UNIVERSIDADE ESTADUAL DE LONDRINA

ANA CRISTINA DA SILVA DO AMARAL HERRERA

"PERFIL OXIDATIVO SISTÊMICO DO CÂNCER DE MAMA E SUA SIGNIFICÂNCIA CLINICO-PATOLÓGICA:

PAPEL DO TGF-BETA 1 E CARACTERIZAÇÃO DOS SUBTIPOS MOLECULARES"

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Defesa de Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para obtenção do título de doutor.

Orientador: Prof. Dr. Rubens Cecchini.

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RESUMO

O câncer de mama consiste em uma doença heterogênea com múltiplos comportamentos biológicos e clínicos. Para o estadiamento desta neoplasia utiliza-se uma classificação molecular baseada na expressão molecular de receptores de estrogênio (ER), progesterona (PR) e do fator de crescimento epidérmico humano (HER-2). Neste estudo, definimos o perfil inflamatório dos principais subtipos moleculares de pacientes com câncer de mama: luminal (ER e PR positivo, HER-2 negativo), HER-2 positivo e triplo negativo (ER, PR e HER-2 negativo). Para avliação do perfil de citocinas foi realizada a determinação da TNF-a, TGF-b, IL-1, IL-12 os níveis plasmáticos de IL-10 e. O perfil oxidativo foi avaliado pela determinação da peroxidação lipídica, capacidade antioxidante total do plasma, os níveis de malondialdeído, carbonilação proteica e óxido nítrico (NO). Os dados clínicos foram correlacionados com os achados inflamatórios. Nossos resultados demonstraram que pacientes portadores do subtipo luminal apresentavam níveis de estresse oxidativo elevado, TNF-α, TGF β-com concentrações elevadas associado com a redução de IL-12. Grupo de HER-2 positivo apresentou maiores níveis de TNF-α, IL-12 e TGF β associado com um aumento do estresse oxidativo. O subtipo triplo-negativo exibiu o perfil mais agressivo de comportamento da doença, com redução tanto TNF-α e TGF β, com altos níveis de lipoperoxidação e NO. A importância clínica dos resultados reside no fato de que o estado inflamatório varia de formas distintas em relação aos subtipos moleculares, podendo futuramente determinar a utilização de terapias alvo.

Palavras-chave: Mama. Câncer. Citocinas. Estresse oxidativo. Receptores hormonais. Patologia experimental.

HERRERA, Ana Cristina da Silva do Amaral. **Perfil oxidativo sistêmico do câncer de mama e sua significância clínico-patológica:** papel do TGF-beta 1 e caracterização dos subtipos moleculares. 2013. 55 f. Tese (Doutorado em Patologia Experimental). Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina. 2013.

ABSTRACT

Breast cancer consists in a disease with multiple biological and clinical behaviors. Based on high throughput technologies data, this disease is currently classified according to the molecular expression of estrogen (ER), progesterone (PR) and human epidermal growth factor (HER-2) receptors. In this study, we defined the inflammatory profile of the main molecular subtypes of breast cancer patients: luminal (ER and PR positive, HER-2 negative), HER-2 enriched (HER-2 positive) and triple negative (ER, PR and HER-2 negative). Cytokines panel was assessed by measurement of TNF-a, TGF-b, IL-1, IL-10 and IL-12 plasmatic levels. Oxidative profile was assessed by determination of lipid peroxidation, total antioxidant capacity of plasma, malondialdehyde levels, carbonyl con- tent and nitric oxide (NO). Clinical data were correlated with inflammatory findings. Our findings demonstrated that patients bearing the luminal subtype displayed high TNF-a, TGF-b and enhanced oxidative stress levels associated with reduced IL-12. HER-2-enriched group exhibited higher levels of TNF-a, IL-12 and TGF-b associated with enhanced oxidative stress. Triple-negative subtype exhibited the most aggressive profile of disease behavior, with reduction in both TNF-a and TGF-b, with high levels of lipid peroxidation and NO. The clinical importance of our findings lies in the fact that the inflammatory status varies in distinct ways due to molecular subtype of breast cancer, opening potential therapeutic targets to future therapies.

Keywords: Breast. Cancer. Cytokines. Oxidative stress. Hormone receptors. Experimental pathology.

LISTA DE ABREVIATURAS E SIGLAS

DNA	ácido desoxirribonucleico
ERNs	espécies reativas de nitrogênio
EROs	espécies reativas de oxigênio
GSH	glutationa reduzida
IFN-γ	interferon gama
IL-1	interleucina 1
IL-10	interleucina 10
IL-12	interleucina 12
INCA	Instituto Nacional do Câncer
MDA	malondialdeído
NO	óxido nítrico
TGF-β 1	fator de crescimento transformador beta 1
TNF-α	fator de necrose tumoral alfa
TNM	sistema de classificação de tumores baseado no tamanho do tumor (T), presença de metástases (M) e positividade de linfonodos (N).
TRAP	capacidade antioxidante total
VEGF	*vascular endothelial growth fator*, fator de crescimento vascular Endotelial

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INTRODUÇÃO GERAL

O câncer de mama é o segundo tipo de neoplasia mais frequente e a principal causa de mortes relacionadas à neoplasias em mulheres do mundo todo. Em 2012 foram diagnosticados 52.680 casos e foram notificados 12.705 óbitos em mulheres no Brasil. A taxa de mortalidade mantem-se elevada no Brasil devido ao diagnóstico geralmente tardio da doença (INCA, 2012).Trata-se de uma doença complexa e heterogênea e seu prognóstico e comportamento biológico dependem de uma série de características tanto do tumor, quanto do hospedeiro (POURZAND et. al. 2011) (SU et. al..2011). Estima-se que 90% das neoplasias mamárias seja esporádicas e resultantes de mutações somáticas acumuladas ao longo da vida (BILIMORIA & MOROW, 1995).

O acúmulo de mutações está relacionado ao surgimento de neoplasias pela ação direta de substâncias altamente reativas que promovem lesões oxidativas capazes de causar danos irreversíveis ao DNA, particularmente os radicais livres (HALLIWELL & GUTTERIDGE, 2006).

Espécies reativas de oxigênio (EROS) ou radicais livres são substâncias produzidas durante a respiração celular. No organismo humano existe um balanço entre a produção de radicais livres e sua neutralização pelos sistemas antioxidantes, visto que as espécies reativas de oxigênio desempenham diversas funções fisiológicas. Quando a produção de EROS é superior à sua neutralização, instala-se uma situação denominada estresse oxidativo. A maior parte dos tecidos é capaz de tolerar níveis moderados de estresse, porém níveis intensos podem levar a alterações metabólicas e danos ás estruturas celulares como lipídeos, proteínas e DNA (HALLIWELL & GUTTERIDGE, 2006).

No câncer o estresse oxidativo pode ser gerado continuamente pela sinalização metabólica resultante do processo inflamatório crônico, através da produção excessiva de EROS, espécies reativas de nitrogênio (ERNS) e aldeídos DNA-reativos que resultam em acúmulo progressivo de mutações e mitogênese favorecendo a patogênese do câncer e etapas da carcinogênese (BARTSCH & NAIR, 2006). Estudos sugerem um papel regulatório dos metabólitos do estresse oxidativo durante o processo de desenvolvimento e progressão tumoral. Produtos do metabolismo oxidativo têm sido descritos como um dos mecanismos protetores anticarcinogênico, sugerindo que o balanço de alguns metabólitos originados pela ação dos radicais livres esteja significantemente associado ao prognóstico da doença (GAGO-DOMINGUES et al., 2007). Desta forma, a participação do estresse oxidativo nos diversos aspectos do câncer depende de características específicas da doença. Estudos do nosso grupo tem demonstrado que fatores como a quimioterapia e estadiamento da doença são capazes de modular os níveis de estresse oxidativo sistêmico em pacientes portadoras de câncer de mama, sugerindo que os níveis de estresse oxidativo possam variar de acordo com outros aspectos clínicos desta doença (PANIS et al., 2011; PANIS et al., 2012; PANIS et al., 2012; PANIS et al., 2012).

A inflamação crônica é um fator de risco bem descrito na patogênese das neoplasias. Em 1863, Virchow postulou que o câncer seria uma doença originada em locais de inflamação (DAVID, 1988). Essa hipótese baseia-se no fato de que quando as células sofrem algum tipo de dano, a resposta inflamatória é ativada para o processo de reparo e cicatrização. Entretanto, quando há persistência da inflamação, ocorrem proliferação e ativação celular persistente que gera mediadores bioativos capazes de promover a carcinogênese (ROBBINS et al., 2000).

Assim como a inflamação por si só pode promover o processo de carcinogênese através da perpetuação do estresse oxidativo e outros fatores pró-tumorais, esta também é capaz de desencadear uma resposta imunológica através da secreção de citocinas e recrutamento de leucócitos. Estudos demonstram que o microambiente tumoral possui uma assinatura imunológica específica e que esta capacidade está diretamente relacionada com um aumento na sobrevida dos pacientes a diversos tipos de câncer, incluindo o câncer de mama (DISIS & PARK, 2009).

Na inflamação crônica ocorre uma polarização mediada por citocinas imunossupressoras do padrão Th2 e células T regulatórias que reprimem a citotoxidade mediada por linfócitos $TCD8^{+}$ e macrófagos através da secreção de interleucinas e fator de crescimento transformador beta (TGF β) que propiciam a perpetuação tumoral. Esta polarização Th2 gera um microambiente pró-tumoral e pró-angiogênese por possibilitar a produção de fator de crescimento vascular endotelial (VEGF) por macrófagos (DENARDO & COUSSENS, 2007).

