based chemotherapy on redox homeostasis in women with HER2-positive breast cancer

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Abstract

Trastuzumab is a targeted therapeutic agent against breast tumors with amplification of the human epithelial growth factor receptor 2 (HER2). Patients with HER2-amplified tumors exhibit altered redox homeostasis; however, the impact of trastuzumab-based chemotherapy on redox processes is still unknown. In this study, we determined the oxidative systemic profile of women with HER2-amplified tumors, who were undergoing trastuzumab-based chemotherapy (CT plus TZ), and compared these with the profiles of healthy controls (CTR) and untreated patients with HER2-amplified breast cancer (CA). The oxidative profile of plasma was assessed by evaluating lipid peroxidation and total antioxidant capacity of plasma (TRAP) and determining the levels of advanced oxidation protein products (AOPPs), nitric oxide (NO), and total thiol. Biochemical markers of cardiac and hepatic damage were also assessed. Our findings showed that AOPP levels were significantly higher in the CT + TZ cohort than the CA group. NO levels were higher in the CT + TZ group than in either CA or CTR groups. Furthermore, CT + TZ treatment augmented TRAP and reduced total thiol in comparison to the CA group. AOPP and the MB fraction of creatine-kinase (CKMB) levels were found to be positively correlated in CT + TZ patients. These results suggest that trastuzumab-associated chemotherapy can modulate the redox status of patients with HER2-positive breast cancer. Furthermore, the findings indicate that trastuzumab-induced toxicity may be mediated in part via induction of protein oxidation.

Key-words: breast cancer, trastuzumab, HER2, oxidative stress.

Introduction

Despite significant advances in our understanding of cancer biology, breast cancer remains the most diagnosed neoplasia [1] and one of the leading causes of deaths among women worldwide [2]. Breast cancer is a heterogeneous disease in which several factors contribute to disease aggressiveness [3]. The amplification/overexpression of human epithelial growth factor receptor 2 (HER2) is an acquired genetic alteration that is well established as an indicator of poor prognosis in breast cancer [4], and it is found in approximately 20–30% of breast tumors [5].

HER2 is a potent oncogene that encodes a transmembrane tyrosine kinase receptor. The oncogenicity of HER2 depends on its dimerization with other HER family members, which allows it to escape normal inhibitory regulation [6]. HER2-containing dimers have long half-lives and exhibit strong and persistent signaling due to both slow ligand dissociation and internalization [7]. Thus, HER2 excessively stimulates survival and mitogenic pathways [8, 9], resulting in overall deregulated signaling [10], reduced apoptosis [11], prolonged cell survival [12], and increased metastatic potential [13].

The sustained signaling induced by HER2 amplification activates several cellular networks, which in turn affects redox homeostasis [14]. Many cancer cells continuously experience oxidative stress due to the presence of significant amounts of reactive oxygen species (ROS) and impaired antioxidant defense system [15]. Abnormal signaling in HER2-overexpressing cancer cells involving ROS are associated with alterations in the PI3K-AKT, NF-κB, and p53 pathways [16, 9, 17]; all of these pathways are implicated in oxidative stress modulation.

Targeted immunotherapy using trastuzumab, a monoclonal antibody, was developed on the basis of the findings of studies designed to identify strategies for

HER2 inhibition. Trastuzumab therapy has been used successfully to treat HER2-amplified malignancies, in particular breast cancer [18]. Trastuzumab exerts its antineoplasic effects by blocking HER2 dimerization [19], which thereby inhibits aberrant receptor signaling [20]. As a consequence, ROS-generating pathways seem to be affected directly [21, 19].

Although in vitro evidence suggests trastuzumab may induce oxidative stress [14], this chemotherapic approach is fairly new; therefore, the impact of this treatment in the oxidative status of women with breast cancer is still unknown. We previously reported the chemotherapeutic drug-induced systemic changes in the oxidative stress profile of breast cancer patients [22, 23, 24], which indicated that modulation of systemic oxidative stress could be a putative in vivo mechanism underlying the activity of anticancer drugs in breast cancer.

In this study, we used highly sensitive methods to investigate the systemic oxidative status of women with HER2-amplified breast tumors and evaluated the effect of trastuzumab-based chemotherapy on this clinical parameter. Our aim was to comprehensively characterize the modulation of redox status by trastuzumab and its impact on clinical features of breast cancer.

Patients and methods

Design of the study and selection of subjects

This study was approved by the Research and Ethics National Council (CAAE 0009.0.268.000-07), and all participants provided informed consent. To determine a significant number of patients for this study, the following formula was applied:

$$n_0 = \frac{Z^2 . p(1-p)}{D^2} \quad n = \frac{n_0}{1 + \frac{n_0}{N}}$$

n_0 = number scaled (384.16), Z = confidence level (1.96), P = probability (50%); D = margin of error (5%); n = sample size, and N = population size (N).

According to the Instituto Nacional de Cancer estimate for breast cancer incidence in Brazil [25], 54 breast cancer cases are reported for 100.000 women in a region of 100.000 eligible women; on the basis of this estimate, significant sample size would 48 patients. In this study, 1008 women diagnosed with breast cancer were screened at the Londrina Cancer Institute, from February 2012 to June 2013. After application of the inclusion and exclusion criteria (described below), the cohort comprised 52 eligible patients. Since the amplification/overexpression of HER2 can be found in 20–30% of breast tumors, the minimal number of individuals in a HER2-amplified/overexpressing cohort is approximately 15 patients.