O fator de crescimento transformador beta 1 (TGF β-1) faz parte de uma família de fatores de crescimento que afetam tanto o tecido mamário normal, quanto o neoplásico. Esta citocina é implicada na regulação da remodelação tecidual e apoptose no desenvolvimento mamário normal (NGUYEN et al., 2011). Evidências apontam que o TGF β-1 participa de todas as etapas da carcinogênese, podendo suprimir ou estimular a progressão tumoral (FLANDERS & WAKEFIELD, 2009). Esta via de sinalização pode estimular a produção de quimiocinas e o desenvolvimento de metástases, pois alterações na sinalização dependente deTGF β-1 estão associadas ao mau prognóstico em pacientes com neoplasias, especialmente de mama, cólon e próstata (BIERIE & MOSES, 2009; BARCELLOS-HOFF & AKHURST, 2009; GIAMPIERI et al., 2009). Apesar do papel de TGF β-1 estar bem

definido *in vitro* no câncer de mama, sua caracterização em níveis circulantes em pacientes e seus aspectos clinico-patológicos ainda são pouco conhecidos.

O tecido tumoral mamário, formado por células estromais benignas, sistema imune e células-tronco residentes, é capaz de influenciar a progressão tumoral e o desenvolvimento de metástases através da secreção de diversos fatores solúveis como interleucinas, proteínas da família do fator de necrose tumoral alfa (TNF-α) e TGF β-1 (DEMICHELLE et al., 2009; MUEHLBERG et al.,2009; WALTER,2009; POCZOBUTT et al., 2010; PELEKANOU et al., 2008; IKUSHIMA & MIYAZONO, 2010). Adicionalmente, o processo de angiogênese tumoral mamária está diretamente ligado ao fator de necrose tumoral alfa (TNF-α) e ao oxido nítrico (NO), moléculas paradoxalmente envolvidas na resposta Th1 de eliminação de tumores. Desta forma, fica evidente o papel controverso dos mediadores da inflamação na modulação da relação tumor-hospedeiro. Nosso grupo tem realizado diversos estudos para melhorar o entendimento dos efeitos dos metabolitos gerados durante a inflamação e estresse oxidativo no câncer de mama (PANIS et al., 2011).

Um outro aspecto importante do câncer de mama se refere ao estadiamento da doença. Em 1942 foi criado o sistema TNM (tumor, nodes, metastasis classification) o qual atribui um prognóstico ao câncer baseado no tamanho do tumor primário, presença e extensão da doença para linfonodos regionais, e na presença de metástases a distância. Atualmente além do sistema TNM, utiliza-se uma classificação molecular do câncer de mama baseada na expressão imunoistoquimica de receptores de estrógeno (RE) e progesterona (RP) e do receptor do fator de crescimento epidermal humano (HER-2).

Com base neste padrão imunoistoquimico o câncer de mama pode ser classificado em 3 grupos: Luminal (A e B), HER-2 positivo e Triplo negativo (VODUC et al., 2010). Os tumores do subtipo luminal são caracterizados por alta expressão de receptores hormonais (RE e RP) e correspondem a 70% das neoplasias de mama. O subtipo HER-2 positivo é caracterizado por superexpressão de receptores HER-2 e expressão negativa de receptores hormonais e correspondem a aproximadamente 15% dos tumores da mama. O subtipo que apresenta o pior prognóstico é o triplo negativo (ou basal-like) e não apresenta expressão de receptores hormonais e de HER-2 (SCHNIT, 2010). As estratégias terapêuticas são determinadas a partir desta classificação molecular, além de fatores clínicos que permitem individualizar o tratamento para atingir os melhores resultados. Nosso entendimento sobre os subtipos de câncer de mama é limitado, especialmente porque a maioria dos resultados se baseiam em analises experimentais e in vitro. Além disso, não há informações sobre as citocinas derivadas de cada subtipo de câncer de mama em humanos. Encontra-se bem documentado o envolvimento dos receptores de estrógeno, progesterona e HER-2 no estresse oxidativo mediando vias de inflamação (KOBIELA et al., 2007; TESAROVA et al.,2007), então é possível hipotetizar que a combinação de cada receptor (que conferem subtipos específicos para cada tumor de mama) pode resultar em diferentes padrões de estímulos por citocinas e também explicar os diferentes comportamentos de cada subtipo, permitindo futuras intervenções terapêuticas.

OBJETIVOS

Neste contexto, o objetivo deste trabalho é avaliar o perfil sistêmico do TGF-β1 em relação aos aspectos clínicos do câncer de mama, bem como caracterizar o padrão de estresse oxidativo plasmático em relação aos principais subtipos de câncer de mama (luminal, HER-2 positivo, e triplo negativo).

METODOLOGIA

Este estudo foi aprovado pelo Comitê de Ética em Pesquisa com Seres Humanos da Universidade Estadual de Londrina e pelo Conselho Nacional de Ética em Pesquisa (CONEP). Todas as mulheres que participaram deste estudo assinaram termos de consentimento livre e esclarecido. Neste estudo foram recrutadas 60 mulheres atendidas pelo SUS diagnosticadas como portadoras de carcinoma ductal infiltrativo de mama atendidas no Instituto do Câncer de Londrina, Londrina-PR, no perído de janeiro de 2009 a agosto de 2011. Voluntárias saudáveis foram convidadas a compor o grupo controle (n = 100), pareadas com as pacientes com câncer por idade e índice de massa corporal. Para caracterização clinicopatológica desta coorte foram considerados os seguintes parâmetros: idade ao diagnóstico da doença, peso, altura, comorbidades, classificação internacional de tumores de mama TNM, status hormonal dos tumores e tipo de regime quimioterápico. Foram utilizados como critérios de exclusão em todos os grupos: histórico de quimioterapia prévia, tabagistas, portadoras de disfunção hepática, renal ou cardíaca, diabetes e outras condições patológicas crônicas que poderiam interferir na análise dos resultados.

As mulheres envolvidas neste estudo foram categorizadas em 2 estudos distintos:

1. Perfil de citocinas e status oxidativo sistêmico em pacientes portadoras dos subtipos de câncer de mama luminal, HER-2 amplificado e triplo negativo.

2. Estudo do perfil dos níveis de TGF-β1 circulantes e sua correlação clinicopatológica.

Amostras de sangue foram obtidas por punção venosa periférica e coletadas em tubos contendo EDTA e armazenadas a -70^0 para análises posteriores. Lâminas histológicas contendo amostras de tecido tumoral obtidas ao diagnóstico (5 μm da biópsia por lâmina) foram empregadas para realização da subtipagem molecular dos tumores de mama por imunoistoquímica, para os anticorpos primários anti-receptor de estrógeno (ER), anti-receptor de progesterona (PR) em anti-receptor do fator epidermal de crescimento humano (HER2), utilizando-se kit comercial (LSAB® Dako). Todos os anticorpos empregados foram diluídos segundo recomendações do fabricante (Dako®). Controles negativos foram obtidos omitindo-se os respectivos anticorpos primários. Para categorização das amostras, seguiram-se os critérios preconizados por guidelines internacionais (Colégio Americano de Patologistas/ASCO). Para marcação de ER e PR, considerou-se positiva amostra contendo pelo menos 10% de marcação nuclear na área de tecido tumoral. Para HER2, somente foram consideradas positivas lâminas com marcação de 3 cruzes, ou lâminas com 2 cruzes com amplificação confirmada pelo teste de hibridização in situ para HER2 (FISH, PharmaDX, Dako®).

As dosagens de TGF-β1, TNF-α, IL-1β, IL-10 e IL-12 foram realizadas no plasma utilizando-se kits comerciais de enzimaimunoensaio (E-Biosciences®). As análises de caracterização do perfil oxidativo sistêmico realizadas foram: lipoperoxidação plasmática e capacidade antioxidante total (TRAP) avaliadas por

quimiluminescência, determinação dos níveis de malondialdeído (MDA) pela técnica colorimétrica de substâncias reativas ao ácido tiobarbitúrico (TBARs), conteúdo de proteínas carboniladas por espectrofotometria em ultravioleta e estimativa dos níveis de óxido nítrico (NO). Análises de marcadores bioquímicos foram realizadas no aparelho Dimension RxL® (Dade-Behring - Siemens Corp.).

Todas as análises laboratoriais descritas foram conduzidas em triplicatas e categorizadas de acordo com o subtipo molecular de câncer de mama. As comparações estatísticas realizadas foram entre o grupo controle e os diferentes aspectos clínicos do grupo de mulheres com câncer. Os dados foram apresentados como médias±erro padrão da média e apresentados graficamente em forma de barras ou dispersão individual acompanhados da mediana de cada grupo. Todas as análises estatíticas foram conduzidas no software GraphPad Prism versão 5.0 (GRAPHPAD Software, San Diego, CA), utilizando a ferramenta on-line *quick calcs* (http://www.graphpad.com/quickcalcs/Grubbs1.cfm) para detecção de indivíduos *outliers*. As diferenças entre os grupos foram avaliadas por análise de variância (ANOVA) seguida de teste de Bonferroni para as curvas de lipoperoxidação e teste t de Student (dados paramétricos) ou teste de Mann-Whitney (dados não-paramétricos) para os demais parâmetros. As curvas de sobrevida foram avaliadas pelo método de Kaplan-Meyer, seguidas do teste de logrank e teste de regressão de Cox. O resultado foi considerado significativo quando $p<0,05$.

RESULTADOS:

Artigo 1: O estudo envolveu 53 mulheres com diagnóstico de câncer e 53 controles saudáveis. A maior parte das pacientes recrutadas neste período apresentou o subtipo luminal (n=35), enquanto que o subtipo triplo negativo foi a minoria (n=9).

Os grupos HER-2 e triplo negativo apresentaram as menores médias de idade ao diagnóstico (47.27 anos e 48.16 anos, respectivamente) quando comparados ao grupo luminal (59.16 anos), ambos com um percentual relevante de pacientes com menos de 40 anos (18.18 e 28.57%, respectivamente). O estadiamento TNM das pacientes com subtipo luminal demonstraram uma distribuição similar entre precoce (TNM I e II, n = 11) e avançado (TNM III e IV n = 24), enquanto o subtipo HER-2 positivo apresentou a maioria das pacientes em estágios avançados (n = 7). Todos os grupos foram tratados posteriormente com regimes de quimioterapia contendo paclitaxel ($175mg/m^2$) e doxorrubicina ($60mg/m^2$). O envolvimento linfonodal foi observado em todos os grupos (luminal = 54%, HER-2 positivo = 50% e triplo negativo = 57%). O grupo triplo negativo foi o que apresentou o maior numero de pacientes com envolvimento linfonodal (70%) e a menor taxa de invasão linfo vascular (43%) em relação ao luminal (54% dos linfonodos positivos, p = 0.0175 e 75% de invasão linfo vascular, p = 0.004) e HER-2 positivo (63% dos linfonodos positivos, p = 0.0053 e 81% de invasão linfo vascular, p = 0.003). Metástases à distância estavam presentes em todos os grupos, mas um número significativo de pacientes no subtipo HER-2 positivo (50% dos casos, p < 0.001). Os subtipos triplo negativo e HER-2 positivo apresentaram as maiores taxa de óbitos (28.6 e 18.2%, respectivamente) ao final de 40 meses.