In this study, a total of 57 women diagnosed with ductal infiltrative carcinoma of the breast bearing HER2 amplified tumors were enrolled to compose two groups: the HER2 overexpressing cancer group formed by women bearing HER2 amplified tumors without any previous radio/chemotherapy (CA group, n=24), and the trastuzumab group, composed by women bearing HER2 amplified tumors which undergo the trastuzumab-based chemotherapy (CT plus TZ group, n=33). The trastuzumab-based regimen consisted of 6 mg/m^2 of intravenous infusion each 21 days with previous ACT scheme (4 cycles of doxorubicin 60 mg/m2 and cyclophosphamide 600 mg/m2 each 21 days during 12 weeks, followed by paclitaxel 175 mg/m^2 each 21 days during 4 weeks, with attacking dosage of 8 mg/m^2 of intravenous trastuzumab). Samples were collected in mean at the cycle 8 to ensure that trastuzumab concentration

in human plasma reached the steady-state [26]. Blood samples were obtained before chemotherapy starting and all patients presented four hours fasting before the plasma collection.

A control cohort was composed by 119 healthy volunteers, matched with the breast cancer cohort by ethnicity, age, body mass index (BMI) and for all excluding factors, without previous history of any type of cancer, chemotherapy, hormonal or antioxidant therapy and chronic diseases. Women were excluded if they were currently smoking, had hepatic, cardiac or renal dysfunction, use of drugs, hypertension, diabetes and other eventual chronic conditions.

Information on lifestyle and medical history were obtained at data collecting. Patients' clinical records were assessed to obtain patients information that included age at diagnosis, BMI, chemotherapic regimen and tumor-node-metastasis classification, as well data regarding tumor pathology (tumor size, histological grade, molecular receptors status and lymph nodal invasion). Nutritional habits of patients were similar to that of the control group. Samples were collected after signing informed consent, obtained from each patient or subject after full explanation of the purpose and nature of all procedures used. The investigation was approved by the local ethical committee. Heparinized blood of all control 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.

Characterization of HER2 overexpression in breast tumoral tissue

Tumor tissue sections (4μM) were histologically analyzed for diagnosis by a pathologist. For immunohistochemical assay, the reaction was performed on 4μm-thick paraffin-embedded sections from tumors by the labeled streptavidin biotin method using commercial kits with microwave accentuation. For each case, negative controls were performed on serial sections, whose were analyzed and classified accordingly positive or negative results [27]. Samples were considered as positive for estrogen (Anti-human estrogen receptor α, clone 1D5, Dako) and progesterone (anti-human progesterone, clone PGR 636, Dako) receptors when at least 10% of the tumor cells nuclei were stained. For HER2 antibody (anti-human HER2-pY-1248, Clone PN2A), the positivity was considered when a strong membrane staining (3+) was detected or FISH analysis amplification of HER2 in samples with moderate (2+) membrane staining was detected. FISH analysis was performed in tissue sections using a Dako commercial kit (HER2 FISH PharmDXTM). HER2 interpretation followed the recommendations from Dako guidelines for breast cancer samples.

Oxidative stress profiling

The pro-oxidant status of plasma was characterized by measuring malondialdehyde (MDA) with high performance liquid chromatography [28], nitrite levels (NO) [29], advanced oxidation protein products (AOPP) [30] and lipid peroxidation evaluated by high-sensitivity chemiluminescence [31].The antioxidant profile was determined by measuring the total antioxidant capacity of plasma (TRAP) by high sensitivity chemiluminescence, uric acid levels (URCA) and total thiol content [32].

Biochemical markers of tissue damage

To investigate the systemic damage of the trastuzumab-based chemotherapy, samples were analyzed to cardiac and hepatic damage markers. After 12 hours fasting, patients underwent the following laboratory blood analysis: cardiac markers (lactate-dehydrogenase (LDH), creatine-kinase fraction B (CKMB) and C reactive protein (CRP) and the hepatic markers (aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and gamma-glutamyl-transpeptidase (GGT). Samples were evaluated by a biochemical auto-analyzer (Dimension Dade AR Dade Behring,

Deerfield, IL, USA), using Dade Behring® kits. Serum high-sensitivity C-reactive protein was measured using a nephelometric assay (Behring Nephelometer II, Dade Behring, Marburg, Germany).

***In vitro* experiments**

Human breast carcinoma HCC1954 (ATCC CRL2338^{TM}) cell lines were maintained in RPMI-1640 media supplemented with FBS. To the experiments, cells were plated into 6-well plates ($2x10^6$) wells an incubated with trastuzumab-containing media (50μg/mL) [26] and trypsinized after 24 hours of culture. The total content of the wells (cells+supernatant) were collected and frozen at -86^0C for *in vitro* oxidative stress analysis, estimated by NO and total thiol levels [32].

Statistical analysis

Analyses were conducted in duplicate sets and data expressed as means±errors of the means. Parameters were compared by Mann-Whitney (non-parametric data) or

Student´s t test (parametric data). Spearman´s correlations were also performed. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, USA). A p value <0.05 was considered significant and represented in graphs by using capital letters, as follow: healthy controls x untreated cancer patients (marked as A), untreated cancer patients x trastuzumab-treated patients (marked as B) and healthy controls x trastuzumab-treated patients (marked as C).

Results

The in vitro impact of trastuzumab treatment in the oxidative status of HER2 positive cell lines is shown in Figure 1. Cells incubated for 24 hours with media containing trastuzumab at the same concentration of human plasma (50 μg/mL) presented reduced levels of NO (58.75±1.49 μM in untreated to 41.22±1.43 μM in trastuzumab, p=0.0137, Figure 1a) and high thiol content (35.95±0.29 μM in untreated and 44.03±1.67 μM in trastuzumab, p=0.0411, Figure 1b) when compared to untreated cells.

Clinicopathological data of patients are presented in Table 1. All women with cancer were diagnosed as carrying ductal infiltrative carcinoma of the breast HER2-amplified. The mean number of trastuzumab cycles at the moment of sample collecting was 8. The mean age at diagnosis was similar to all groups evaluated (47.77 years to control, 49.5 years to CA group and 48.12 years to CT plus TZ patients). Most of patients presented clinical early disease (TNM stages IIa and IIb). Analysis of the histological grade revealed a large percent of undifferentiated tumors in both cancer groups (CA and CT plus TZ groups).

Pro-oxidant parameters are grouped in Figure 2. MDA levels (Figure 2a) were lower in CT pluz TZ group when compared to both CA patients (154.9±27.43nM in TZ and 316.5±29.32nM in CA, P=0.0004) and healthy controls (154.9±27.43nM in TZ to 345±20.23nM in CTR, P<0.0001). CA versus controls did not show any significant difference (P=0.6991). AOPP levels (Figure 2b) were reduced in CA group when compared to controls (from 341.9±18.90µM in CTR to 194.5±4.042µM in CA, P<0.0001) and augmented in CT plus TZ group in relation to CA patients (from 194.5±4.042µM CA to 333.9±39.36µM in TZ, P=0.0119). AOPP levels in CT plus TZ group was similar to controls (P=0.8409). NO in plasma (Figure 2c) was found reduced in CA group in relation to controls (from 17.58±1.222µM in CA to 27.26±1.252µM in CTR, P<0.0001), while CT plus TZ patients exhibited higher levels than controls (32.77±2.578µM in CT plus TZ and 27.26±1.252µM in CTR, P=0.0492) and CA patients (32.77±2.578µM in CT plus TZ and 17.58±1.222µM in CA, P<0.0001). Two-way analysis of the profile of lipid peroxidation curves (Figure 2d) revealed that CA patients presented lower lipid peroxidation than controls (P=0.0003). CT plus TZ group presented enhanced lipid peroxidation profile when compared to both controls (P<0.0001) and CA group (P<0.0001).

The antioxidant status is presented in Figure 3. CA patients showed reduced antioxidant capacity (Figure 3a) when compared to controls (278.5±22.08 nMTrolox in CA and 2121±173.0nMTrolox in CTR, P<0.0001). CT plus TZ enhanced TRAP levels in relation to untreated patients (1393±143.4 nMTrolox in CT plus TZ and 278.5±22.08 nMTrolox in CA, P<0.0001). TRAP levels were similar between controls and CT plus TZ groups (P=0.2533). When TRAP values were expressed by uric acid levels (Figure 3b) we still observed reduced antioxidant capacity in CA group (66.48±5.269nM Trolox/g x dL-1 URCA in CA to 711.7±45.23 nMTrolox/g x dL-1 URCA in CTR,

P<0.0001). CT plus TZ presented reduced levels of TRAP/URCA in relation to controls (310.9±32.00 nMTrolox/g x dL-1 URCA in CT plus TZ and 711.7±45.23 nMTrolox/g x dL-1 URCA in CTR, P<0.0001) and higher levels in comparison to CA patients (310.9±32.00 nMTrolox/g x dL-1 URCA in CT plus TZ and 66.48±5.269 nMTrolox/g x dL-1 URCA in CA, P<0.0001). TRAP levels expressed by thiol content (Figure 3c) also resulted in the similar results to CTR x CA group (2.759±0.3198 nMTrolox/μM thiol in CA and 243.0±17.46 nMnMTrolox/μM thiol in CTR, P<0.0001), CA x CT plus TZ (82.29±12.10 nMTrolox/μM thiol in CT plus TZ and 2.759±0.3198 nMTrolox/μM thiol in CA, P<0.0001) and CTR x CT plus TZ (243.0±17.46nMTrolox/μM thiol in CTR and 82.29±12.10nMTrolox/μM thiol in CT plus TZ, P=0.0003). Table 2 shows the plasmatic levels of uric acid and thiol content in all evaluated groups. Uric acid did not vary in any group. Thiol content was significantly higher in CA patients than in controls (105.9±5.046 μM in CA and 10.01±0.401 μM in controls, p<0.001). CT plus TZ displayed thiol content higher than controls (20.80±2.053 μM in TZ and 10.01±0.401 μM in CTR, P<0.0001), but reduced in relation to CA group (20.80±2.053 μM in CT plus TZ and 105.9±5.046 μM in CA, P<0.0001).

Biochemical profile of cardiac and hepatic markers of damage are shown in Figure 4. Hepatic markers (Figures 4a, 4b and 4c) showed augmented in CA group when compared to controls for AST (60.30±9.876U/L in CA and 21.62±0.7253U/L in CTR, P<0.0001), ALT (48.65±4.246U/L in CA and 26.92±0.5721U/L in CTR, P<0.0001) and GGT (146.4±35.45U/L in CA and 16.75±0.8449U/L in CTR, P<0.0001). CT plus TZ group presented reduced levels of ALT (31.87±1.502U/L in CT plus TZ and 48.65±4.246U/L in CA, P=0.0005) and GGT (23.81±2.414U/L in CT plus TZ and 146.4±35.45U/L in CA P=0.0004) in relation to CA group. In relation to controls, AST (39.43±3.140U/L in CT plus TZ and 21.62±0.7253U/L in CTR, P<0.0001), ALT

(31.87±1.502U/L in CT plus TZ and 26.92±0.5721U/L in CTR, P=0.0008) and GGT (from 23.81±2.414U/L in CT plus TZ and 16.75±0.8449U/L in CTR, P<0.0072) were significantly elevated in CT plus TZ patients.