Os tumores do grupo luminal apresentaram a maior média de tamanho (3.87± 0.66 cm) em relação ao grupo HER-2 positivo (1.63±0.44 cm, p = 0.0385) e o grupo triplo negativo (2.35± 0.65 cm, p = 0.044). Por outro lado, os tumores triplo negativos tiveram o maior grau histológicodo que os tumores do grupo luminal e do grupo HER-2 positivo (p = 0.0035).

O perfil de citocinas demonstrou que os grupos luminal e HER-2 positivo apresentaram concentrações significativamente mais altas de TNF-α do que o grupo controle, com p = 0.0122 e 0.0108, respectivamente. O grupo triplo negativo revelou uma importante redução nas concentrações de TNF-α quando comparado ao grupo luminal (p = 0.0429). As concentrações de IL-12 estiveram reduzidos no grupo luminal em relação ao grupo controle (p = 0.0014. O grupo HER-2 apresentou concentrações mais altas de IL-12 quando comparado ao grupo luminal com p = 0.0290. As concentrações de TGF-β estiveram aumentadas nos grupos luminal e HER-2 positivo em relação ao grupo controle. O grupo triplo negativo apresentou concentrações mais baixas do que o grupo luminal (p = 0.0176). IL-1 e IL-10 não apresentaram diferenças significativas.

A caracterização do perfil oxidativo demonstrou que o grupo luminal apresentou níveis significativamente altos de malondialdeído (p = 0.0356) e redução na capacidade antioxidante total (p = 0.0047). O grupo HER-2 positivo apresentou os maiores níveis de malondialdeído (p < 0.0001), altos níveis de proteína carbonílica (p = 0.0867) e redução na capacidade antioxidante total (p = 0.0251). O grupo HER-2 positivo também apresentou diferenças nos níveis de malondialdeído quando comparado ao grupo luminal (161 ± 20.2 nM, p = 0.0155) e ao grupo triplo negativo (p = 0.0275). O grupo triplo negativo apenas apresentou níveis significativos de nitrito (p = 0.0829), sem qualquer variação em outros parâmetros oxidativos .

O perfil de lipoperoxidação foi avaliada por quimiluminescência de alta sensibilidade. Pacientes do subtipo luminal revelaram maiores níveis de lipoperoxidação quando comparado ao grupo controle em relação ao perfil total da curva, avaliados por ANOVA two-way (p <0,001) e quantitativamente em 26 pontos quando analisadas pelo teste de Bonferroni (p <0,001). Teste de Mann-Whitney

apontou aumento significativo nas curvas medianas ($p < 0,0001$). Nenhuma diferença foi detectada na área sob a curva de integração de todos os grupos (17.995 ± 6.572 RLU nos controles, 37.773 ± 8.067 RLU em luminal, 21.669 ± 6.554 em HER-2 positivo e 57.004 ± 16.962 RLU em triplo negativos). Pacientes HER-2 positivas não exibem significativa lipoperoxidação em qualquer uma das avaliações estatísticas realizadas, enquanto as triplo negativos exibiram uma curva com perfil significativamente distinto por two-way ANOVA ($p < 0,01$) e estatisticamente diferente da média sob a curva ($p < 0,001$). Todos os subtipos apresentaram diferentças significativas quando comparados entre si ($p < 0,001$).

Finalmente, algumas correlações foram encontradas entre parâmetros clínicos e plasmáticos nos pacientes, evidenciados pelo teste de Spearman. O grupo luminal apresentou correlação positiva entre a idade ao diagnóstico e a capacidade antioxidante ($p = 0.0016$). O grupo HER-2 positivo demonstrou que a idade ao diagnóstico foi positivamente correlacionada a lipoperoxidação ($R = 0.8420$, $p = 0.0175$), e os níveis de oxido nitrico foram altamente associados ao tamanho do tumor ($R= 1$, $p < 0.0001$). O subtipo triplo negativo apresentou alta correlação entre grau histológico do tumor e níveis de TGF-β ($R = 0.9719$, $p = 0.0020$).

Artigo 2: A média de idade ao diagnóstico foi de 59,1 anos e todas as mulheres recrutadas foram diagnosticadas com carcinoma ductal infiltrante da mama. O status do receptor molecular apresentou uma distribuição freqüentemente observada na população em geral . O estadiamento TNM exibida uma distribuição equivalente entre estagio inicial (TNM I e II) e doença avançada (III e IV) .

Os níveis de TGF - β no plasma foram examinados e classificados em conformidade com os parâmetros clínicos . Pacientes avaliados por estadiamento

TNM não apresentaram diferenças nos níveis de TGF- β . No que diz respeito à classificação molecular dos subtipos de câncer de mama foi observado que os doentes com tumores triplo-negativos apresentavam níveis significativamente mais baixos de TGF- β quando comparado com o grupo geral das mulheres com câncer de mama ($p = 0,0338$) .

A remoção cirúrgica da massa tumoral primária não afetou o nível de TGF- β1 no plasma quando comparados com pacientes ainda apresentavam tumor primário. Os níveis de TGF - β1 plasmáticos foram também diminuídos em pacientes com metástases a distância, quando comparado com aqueles com doença não metastática ($p = 0,0442$). Mulheres com diagnóstico da doença em idade precoce (< 45 anos de idade) apresentaram maiores níveis de TGF- β quando comparados àqueles que tinham de início tardio (idade ≥ 45 anos , $p = 0,0036$) .Observou-se que o nível de TGF- β1 dependeu do tipo de regime quimioterapêutico , uma vez que os doentes submetidos a quimioterapia com doxorrubicina como monoterapia apresentaram uma redução significativa na concentração plasmática TGF- β1 quando comparados com pacientes que receberam o paclitaxel como monoterapia ($p = 0,0088$) .

Níveis de TGF- β1 em pacientes com câncer de mama foram maiores em comparação com controles saudáveis ($p = 0,0435$) . Além disso , a avaliação do TGF- β1 em pacientes após o tratamento com paclitaxel mais doxorubicina , comparadas antes e após a infusão mostrou que a quimioterapia prontamente reduzida de TGF- β1 para níveis menores do que os observados em pacientes com câncer de mama , sem qualquer tratamento ($p = 0,0494$) . Em relação à resposta a quimioterapia, os níveis de TGF - β1 não diferiram em pacientes responsivos e aqueles com doença resistente .

A análise do perfil de sobrevida indica que os níveis de TGF- β1 na coorte de pacientes que sobreviveram e o grupo que foram a óbito não diferiram

significativamente . Para compreender o impacto dos níveis de TGF- β1 em taxas de sobrevivência , os pacientes foram classificados como tendo baixa (≤ 20 pg / ml) , intermediária (21-39 pg / ml) ou alta (≥ 40 pg / ml). Embora não foram encontradas diferenças significativas , os pacientes com baixos níveis de TGF- β1 (p = 0,1797) tenderam a apresentar uma taxa de sobrevida menor quando comparado com pacientes portadores de níveis elevados de TGF- β1 .

Uma visão geral da correlação entre os níveis de TGF - β1 e parâmetros de estresse oxidativo mostrou uma correlação significativamente positiva entre o nível de TGF- β1 em plasma e o conteúdo antioxidante eritrocitário , representada por níveis de GSH (p = 0,0231) .

CONCLUSÃO

Estes dados sugerem que níveis reduzidos de TGF-beta1 estão associados à aspectos clínicos de pior prognóstico no câncer de mama. Além disso, estes resultados indicam que o perfil oxidativo sistêmico está diretamente ligado ao subtipo molecular de câncer de mama apresentado por cada paciente.

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ORIGINAL ARTICLE

Molecular subtype is determinant on inflammatory status and immunological profile from invasive breast cancer patients

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Abstract Breast cancer consists in a chronic inflammatory disease with multiple biological and clinical behaviors. Based on high throughput technologies data, this disease is currently classified according to the molecular expression of estrogen (ER), progesterone (PR) and human epidermal growth factor (HER-2) receptors. In this study, we defined the inflammatory profile of the main molecular subtypes of breast cancer patients: luminal (ER and PR positive, HER-2 negative), HER-2 enriched (HER-2 positive) and triple negative (ER, PR and HER-2 negative). Cytokines panel was assessed by measurement of TNF-α, TGF-β, IL-1, IL-10 and IL-12 plasmatic levels. Oxidative profile was assessed by determination of lipid peroxidation, total antioxidant capacity of plasma, malondialdehyde levels, carbonyl content and nitric oxide (NO). Clinical data were correlated with inflammatory findings. Our findings demonstrated that patients bearing the luminal subtype displayed high TNF-α, TGF-β and enhanced oxidative stress levels associated with reduced IL-12. HER-2-enriched group exhibited higher levels of TNF-α, IL-12 and TGF-β associated with enhanced oxidative stress. Triple-negative subtype exhibited the most aggressive profile of disease behavior, with reduction in both TNF-α and TGF-β, with high levels of lipid peroxidation and NO. The clinical importance of our findings lies in the fact that the inflammatory status varies in distinct ways due to molecular subtype of breast cancer, opening potential therapeutic targets to future therapies.

Keywords Breast cancer · Molecular subtypes · TNF-α · TGF-β · Oxidative stress

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Introduction

Breast cancer is the leading cause of cancer-related deaths among women worldwide, accounting for 14 % of cancer deaths in 2008 [1]. It is a complex and heterogeneous disease, and its prognosis and biological behavior depend on several tumor and host characteristics [2, 3]. Inflammatory status plays a pivotal role in the immunomodulation of the tumor environment, as cytokines can exert a stimulatory or suppressive effect, affecting prognosis and the response to therapy [4].

Recent studies have revealed data that were used by researchers to propose a breast cancer molecular classification system based on several parameters, especially tissue expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2). On the basis of immunohistochemical staining and gene expression profiles, breast tumors are grouped into 3 major subtypes [5]. Luminal tumors (A and B) are characterized by high expression levels of hormonal receptors (ER and PR) and comprise approximately 70 % of invasive breast cancers. The luminal A subtype has a better prognosis than the luminal B subtype owing to a lower level of expression of the proliferation marker Ki-67. The HER-2-positive subtype is characterized by high expression levels of HER-2 and negative expression of ER and PR and represents approximately 15 % of invasive breast cancers. On the other hand, triple-negative tumors

(or basal-like) show negative expression of HER-2, ER and PR and are described as the subtype with the worst prognosis [6]. The design of breast cancer therapy strategies is based on this classification as well as clinical factors, which are used to categorize patients to achieve the most adequate and effective treatment response.

Breast cancer is an inflammatory disease and its progression involves changes in redox metabolism, which lead to cellular injury and irreversible damage to DNA caused by reactive species and cytokines [7]. Our group has recently performed a series of studies to improve the understanding of the effects of metabolites generated during inflammation and oxidative stress in breast cancer and to clarify the correlation between the cytokine profile and several aspects of the disease [8–10]. Our understanding of human breast cancer subtypes is limited, mainly because most research results are based on in vitro analyses. Furthermore, there is no information about the cytokines derived from human breast cancer subtypes.

Because the involvement of estrogen, HER-2 and progesterone receptors [11–13] in oxidative stress-mediated inflammatory pathways has been well delineated, it is possible to hypothesize that the combination of such receptors (which confer specific molecular subtypes to human breast tumors) could result from the distinct patterns of cytokine stimuli and also explain the singular behavior of each tumor subtype, allowing future therapeutic interventions. Improving our knowledge of these processes is important as certain breast cancer subtypes present a very aggressive behavior and often result in increased mortality due to the lack of therapeutic options.

The present study evaluated the repertoire of cytokines and the oxidative stress profile in correlation with the inflammatory status of the major breast cancer subtypes (luminal, HER positive and triple negative). We hypothesized that the differences among subtypes could be associated with distinct cytokine profiles and oxidative status. To address this question, we assessed the plasma levels of the cytokines tumor necrosis factor (TNF-α), transforming growth factor (TGF-β), interleukin 1 (IL-1), interleukin 12 (IL-12) and interleukin 10 (IL-10) and determined the oxidative status of breast cancer subtypes by measuring the oxidative damage to lipids and proteins, in addition to nitric oxide levels. We also correlated these findings with clinical data regarding disease prognosis.

Methods

Patient selection and study design

This study investigated a total of 53 women diagnosed with ductal infiltrative carcinoma of the breast, recruited at Londrina Cancer Institute, Londrina, Paraná, Brazil, from January 2009 to August 2011. Further, 53 healthy women were selected as age-paired volunteers to compose the healthy control group. This study was approved by Research and Ethics National Council (CAAE 0009.0.268.000-07) and all practice approved by Institutional board. All participants signed informed consent terms. Patients' clinical history was assessed and delineation included age at diagnosis, weight (to exclude overweight and obese individuals), comorbidities, TNM staging (tumor-node-metastasis classification of breast cancer) and hormonal status. Patients were divided into 3 cohorts, classified according to immunohistochemical criteria for defining breast cancer intrinsic subtypes, based on molecular receptors profile expression [5]: Luminal group ($n = 35$), HER-2-enriched group ($n = 11$) and triple negative ($n = 7$). Formalin-fixed paraffin-embedded biopsies from patients were immunostained with primary antibodies to ER, PR and HER-2 and identified by light microscopy as positive or negative based on stained area and intensity. Control group ($n = 53$) consisted from healthy women, paired with cancer patients 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, presented overweight or obesity, use of drugs, hypertension, sedentarism, diabetes and other eventual chronic conditions. Sample was obtained from whole blood collected in heparinized vacuum tubes (10 mL), and plasma was separated from centrifugation at $1{,}400\times g$ during 5 min. Aliquots were stored at -76 °C until analysis.

Cytokines analysis

Transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin 10 (IL-10) and interleukin 12 (IL-12) levels were determined in plasma using a commercial antibody specific RSG ELISA kit (eBiosciences, USA) employing internal controls, as directed by manufacturer and analyzed with a ELISA microplate reader at 490 nm. Limit detection was 2 pg/mL. The results were calculated in pg/mL by fitting to a standard curve obtained using human recombinant cytokines.

Oxidative stress profile determination

Total antioxidant capacity of the plasma (TRAP) was determined employing 2,2′azobis (ABAP) as a free-radical generator and luminol to amplify photons detection [10, 14]. Plasma samples were diluted 1:50 in glycine buffer 0.1 M, pH 8.6 at 37 °C. ABAP solution was obtained

dissolving 54.24 mg in 1 mL of ultra pure distillated water. Soluble vitamin E (Trolox) was employed as a standard antioxidant (2.5 mg in 5 mL of glycine buffer 0.1 M, pH 8.6 at 37 °C) and luminol solution as an amplificatory co-factor (3.98 mg in 250 µL of KOH 1 M added to 10 mL of glycine buffer and diluted 1:10 at time of reaction). Chemiluminescence curves were obtained in Glomax luminometer (Promega) and the results expressed in nM of Trolox.

Plasmatic lipoperoxidation [9, 15] was evaluated by adding 125 µL of sample in 865 µL of phosphate buffer 10 mM pH 7,4, with addition of 10 µL of *t*-butyl 3 mM solution. The reading of the reaction was carried out in a Glomax luminometer (Promega). The results were expressed in relative light units (RLU) after mathematical treatment of the entire curve obtained in Origin Lab 7.5 (smoothing, interpolation and area under the curve integration).

Sample nitrite was measured as estimative of NO levels and determined as previously described [16]. Briefly, plasma samples were deproteinized, incubated with cadmium granules and mixed with Griess reagent. The absorbance of samples was determinated at 550 nm in a microplate reader.

To estimate plasmatic malondialdehyde (MDA), plasma was oxidized in the presence of $FeCl_3$ and ascorbic acid and deproteinized with trichloric acetic acid reacted at heat with thiobarbituric acid to form MDA adducts [9]. Adducts were extracted with *n*-butanol. Organic phase was read at 535 and 572 nm in spectrophometer (Shimadzu UV-1650PC), and concentrations were obtained from the difference between absorbance, considering molar extinction coefficient of MDA at 535 nm. The results were expressed in nmol/L.

Carbonyl content was measured as estimative of oxidative injury to plasmatic proteins [17]. Plasma aliquots were incubated on ice with dinitrophenylhydrazine and deproteinized in trichloric acetic acid. After centrifugation, the pellets were treated with an ethanol/water solution (1:1). The final precipitates were dissolved in guanidine, incubated during 24 h at 37 °C and the carbonyl content calculated by obtaining sample spectra at 355–390 nm of samples. The obtained peaks were employed to calculate carbonyl concentration using a molar extinction coefficient of 22 $M^{-1}cm^{-1}$. Results were expressed in nmol/L/mg total protein content [18].

Statistical analysis

Analyses were carried out in duplicate sets, and data expressed as means ± errors of the means. CL curves profiles were compared using two-way analysis of variance (ANOVA). Bonferroni's test was employed to analyze differences between each points of the curve. Mann–Whitney's test was used to evaluate the difference between curves medians. Other parameters were compared by Student's t-test (parametric data) or Mann–Whitney's test (non-parametric data). $p < 0.05$ was considered significant. Spearman's test was also performed to verify possible correlation among parameters. All statistical analyses were performed using GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA). * Symbol indicates statistical significance when compared to controls and # when compared among breast cancer subtype groups.

Results

A schematic design of the study is presented in Fig. 1. The current study enrolled a total of 53 breast cancer patients and 53 healthy volunteers' controls. As shown in Table 1, most of the patients recruited at the period of the study presented a luminal profile ($n = 35$), while triple-negative patients corresponded to the minority ($n = 9$). HER-2-enriched and triple-negative group had lower mean age at disease diagnosis (47.27 years and 48.16 years, respectively) when compared to luminal group (59.16 years), both with a relevant percent of patients below 40 years (18.18 and 28.57 %, respectively). TNM staging of luminal patients demonstrated a similar distribution in relation to early (TNM I and II, $n = 11$) and advanced (TNM III and IV, $n = 24$) stages, while HER-2-enriched presented most of patients in advanced stages ($n = 7$). All groups of patients were similarly treated with paclitaxel (175 mg/m^2 i.v infusion, 1 h) or doxorubicin (60 mg/m^2 i.v infusion, 1 h) chemotherapic regimens. Lymph node involvement was observed in all groups (54 % to luminal, 50 % to HER enriched and 57 % to triple-negative group). Triple-negative patients revealed the greater number of patients with lymph node commitment (70 %) and the lowest lymph vascular invasion (43 %) in relation to luminal (54 % of positive lymph nodes, $p = 0.0175$ and 75 % of lymph vascular invasion, $p = 0.004$) and HER-2-enriched patients (63 % of positive lymph nodes, $p = 0.0053$ and 81 % of vascular invasion, $p = 0.003$). Distant metastasis was present in all groups, but a significative number of patients were observed in HER-2-enriched group (50 % of the cases, $p < 0.001$). Triple-negative and HER-2-enriched group also illustrated the largest percent of patients' death (28.6 and 18.2 %, respectively).

Tumor characterization (Fig. 2) denoted that luminal tumors had the highest mean tumor size (3.87 ± 0.66 cm) in relation to HER-2-enriched (1.63 ± 0.44 cm, $p = 0.0385$) and triple-negative (2.35 ± 0.65 cm, $p = 0.0441$) groups. On the other hand, triple-negative tumors revealed higher histological grade (2.8 ± 0.2) than luminal (2 ± 0.09, $p = 0.0452$) or HER-2-enriched patients (2.16 ± 0.3, $p = 0.0035$).