Cardiac markers (Figures 4d, 4e and 4f) showed augmented only to C reactive protein in CA group when compared to controls (6.495±1.190mg/dL in CA and 2.075±0.2763 mg/dL in CTR, P<0.0001). CT plus TZ presented reduced levels of C reactive protein (2.380±0.3079 mg/dL in CT plus TZ and 6.495±1.190 mg/dL in CA, P= 0.0032) and augmented levels of CKMB (18.24±3.280 U/L in CT plus TZ and 1.591±0.424 U/L in CA, P<0.0001) and LDH (337.4±30.03 U/L in CT plus TZ and 227.6±27.10 U/L in CA, P=0.0014) in relation to CA group. In comparison to controls, CT plus TZ patients exhibited augmented levels of C reactive protein (2.380±0.307 mg/dL in CT plus TZ and 2.075±0.2763 mg/dL in CTR, P=0.0419), CKMB (18.24±3.28 U/L in CT plus TZ and 2.847±0.30 U/L in CTR, P<0.0001) and LDH (337.4±30.03 U/L in CT plus TZ and 165.4± 5.44 U/L in CTR, P<0.0001).

Spearman`s correlation analysis (Figure 5) showed a significant positive correlation between AOPP and CKMB levels in CT plus TZ patients (r = 0.4537, p = 0.0091).

Discussion

Oxidative stress in breast cancer induces two main types of response, which are each dependent on specific metabolites. Accumulating evidence shows that moderate levels of oxidative stress can have a proliferative effect on the cell cycle, while high levels are deleterious as they damaging cellular structures [33-36].

Chemotherapeutic agents commonly used against breast cancer can induce ROS production as part of their antineoplasic mechanisms [37, 38]. Because trastuzumab is suggested to have pleiotropic effects, we investigated the impact of trastuzumab-based treatment on the systemic redox status of breast cancer patients with HER2-amplified tumors. In vitro evidence suggests that HER2 stimulation attenuates oxidative stress and that inhibition of HER2 by trastuzumab triggers oxidative stress by causing cytosolic calcium misbalance [39].

To investigate the impact of trastuzumab on the redox status of patients, we treated HER2-positive cells in vitro with a trastuzumab dosage equivalent to that reached in the plasma during its infusion in humans [26]. Our data showed that trastuzumab alters the redox status in vitro by reducing NO levels and augmenting the thiol content of HER2-positive breast cancer cells, causing oxidative stress under a physiological dosage of trastuzumab. Based on this, we examined the data from women with HER2 tumors, who were undergoing trastuzumab-based treatment.

We first evaluated the effect of trastuzumab on the level of lipid and protein oxidation. Trastuzumab treatment induced a global pro-oxidative status, as evidenced by the high lipid peroxidation and enhanced formation of advanced products of protein oxidation. Lipid peroxidation is a very complex chain of reactions, which results in the formation of several low-molecular-weight metabolites that form adducts with cellular structures such as protein and DNA [40]. Several factors can induce lipid peroxidation, including the NO-derived metabolite peroxynitrite [41]. In this study, we observed high lipid peroxidation in association with elevated NO in plasma from patients undergoing trastuzumab-based therapy. The pro-oxidative status observed here indicates trastuzumab-induced lipid peroxidation may be due to the conversion of large amounts of plasma NO to peroxynitrite [42].

Furthermore, we found reduced levels of MDA in patients receiving trastuzumab therapy, which indicated that this drug affects the lipid peroxidation equilibrium in plasma. Recent studies have highlighted the anticarcinogenic potential of lipid peroxidation-derived metabolites [33, 34, 35, 36]. Taken together, these findings suggest that trastuzumab is an important redox status modulator in patients, owing to its ability to increase NO-mediated lipid peroxidation and modulate the production of lipid peroxidation-derived products such as MDA.

Lipid and protein oxidations directly affect antioxidant defenses [43]. Therefore, we investigated the systemic antioxidant status of patients undergoing trastuzumab therapy. Our findings demonstrated that trastuzumab restores the total antioxidant capacity of plasma of patients with HER2-amplified breast cancer. Because uric acid and thiol content are the most abundant antioxidants evaluated by TRAP analysis [44], we also evaluated the antioxidant profile of plasma by correcting TRAP values in relation to these compounds. Our findings show that the ability of trastuzumab to restore the antioxidant capacity to control levels is independent of uric acid and thiol levels, suggesting that other antioxidants may be mobilized during treatment.

Despite the improved survival of patients treated with trastuzumab [5], side effects including cardiac and hepatic toxicities have been reported. In this context, we evaluated the biochemical profile of HER2 patients undergoing trastuzumab therapy by evaluating the markers of cardiac, hepatic, and renal damage, and we found that the levels of hepatic and cardiac enzymes were altered. Specifically, in most cases, the levels of these enzymes were higher than in that in healthy controls but lower than that in untreated patients. Hepatic alterations are reported as consequence of both the cancer pathophysiology and chemotherapy [45]. Despite these biochemical alterations, no cardiac or hepatic dysfunctions were clinically reported.

In approximately 75% of HER2 patients undergoing the trastuzumab therapy, the CKMB and LDH levels were significantly higher than the normal reference values, highlighting the occurrence of cardiac injury at the cellular level. These findings reinforce the physiological role of HER2 signaling in the maintenance of the integrity of the cardiac cells [46]. Oxidative stress in cardiac tissue may contribute to cardiac dysfunction following trastuzumab regimens, particularly when associated with doxorubicin chemotherapy [39]. In this study, we observed a positive correlation between CKMB and the advanced oxidation protein products in the plasma. This suggests that protein oxidation may be related to alterations in cardiomyocyte permeability during trastuzumab treatment. In fact, blockade of HER2 impairs the ability of cells to activate survival pathways, which renders cardiomyocytes sensitive to ROS and leaves cellular structures sensitive to oxidation [14]. Furthermore, HER2 patients under chemotherapeutic regimens can suffer from cardiac toxicity induced by several oxidative stress-dependent mechanisms. These include impairment of the neuregulin-angiotensin I network, failure of mitochondrial electron transport, inhibition of MAP/ERK signaling, and activation of the NADPH oxidase system; all these events are associated with reduced antioxidant capacity of cardiomyocytes [47, 48].

In conclusion, our data show that trastuzumab modulates the systemic redox status of women with HER2-amplified breast cancer, as well as a possible interaction between protein oxidation and cardiac damage in this condition. Trastuzumab-based chemotherapy appears to improve the antioxidant capacity through elevation of thiol levels and reduction of ROS levels. These data may help the development of less toxic cancer therapies in the future. Finally, our data underscore the need for long-term monitoring of cardiac lesion markers in patients on trastuzumab regimens, even in the absence of overt clinical symptoms.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1

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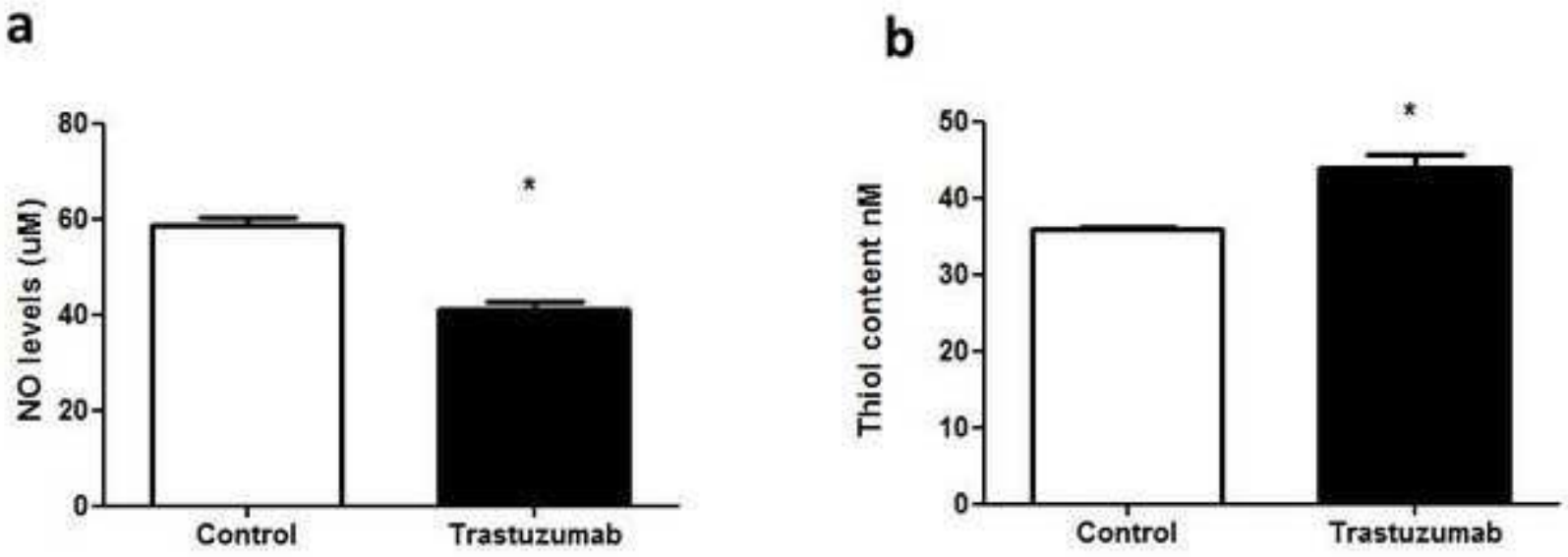

Figure 1 – *In vitro* impact of trastuzumab on the oxidative profile of HER2 positive breast cancer cells. HCC1594 cell lines were incubated during 24 hours with or without a trastuzumab dosage similar to that observed in the steady-state of trastuzumab in human plasma (50 µg/mL). Nitric oxide (a) and Thiol content (b) were measured as oxidative stress parameters. Control = HCC159 cells plus RPMI media, Trastuzumab = HCC1594 cells plus RPMI media containing trastuzumab 50 µg/mL . * indicate statistical difference ($p<0.05$).