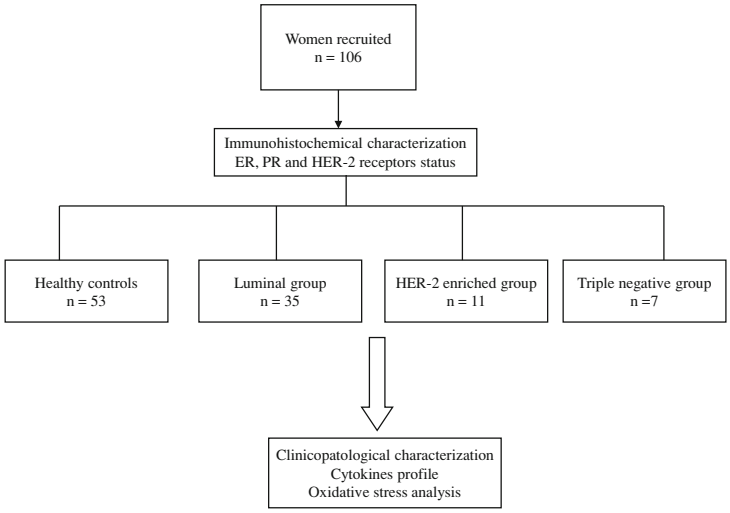

Fig. 1 Schematic design of the study. Luminal = ER and PR positive tumor patients, HER = ER and PR negative plus human epidermal growth factor receptor 2–enriched tumor patients, TN = triple-negative patients (estrogen, progesterone and HER-2 negative receptors)

Table 1 Clinicopathological characterization of patients

	Luminal	HER enriched	Triple negative
Number of patients	$n = 35$	$n = 11$	$n = 7$
Mean age at diagnosis (years)	59.16	47.27	48.16
<40 years	0	18.18 %	28.57 %
>40 years	100 %	81.81 %	71.43 %
TNM staging			
I	$n = 1$	–	–
II	$n = 10$	$n = 4$	$n = 3$
III	$n = 12$	$n = 5$	$n = 2$
IV	$n = 12$	$n = 2$	$n = 2$
Systemic chemotherapy treatment			
Paclitaxel 175 mg/m^2	54 %	50 %	57 %
Doxorubicin 60 mg/m^2	36 %	50 %	43 %
Positive lymph nodes	54 %	63 %	70 %
Lymphovascular invasion	75 %	81 %	43 %
Distant metastasis			
Yes	25 %	50 %	28 %
No	75 %	50 %	72 %
Number of deaths	$n = 4$	$n = 2$	$n = 2$

n number of patients, *HER-2* human epidermal growth factor receptor 2 patients, *TNM* tumor-node-metastasis classification

Cytokines profile (Fig. 3) showed that luminal (28.68 ± 7.74 pg/mL) and HER+2 (31.48 ± 11.96 pg/mL) subtypes presented significantly higher levels of TNF-α than controls (9.47 ± 1.55 pg/mL), $p = 0.0122$ and 0.0108, respectively, Fig. 3a). TN group revealed an important reduction in TNF-α levels, when compared to luminal subtype (3.2 ± 2.29 pg/mL, $p = 0.0429$). IL-12 levels (Fig. 3c) were reduced in luminal group in relation to controls (33.4 ± 0.89 pg/mL in controls and 29.72 ± 0.51 pg/mL in luminal, $p = 0.0014$). HER+2 subtype displayed higher IL-12 levels than luminal group (29.72 ± 0.51 pg/mL in luminal and 36.16 ± 3.38 pg/mL in HER+2, $p = 0.0290$). TGF-β levels (Fig. 3e) were augmented in luminal (19.8 ± 3 pg/mL, $p = 0.0250$) and HER+2 (30.2 ± 8.3 pg/mL, $p = 0.0092$) subtypes in relation to controls (10.5 ± 2.2 pg/mL). TN subtype

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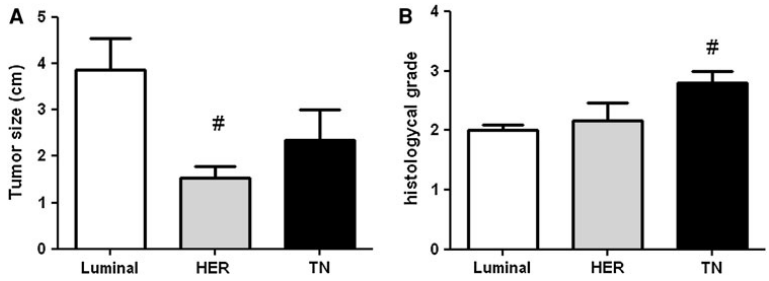


Fig. 2 Tumor characterization. **a** Tumor size and **b** histological grade from evaluated patients. Luminal = ER and PR positive tumor patients, HER = ER and PR negative plus human epidermal growth factor receptor 2–enriched tumor patients, TN = triple-negative patients (estrogen, progesterone and HER-2 negative receptors). #Statistical difference between groups ($p < 0.05$)

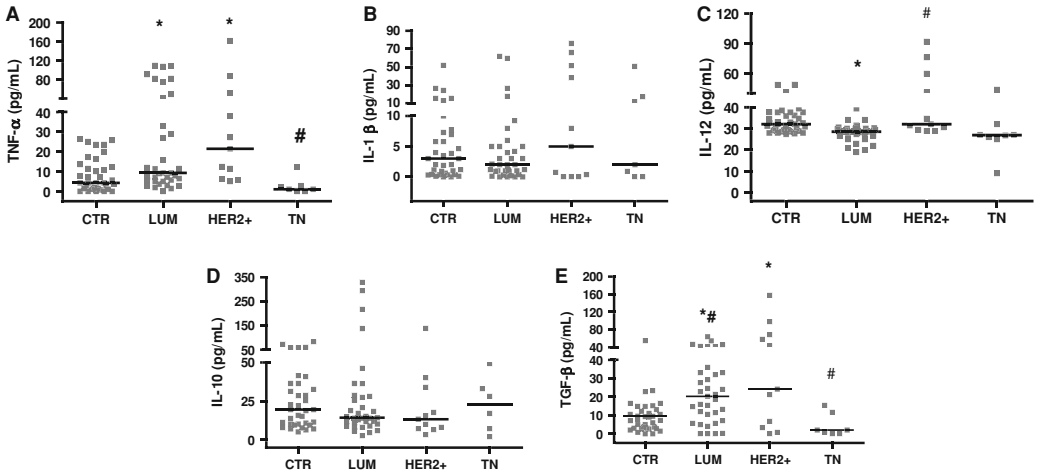


Fig. 3 Cytokines status from breast cancer subtypes. **a** TNF-α, **b** IL-1β, **c** IL-12, **d** IL-10 and **e** TGF-β plasmatic levels. Groups were compared by Mann–Whitney's test. Data are presented as individual distribution (*gray scatters*) and medians (*black line*). *Statistical difference when related to control and # when compared among subtypes ($p < 0.05$). CTR = Healthy controls, LUM = ER and PR positive tumor patients, HER2+ = ER and PR negative plus human epidermal growth factor receptor 2–enriched tumor patients, TN = triple-negative patients (estrogen, progesterone and HER-2 negative receptors)

exhibited lower levels than luminal group (5.03 ± 2.76 pg/mL, $p = 0.0176$). IL-1 and IL-10 levels did not show any significative alteration (Fig. 3b, d).

Oxidative profile characterization (Fig. 4) demonstrated that luminal patients displayed significative high levels of malondialdehyde (from 109.7 ± 10.83 nmol/L in controls to 161.5 ± 20.19 nmol/L, $p = 0.0356$) and reduced antioxidant capacity (from 440.3 ± 45.37 nmol/L in controls to 285.2 ± 22.7 nmol/L, $p = 0.0047$). HER-2-enriched group had the largest malondialdehyde level (from 440.3 ± 45.37 nmol/L in controls to 286 ± 47.67 nmol/L, $p < 0.0001$), high carbonyl protein content (from 77.58 ± 6.1 nmol/L/mg total protein in controls to 95.76 ± 10.44 nmol/L/mg protein, $p = 0.0867$) and reduced antioxidant capacity (from 440.3 ± 45.37 nmol/L in controls to 286 ± 47.67 nmol/L, $p = 0.0251$). HER+2 was also different in MDA levels from luminal (161 ± 20.2 nM, $p = 0.0155$) and TN subtypes (81.71 ± 24.8 nM, $p = 0.0275$). Triple-negative patients only showed significative levels of nitrite (from 17.38 ± 1.18 μM in controls to 20.61 ± 1.3 μM, $p = 0.0829$), without any variation in other oxidative parameters (81.71 ± 24.79 nmol/L to malondialdehyde levels, 329.9 ± 110.5 nmol/L to antioxidant capacity and 66.82 ± 10.97 to carbonyl content).

Lipid peroxidation profile was assessed by high sensitivity chemiluminescence (Fig. 4e). Luminal patients revealed higher levels of lipid peroxidation when compared to controls in relation to total profile of the curve, evaluated by two-way ANOVA ($p < 0.001$) and quantitatively in 26 points when analyzed by Bonferroni's test ($p < 0.001$). Mann–Whitney's test pointed a high significative difference in curves medians ($p < 0.0001$). No difference was

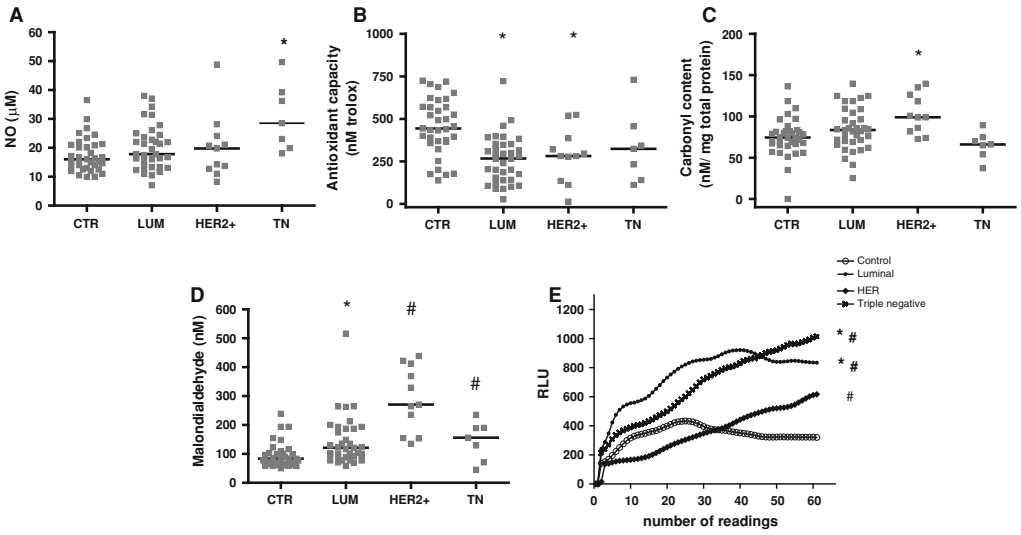


Fig. 4 Oxidative stress profile. **a** Nitrite levels measured as estimative of NO levels, **b** total antioxidant capacity, **c** carbonyl content, **d** malondialdehyde levels and **e** lipid peroxidation of plasma from breast cancer subtypes. Data are presented as individual distribution (*gray scatters*) and medians (*black line*). Groups were compared by Mann–Whitney's test. *Statistical difference when related to control and # when compared among subtypes ($p < 0.05$). Control = Healthy control, Luminal = ER and PR positive tumor patients, HER = ER and PR negative plus human epidermal growth factor receptor 2–enriched tumor patients, TN = triple-negative patients (estrogen, progesterone and HER-2 negative receptors)

detected in area under the curve integration of all groups (17,995 ± 6,572 RLU in controls, 37,773 ± 8,067 RLU in luminal, 21,669 ± 6,554 in HER-2 enriched and 57,004 ± 16,962 RLU in triple negatives), although the luminal and triple-negative groups have been a strong tendency to be higher agreeing with the same statistical profile revealed by the other tests. HER-2-enriched patients did not display significative lipid peroxidation in any of statistical evaluations, while triple negatives exhibited a significative distinct curve profile by two-way ANOVA ($p < 0.01$) and statistically different median of the curve ($p < 0.001$). All subtypes presented significantly different when compared among each other ($p < 0.001$).

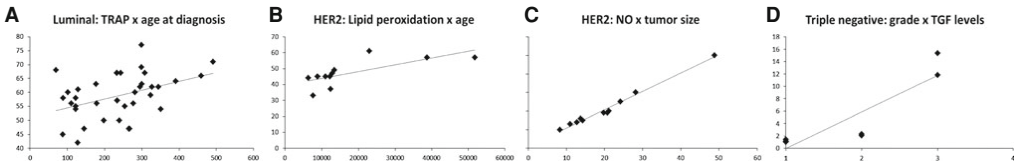
Finally, some correlations were found among clinical and plasmatic parameters from patients evidenced by Spearman's test (Fig. 5). Luminal patients presented a positive correlation between age at diagnosis and antioxidant capacity ($p = 0.0016$, Fig. 5a). HER-2-enriched patients demonstrated that age at diagnosis was positively correlated to lipid peroxidation ($R = 0.8420$, $p = 0.0175$, Fig. 5b), and nitric oxide levels were highly associated with tumor size ($R = 1$, $p < 0.0001$, Fig. 5c). Triple-negative subtype presented a high significative correlation between tumor histological grade and TGF-levels ($R = 0.9719$, $p = 0.0020$, Fig. 5d).

Discussion

The clinical management of breast cancer patients is determined by several prognostic factors, in particular ER, PR, and HER-2 status. Further, since inflammatory signaling constitutes a pivotal event in cancer, we focused for the first time on providing an overview of the major molecular subtypes of breast cancer in relation to the inflammatory status of breast cancer patients. We showed the existence of a distinct inflammatory profile associated with each specific breast cancer subtype.

Analysis of clinicopathological parameters revealed that the patients enrolled in the present study showed a variety of clinical factors associated with poor prognosis in breast cancer, such as earlier age at diagnosis [19, 20], high number of positive lymph nodes [21, 22], undifferentiated histological grade [23] and presence of lymphonodal metastasis [24, 25].

Our findings demonstrated that patients bearing the luminal subtype displayed high TNF-, TGF- and oxidative stress levels associated with reduced IL-12 levels. The HER-2-positive group exhibited higher levels of TNF-, IL-12 and TGF-, which were associated with enhanced oxidative stress. The triple-negative subtype showed the most aggressive disease behavior profile, with reduced



Spearman`s correlation	*P value*	*R value*
Luminal		
Antioxidant capacity x age at diagnosis	0.0016	0.7599
HER-2 enriched		
Nitric oxide levels x tumor size	P<0.0001	1.000
Age at diagnosis x lipid peroxidation	0.0175	0.8420
Triple negative		
Histological grade x TGF-β levels	0.0020	0.9719

Fig. 5 Spearman's correlation. Only significant correlations are presented ($p < 0.05$). **a** Luminal group correlation between antioxidant capacity and age at diagnosis. **b** and **c** HER2-enriched group correlations between nitric oxide and tumor size (**b**) and age at diagnosis and lipid peroxidation levels (**c**). **d** Triple-negative group correlation between histological grade and TGF-beta levels. Groups were compared by Mann–Whitney's test. HER = epidermal growth factor receptor 2–enriched tumor patients

TNF- and TGF-levels, a high degree of lipid peroxidation, and elevated nitric oxide levels.

One important focus of this study was to evaluate the inflammatory status of patients bearing each of the 3 major breast cancer subtypes through the assessment of a panel of cytokines and oxidative stress parameters. Enhanced oxidative stress was further detected in all evaluated subtypes, suggesting a sustained pro-inflammatory status associated with the expression of specific cytokines. A similar pattern of inflammatory responses was detected in both luminal and HER2 subtypes, while a very distinct profile was observed in the triple-negative samples.

We have recently demonstrated that patients with infiltrating breast cancer have high levels of oxidative stress that are enhanced by disease progression to metastasis [8]. Impairment of the antioxidant capacity as well as enhanced lipid peroxidation was observed in breast cancer patients, especially in the advanced stages, providing new evidence to support the involvement of the inflammatory status resulting from oxidative stress and cytokine imbalance. However, the association between breast cancer molecular subtypes and these parameters remains unclear.

Luminal subtype patients presented with high levels of TNF-, a pro-inflammatory cytokine closely related to oxidative stress responses and cancer progression [8, 27]. Furthermore, a sustained increase in TGF-levels is associated with oxidative changes in proteins, leading to the generation of advanced oxidation products [28]. These data suggest that the cytokine pattern observed in luminal patients may be an additional source of oxidative stress beyond estrogen receptors [29], as TNF-interferes with a wide range of pathways that involve both oxidative stress and TGF-signaling [30, 31].

Interestingly, the HER-2-overexpressing subtype showed an inflammatory pattern similar to that observed in luminal subtype patients, with high TNF- and TGF-levels associated with enhanced oxidative responses. Enhanced TGF-β signaling in HER-2-overexpressing tumors causes increased cell proliferation, survival, and invasive capabilities [26, 32]. These findings indicate that although luminal and HER-2-enriched subtypes present distinct patterns of molecular receptors and signal transduction, the cytokine profile associated with the tumor microenvironment is very similar, besides low molecular products formed as a consequence of oxidative stress that are involved in cellular proliferation and apoptosis mechanisms. However, patients with HER-2-positive cancer also showed high levels of IL-12 when compared to the luminal group, suggesting that this cytokine could be involved in HER-2 amplification, signaling, or host responses against HER-2 overexpression. In fact, studies have demonstrated that IL-12 is capable of inducing a progressive decrease in HER-2 expression in HER-2-positive tumors [33].

Contrary to the luminal and HER-2 patients, the triple-negative cohort showed reduced TNF- and TGF-levels, with enhanced lipid peroxidation and high NO levels. Tumor-associated macrophages from the triple-negative subtype express high levels of TGF-receptors [4]. Furthermore, attenuation of TGF- has been reported to promote oncogenic transformation and integrate a network of

autocrine signaling that mediates malignant transformation in triple-negative breast cancer [31], explaining the reduced levels observed in our study.

The role of TNF- in triple-negative tumors suggests that this cytokine plays a role in tumor invasion and metastasis [34, 35]. Clinical trials have been conducted aiming to block the TNF-signaling pathway in triple-negative patients that do not respond to conventional chemotherapy [36]. This scenario suggests that TGF- is a necessary mediator that contributes to the aggressive phenotype of triple-negative tumors, and the associations with TNF- and NO ensure growth via angiogenic pathways and metastatic spread [37], predicting the poor survival observed in estrogen receptor-negative breast cancers [38].

Analysis of clinical parameters revealed a significant correlation between age at diagnosis and the antioxidant capacity of the plasma from luminal patients, indicating that older age is associated with higher levels of TNF-associated protein (TRAP). Another interesting finding was the highly significant correlation between NO levels and tumor size detected in HER-2-positive patients. NO is tightly associated with tumor growth due to its pro-angiogenic properties, which result in accelerated tumor growth and metastasis [37]. Therefore, HER-2 overexpression could represent an additional mechanism to ensure tumor survival and growth mediated by NO signaling and further associated with oxidative stress and age at diagnosis. In triple-negative patients, histological grade showed a positive correlation with TGF-levels, suggesting that this cytokine may be associated with tumor differentiation.

The shortcomings of our study included a small sample size and the lack of prolonged patient follow-up to evaluate the impact of oxidative stress on breast cancer mortality according to the distribution of tumor subtypes. Despite these limitations, our findings are of clinical importance because they demonstrate that the inflammatory status varies in distinct ways according to the molecular subtype of breast cancer. In fact, little is known about the significance of the expression of surface receptors in breast cancer and its impact on tumor metabolism and host homeostasis. Considering the relevance of hormone receptors and HER-2 to tumor behavior and the systemic treatment of patients, we believe that our findings could contribute to a better understanding of tumor biology. Our findings could also contribute to help correlate the impact of inflammatory status on breast cancer subtypes to other types of tumors in the body.