Figure 2

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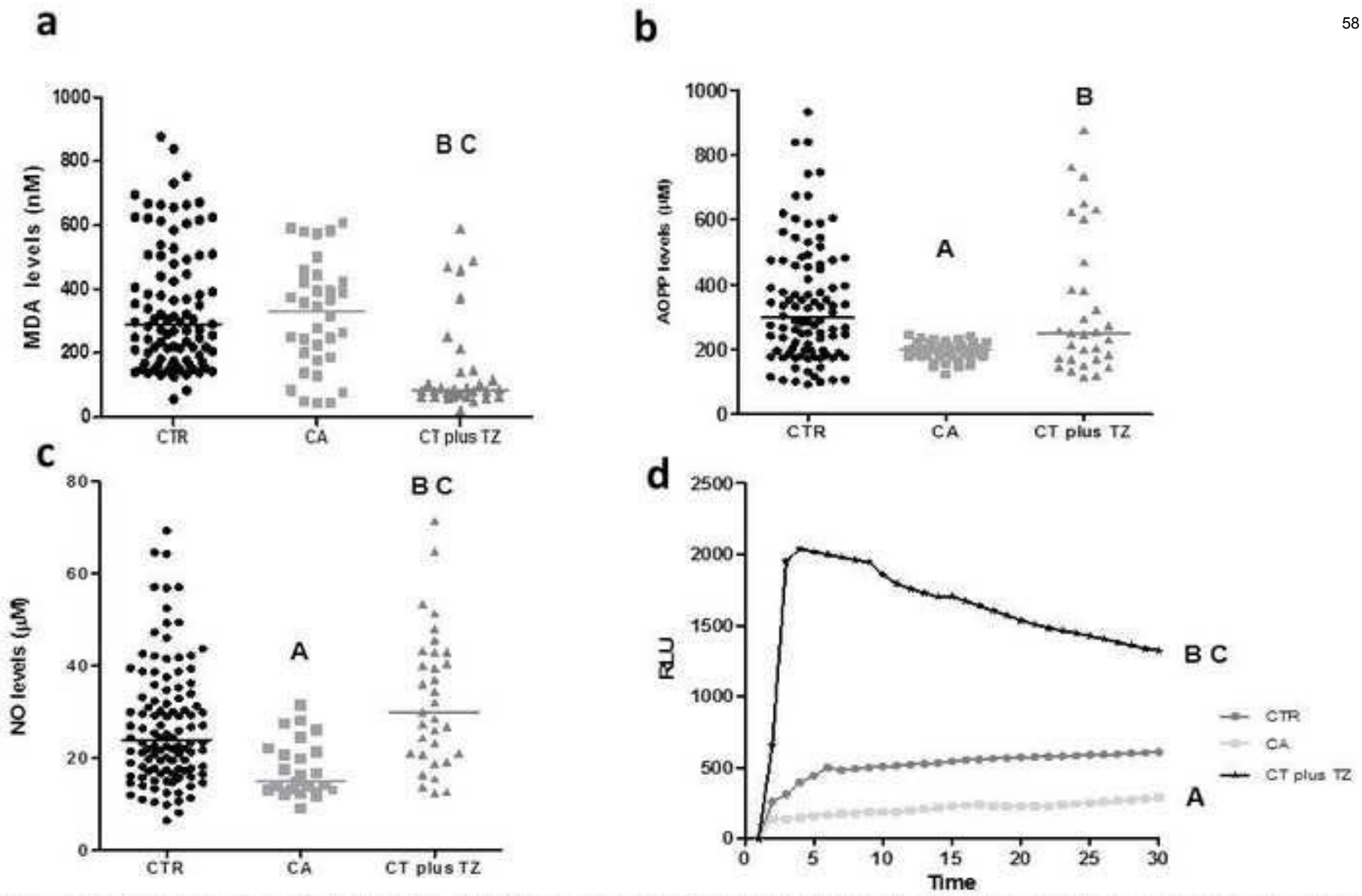

Figure 2: Pro-oxidant parameters in plasma. Malondialdehyde (a), advanced oxidation protein products (b), nitric oxide (c) and lipid peroxidation profile (d) were evaluated as indicative of pro-oxidative status in plasma. Results are represented as individual dispersion data and medians, excepting for the lipid peroxidation profile. MDA = malondialdehyde, AOPP = Advanced oxidation protein products; RLU = relative light unities. A p value <0.05 was considered significant and represented in graphs by using capital letters, as follow: healthy controls x untreated cancer patients (marked as A), untreated cancer patients x trastuzumab-treated patients (marked as B) and healthy controls x trastuzumab-treated patients (marked as C). CTR: healthy control group, CA = women bearing HER2 breast cancer without any chemotherapy, CT plus TZ: women bearing HER2 breast cancer undergoing trastuzumab-based chemotherapic scheme.

Figure 3

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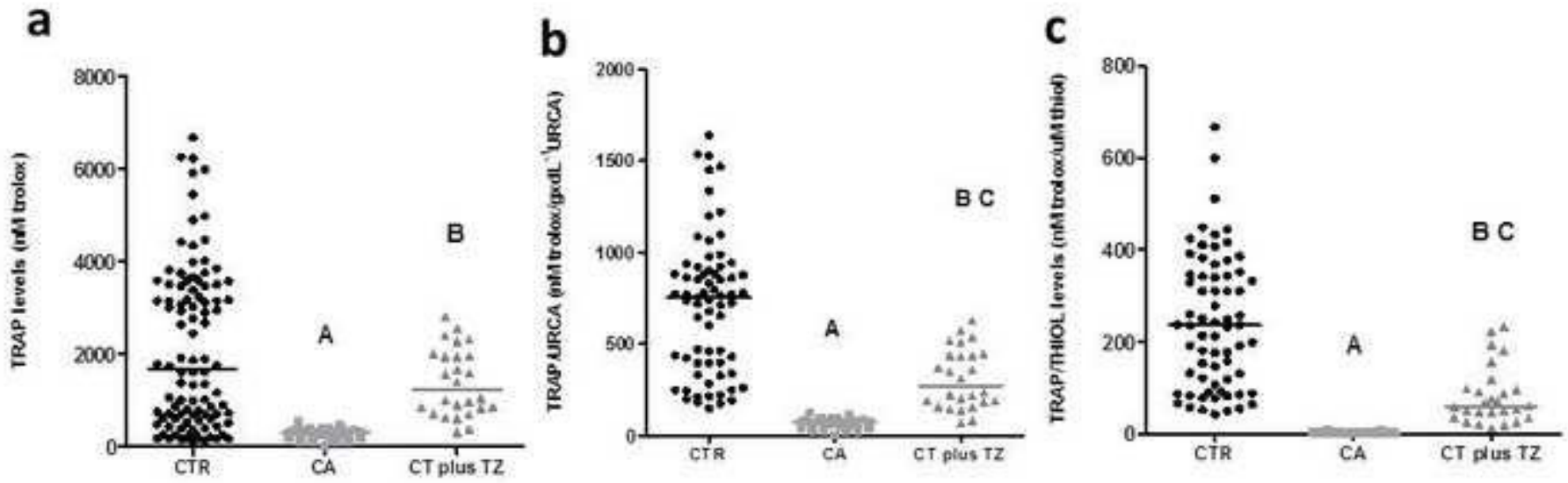