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Conflict of interest The authors state no conflict of interest.

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Declaração de conflito de interesse

O trabalho intitulado *"Screening of circulating TGF-β1 level and its clinicopathological significance in human breast cancer"* publicado no periódico *Anticancer Research* apresenta divisão da primeira autoria, conforme depositado na própria publicação (página 1). Isto significa que a candidata **Ana Cristina da Silva do Amaral Herrera** teve contribuição equivalente ao primeiro autor do trabalho.

Adicionalmente, este trabalho não faz parte de nenhuma defesa anterior de tese ou dissertação submetida para obtenção de título neste programa de pós-graduação. Portanto, não há conflitos de interesse a declarar.

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☐ Epidemiological

Screening of Circulating TGF-β Level and Its Clinicopathological Significance in Human Breast Cancer

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Abstract. *Background: Transforming growth factor beta 1 (TGF-β1) participation in breast cancer development and metastasis is well established, however, the clinical meaning of the circulating level in women with breast cancer is poorly understood. Aim: To characterize the level of TGF-β1 in plasma from women with breast cancer and associated it with the main clinical factors associated with disease prognosis. Materials and Methods: TGF-β1 levels were measured by Enzyme-linked immunoassay (ELISA) kit. Clinicopathological data was assessed. Results: Women bearing triple-negative tumors presented significantly reduced levels of this cytokine when compared to the other subtypes (p=0.0338). Patients with metastases exhibited lower levels of TGF-β1 than the non-metastatic cohort (p=0.0442). Patients with early-onset disease had the highest plasma TGF-β1 level (p=0.0036). Doxorubicin chemotherapy induced a reduction in TGF-β1 level, promptly after drug infusion (p=0.0494). Patients with TGF-β1 levels lower than 20 pg/ml exhibited a tendency to have a reduced overall survival in a 40-month follow-up. Conclusion: Lower levels of circulating TGF-β1 are associated with a poor disease prognosis.*

Transforming growth factor beta (TGF-β) is a family of growth factors that affect both normal and neoplasic processes in the mammary gland. This pleiotropic cytokine is well implicated in regulating tissue remodeling and apoptosis in normal breast development (1). The role of TGF-β1, the most abundant isoform of TGF-β, in normal breast is based on its tumor suppressor functions. However, in breast cancer, this cytokine has tumor-promoting functions, especially in cells that evade TGF-β1 regulating properties during metastatic progression (2).

Effects of TGF-β1 can be observed systemically beyond the tumor microenvironment, since virtually all cells present TGF-β receptors (1). TGF-β also displays a redox-sensor function, as evidenced during the experimental radiation response (3). It has been implicated in several processes mediated by reactive species, particularly inflammation, aiming at the restoration of homeostasis. This redox-sensor activity seems to further regulate the reactive oxygen species (ROS) production in chronic processes (3).

Some important evidence regarding this cytokine has been reported in human breast cancer. Analysis of gene expression signatures reveal that TGF-β signaling regulates the expression of some chemokines during disease progression and is associated with poor patient prognosis, mainly in those with estrogen-positive tumors (4). In this way, the impact of TGF-β in cancer has become so evident that an anti-TGFβ therapy has been explored and improved (5-10). Over the years, the role of TGF-β in mammary tumorigenesis has been established *in vitro* (11-13), as well by *in situ* studies in resected tumors from patients, specially in metastatic conditions (14-16). Furthermore, the members of the TGF family are also predictors of poor response to chemotherapy in women with breast cancer (17).

Although the role of TGF-β in breast tumors has been successfully established, the characterization of its circulating levels in patients and its clinicopathological meaning in human breast cancer is poorly understood. Thus, the aim of this study was to provide a comprehensive characterization of TGF-β1 circulating levels in plasma from breast cancer women concerning the main factors associated with disease prognosis, as disease stage, tumor molecular subtype, metastatic status, age at diagnosis, chemotherapic regimen/response, as well to come up with the 40 months survival profile of patients accordingly to its levels.

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Key Words: Breast cancer, TGF-β1, prognostic factors.

Materials and Methods

Patient selection and design of the study. This prospective study enrolled a total of 101 women. A group of 61 women diagnosed with ductal infiltrative carcinoma of the breast were recruited at the Londrina Cancer Institute, Londrina, Paraná, Brazil, from January 2009 until September 2011, and 40 healthy women were selected as age-paired volunteers to comprise the healthy control group. This study was approved by the Research and Ethics National Council (CAAE 0009.0.268.000-07) and all practices were approved by the Institutional board. All participants provided informed consent. Patients' clinical history was assessed and included age at diagnosis, weight, comorbidities, TNM staging (tumor-node-metastasis classification of breast cancer) and hormonal status.

Patients were divided into cohorts classified accordingly to clinical and biochemical parameters regarding breast cancer prognosis and progression, as follows: disease stage, molecular subtype of breast tumor, presence or absence of primary tumor, occurrence of metastasis, age at diagnosis, type of chemotherapy and its impact after infusion, chemotherapy resistance, incidence of death and survival rates.

The control group consisted of healthy women, age-paired with patients (34-78 years), without previous history of any type of cancer, chemotherapy, hormonal or antioxidant therapy, or chronic diseases. Women were excluded if they were currently smoking, had hepatic, cardiac or renal dysfunction, obesity, use of drugs, hypertension, sedentarism, diabetes and other eventual chronic conditions. Sample was obtained from whole blood collected before chemotherapy infusion in heparinized vacuum tubes (10 ml) and plasma was separated by centrifugation at 1400 × *g* during 5 minutes. Aliquots were stored at –76˚C until analysis.

Immunohistochemical staining of tumor samples. Patients were divided in three cohorts, classified accordingly to immunohistochemical criteria for defining breast cancer intrinsic subtypes, based on molecular receptors expression profile (18): Luminal, HER-2 enriched and triple negative groups. Formalin-fixed paraffin embedded biopsies from patients were immunostained with primary antibodies to estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2 (HER-2) (Dako, Denmark) and identified by light microscopy as positive or negative based on stained area and intensity.

TGF-β1 analysis. TGF-β1 levels in plasma were determined using a commercial antibody-specific RSG ELISA kit (eBiosciences, San Diego, USA) employing internal controls, as directed by manufacturer and analyzed with a ELISA microplate reader at 490 nm. Plasma samples were previously acidified to activate latent TGF-β1 to its immunoreactive form. The limit quantification was 2 pg/ml. The results were calculated in pg/ml by fitting to a standard curve obtained using human recombinant TGF-β1.

Oxidative stress profile. Total antioxidant capacity of the plasma (TRAP) and erythrocytic reduced glutathione content (GSH) were used to determine the antioxidant profile, while nitric oxide levels (NO) and plasmatic malondialdehyde (MDA) represented the pro-oxidative status. All methods were performed as previously published (18, 19).

Statistical analysis. Analyses were carried out in duplicate sets and data expressed as the distribution of individual values and the respective medians. Parameters were compared by Mann-Whitney test.

Table I. *Clinicopathological characterization of patients.*

Age at diagnosis (years)	
Over 45	n=15
Below 45	n=46
Median age (range)	59.1 (31-78)
Histological type	
Ductal	100%
Lobular	None
Mixed	None
Molecular receptor status	
ER	70%
PR	60%
HER-2/neu	24%
TNM classification	
I	5%
II	30%
III	30%
IV	35%

ER (estrogen receptor); PR (progesterone receptor); HER2/neu (human epidermal growth factor receptor 2); TNM (tumor, node, metastasis classification).

Survival rates were evaluated by Kaplan-Meier method and the log-rank test was performed. A value of $p<0.05$ was considered significant. Spearman's test was also performed to verify possible correlation among parameters. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, USA).

Results

Clinicopathological characterization of the patient cohort is supplied in Table I. The median age at diagnosis was 59.1 years and all enrolled women were diagnosed with infiltrative ductal carcinoma of the breast. The molecular receptor status presented a distribution frequently observed in the general population. TNM staging displayed an equivalent distribution between early (TNM I and II) and advanced (III and IV) disease.

TGF-β levels in plasma were screened and categorized accordingly to clinical parameters. Patients evaluated by TNM staging did not exhibit any differences in TGF-β levels (Figure 1A). With respect to the molecular classification of breast cancer subtypes (Figure 1B), it was observed that patients bearing triple-negative tumors presented significantly lower levels of TGF-β when compared to the general cohort of women with breast cancer ($p=0.0338$). The surgical removal of the primary tumor mass did not affect the TGF-β1 level in plasma when compared to patients still bearing the primary tumor (Figure 1C). Plasmatic TGF-β1 levels were also diminished in patients presenting distant metastasis when compared to those with non- metastatic disease ($p=0.0442$). Women presenting early-onset (<45 years of age) of disease had higher levels of TGF-β when compared to those that had late-onset (age ≥45 years, $p=0.0036$).