Figure 3: Plasmatic antioxidant profile. Total antioxidant capacity of plasma (a), total antioxidant capacity of plasma in relation to uric acid levels (b), nitric oxide (c) and total antioxidant capacity of plasma in relation to thiol content (d) are presented in the plasmatic antioxidant profile. Results are represented as individual dispersion data and medians . A p value <0.05 was considered significant and represented in graphs by using capital letters, as follow: healthy controls x untreated cancer patients (marked as A), untreated cancer patients x trastuzumab-treated patients (marked as B) and healthy controls x trastuzumab-treated patients (marked as C). CTR: healthy control group, CA = women bearing HER2 breast cancer without any chemotherapy, CT plus TZ: women bearing HER2 breast cancer undergoing trastuzumab-based chemotherapic scheme.

Figure 4

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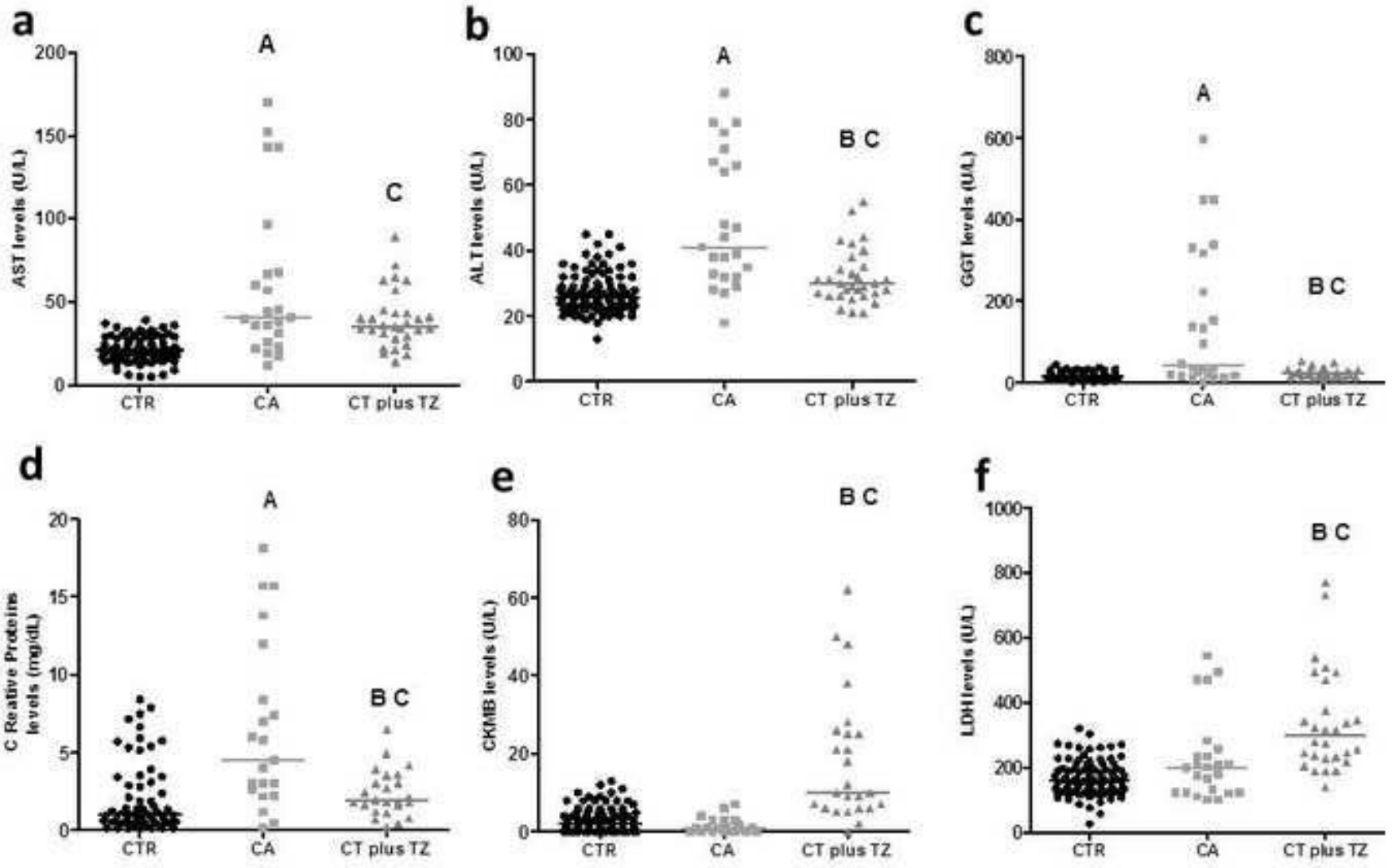

Figure 4- Cardiac and hepatic markers. Hepatic markers are represented by AST (a), ALT (b) and GGT (c). Cardiac markers are represented by C reactive protein (d), CKMB (e) and LDH (f) levels. Results are represented as individual dispersion data and medians . A p value <0.05 was considered significant and represented in graphs by using capital letters, as follow: healthy controls x untreated cancer patients (marked as A), untreated cancer patients x trasturumab-treated patients (marked as B) and healthy controls x trasturumab-treated patients (marked as C). CTR: healthy control group, CA = women bearing HER2 breast cancer without any chemotherapy, CT plus TZ: women bearing HER2 breast cancer undergoing trasturumab-based chemotherapic scheme. AST=aspartate aminotransferase, ALT=alanine aminotransferase, GGT=gamma glutamyl transferase CKMB= MB fraction of creatine kinase, LDH=lactate dehydrogenase.

Figure 5
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Parameter	R value	P value
AOPP X CKMB	0.4537	0.0091*

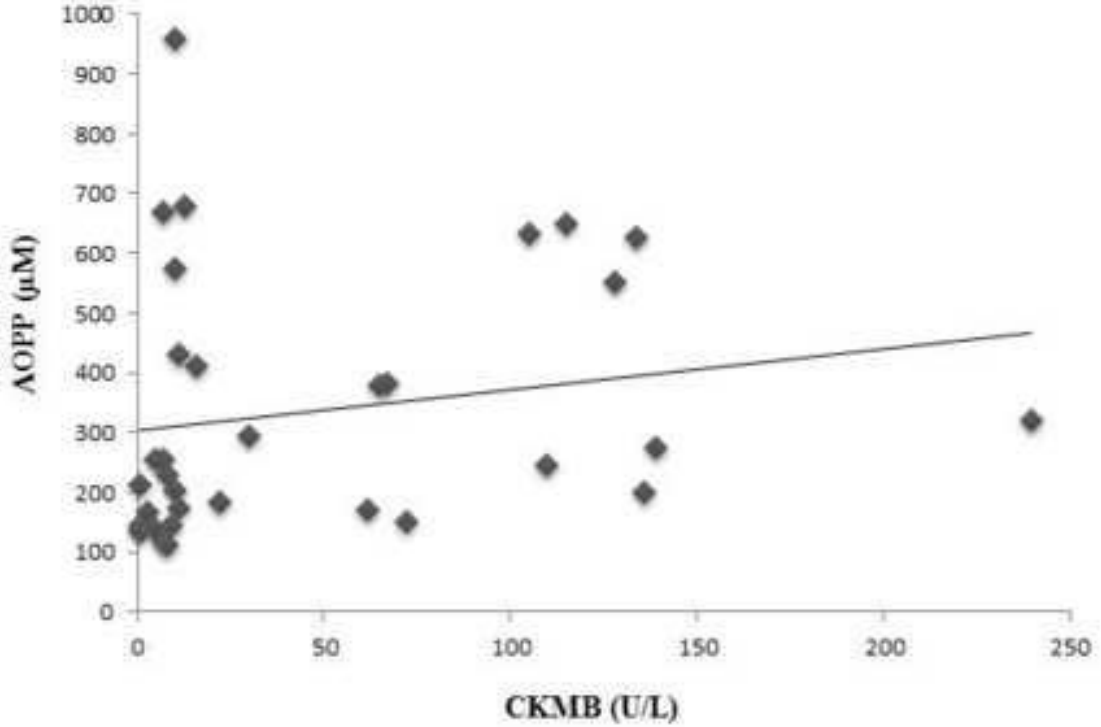

Figure 5- Correlation analysis between CKMB and AOPP levels in plasma from patients undergoing trastuzumab-based chemotherapy. Results are represented as individual dispersion data and evaluated by Spearman´s correlation test. A p value <0.05 was considered significant. CKMB= MB fraction of creatine kinase, AOPP = advanced oxidation protein products. * indicates statistical significance ($p < 0.05$).

Table1

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Table 1 – Clinicopathological data of patients cohort.

	CTR	CA	CT plus TZ
Number of patients	n= 119	n= 24	n=33
Median age at diagnosis in years (range)	47.77 (21-81)	49.5 (29-72)	48.12 (26-67)
Histologycal type			
Ductal	---	100%	100%
Lobular	---	none	none
Mixed	---	none	none
Clinical disease			
Local	---	100%	90%
Metastatic	---	---	10%
Histological grade			
1	---	0.8%	---
2	---	37.5%	20%
3	---	61.7%	80%

Legend: CTR: healthy control group, CA = women bearing HER2 breast cancer without any chemotherapy, CT plus TZ: women bearing HER2 breast cancer undergoing trastuzumab-based chemotherapic scheme.

Table2

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Table 2– Uric acid and total thiol levels in plasma.

	CONTROL	CA	CT plus TZ
Uric Acid (mg/dL)	4.077±0.2734	4.070±0.1681	4.479±0.3265
Thiol content (μM)	10.01±0.4017	105.9±5.046[A]	20.80±2.053[B,C]

Legend: Results are represented as mean ± standard errors of the mean. A p value <0.05 was considered significant and represented by using capital letters, as follow: healthy controls x untreated cancer patients (marked as A), untreated cancer patients x trastuzumab-treated patients (marked as B) and healthy controls x trastuzumab-treated patients (marked as C). CTR: healthy control group, CA = women bearing HER2 breast cancer without any chemotherapy, CT plus TZ: women bearing HER2 breast cancer undergoing trastuzumab-based chemotherapic scheme.

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