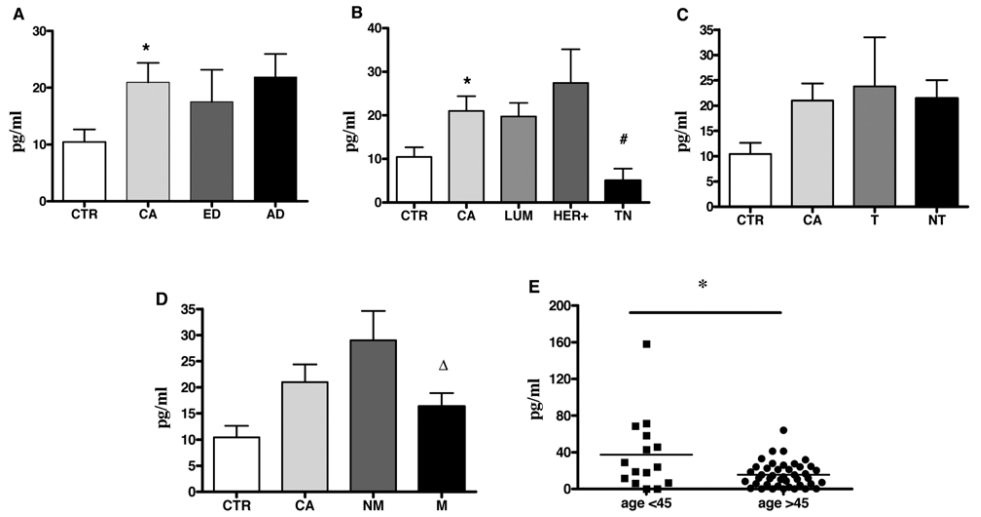

Figure 1. *Clinical parameters and transforming growth factor beta 1 (TGF-β1) level from patients diagnosed with ductal infiltrative carcinoma of the breast. Plasmatic TGF-β1 level was distributed according to TNM staging classification (A), breast tumor subtype (B), presence (T) or absence (NT) of the primary tumor (C), occurrence (M) or not (NM) of distant metastasis (D) and age at diagnosis of disease (E). Groups were compared by Mann-Whitney test. *statistical difference when compared to control, #when compared to all patients diagnosed with breast cancer (p<0.05), D significant difference between metastatic (M) and non-metastatic (NM) groups; *signficant difference between patients classified according to age at diagnosis. CTR (healthy control group); CA (all patients bearing ductal breast cancer enrolled in the study); ED (early stage patients, TNM I and II), AD (advanced stage patients, TNM III and IV); LUM (patients bearing luminal breast tumors); HER+ (patients bearing HER-2 amplified tumors); TN (patients bearing triple negative tumors).*

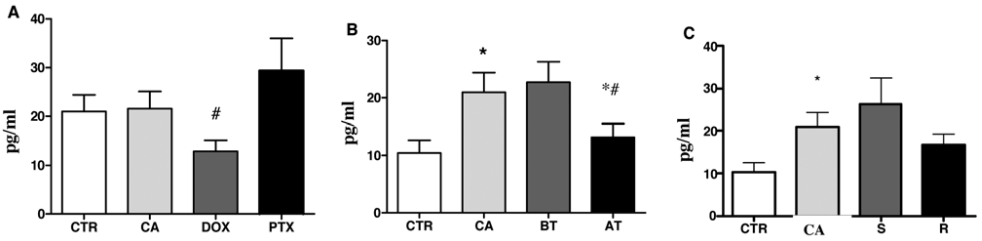

Figure 2. *Chemotherapy evaluation and transforming growth factor beta 1 (TGF-β1) level from patients diagnosed with ductal infiltrative carcinoma of the breast. Plasmatic TGF-β1 level was distributed accordingly to Doxorubicin (DOX) or Paclitaxel (PTX) treatment (A); before (BT) or after (AT) chemotherapy (B); and sensitivity (S) or resistant (R) to treatment (C). Groups were compared by Mann-Whitney test. *significant difference between CA and AT groups; #significant difference between AT and BT groups, and DOX and PTX groups (p<0.05). CTR (healthy control group); CA (all patients bearing ductal breast cancer enrolled in the study).*

Figure 2A shows that the TGF-β1 level depended on the type of chemotherapeutic regimen, since patients undergoing doxorubicin chemotherapy as monotherapy presented a significant reduction in plasmatic TGF-β1 when compared to patients that received only paclitaxel as treatment (p=0.0088). TGF-β1 levels in patients with breast cancer were higher compared to these in healthy controls (p=0.0435, Figure 2B). Furthermore, the evaluation of TGF-β1 in patients after treatment with paclitaxel plus doxorubicin, compared before and after infusion showed that chemotherapy promptly reduced

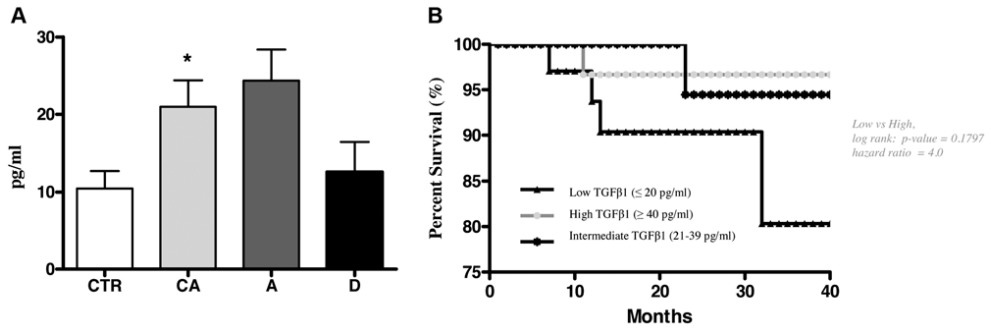

Figure 3. *Survival profile and transforming growth factor beta 1 (TGF-β1) level from patients diagnosed with ductal infiltrative carcinoma of the breast. Plasmatic TGF-β1 level was distributed according to analysis of the number of alive and dead patients (A). Overall survival of patients based on TGF-β1 level, evaluated during a 40-month follow-up (B). Groups were compared by Mann-Whitney's test. *indicate statistical difference when compared to control and # when compared to all patients diagnosed with breast cancer (p<0.05). CTR (Healthy control group); CA (all patients bearing ductal breast cancer).*

TGF-β1 to levels to less than those observed in patients with breast cancer without any treatment (p=0.0494). In relation to the chemotherapy response (Figure 2C), TGF-β1 levels did not differ in patients with responsive and those with resistant disease.

Analysis of the survival profile (Figure 3) indicates that the TGF-β1 levels in the cohort of patients who survived and the group that died did not differ significantly (Figure 3A). To understand the impact of the TGF-β1 levels on survival rates (Figure 3B), patients were categorized as having low (≤20 pg/ml), intermediate (21-39 pg/ml) or high (≥40 pg/ml) circulating levels. Although no significant differences were found, patients with lower levels of TGF-β1 (p=0.1797) tended to present the lowest survival rate when compared with patients bearing high TGF-β1 levels.

An overview of the correlation between TGF-β1 levels and oxidative stress parameters is presented in Table II. A significant positive correlation was observed between the TGF-β1 level in plasma and the erythrocytic antioxidant content, represented by GSH levels (p=0.0231).

Table II. *Spearman's correlation of transforming growth factor beta 1 (TGF-β1) level and oxidative status of patients with breast cancer.*

Correlation	R value	*p*-Value
GSH X TGF-β	0.3380	0.0231*
NO X TGF-β	–0.1471	0.2981
TRAP X TGF-β	–0.0583	0.6841
MDA X TGF-β	–0.2412	0.1105

Groups were compared by Mann-Whitney test. *Statistical significant difference (p<0.05). GSH (reduced glutathione); NO (nitric oxide); TRAP (total antioxidant capacity of plasma); MDA (malondialdehyde).

Discussion

The prognostic utility of TGF-β in human breast cancer has been recently described. TGF-β has been implicated in overall survival shortening (20), early tumor progression (21) and enhanced breast cancer susceptibility (22). Nevertheless, an overview of this circulating cytokine in a context including the most relevant prognostic and clinical aspects of breast cancer disease is still lacking. Our study provides information regarding some of the known aspects and novel insights into aspects of TGF-β1 in breast cancer for the first time. Relevant findings include the occurrence of lower TGF-β1 levels detected in patients bearing the triple-negative tumor subtype, as well in those carrying metastatic disease and submitted to doxorubicin chemotherapy. On the other hand, high plasmatic levels were found in patients with early-onset disease (with age at diagnosis below 45 years). When stratified by TGF-β1 levels, it was detected that patients exhibiting low circulating levels of this cytokine (lower than 20 pg/ml) tended to a reduced overall survival when compared to those that presented high TGF-β1 levels (up to 40 pg/mL).

A global analysis of circulating TGF-β1 in women carrying breast tumors revealed the occurrence of high levels of this cytokine when compared to healthy controls. Alterations in TGF-β signaling affect a variety of aspects of breast cancer development and progression, justifying its role according to disease staging (23). Sustained TGF-β expression in breast cancer is mainly associated with the advanced stages of disease (24), in agreement with the distribution of most our patient cohort (about 65% in TNM stages III and IV).

Our results further indicate the existence of a differential profile of circulating TGF-β1 accordingly to the molecular subtype of breast cancer. Patients bearing triple-negative tumors had a significantly lower TGF-β1 levels when compared to both those with the luminal/HER2 subtypes and healthy controls. Although the estrogen pathway has a regulatory effect on TGF-β signaling in the breast, evidence supports the deregulation of such networks in breast cancer (25), reflected by our findings of reduced circulating TGF-β in the triple negative cohort. A well-established fact, also described here, is that patients with metastatic disease present enhanced TGF-β1 levels when compared to those with non-metastatic and to healthy individuals. The metastatic potential induced by this cytokine is mainly related to its capacity to polarize the epithelial mesenchymal transition of mammary epithelial cells and ensure its malignant phenotype (26).

We further demonstrated that the circulating level of TGF-β1 is significantly different in relation to the age at diagnosis. Patients presenting early-onset disease (under 45 years) exhibited higher levels of this cytokine in relation to older patients. High expression of both extracellular TGF-β and TGF-βR2 have been identified in breast tumors and strongly associated with earlier age at onset (27), corroborating our findings.

Studies demonstrate that alterations in TGF-β signaling affects the toxicity of doxorubicin and enhances its antitumoral activity (28-30). We showed that patients undergoing doxorubicin-based chemotherapy display a reduction of circulating TGF-β1 when compared to untreated or paclitaxel-treated cohorts, but no alteration in the sensitivity profile was found. This profile was observed promptly after doxorubicin infusion, indicating that this drug is able to promote a swift reduction in circulating levels of TGF-β1. The mechanism underlying this fact is not known.

Although not significant, we observed a tendency for reduced overall survival in women presenting TGF-β levels lower than 20 pg/ml. Alterations in TGF-β signaling are associated with poor outcome in breast cancer. A large cohort study demonstrated the occurrence of dysfunctions in the components of the TGF-β cascade in tumors and associated some of these aspects with shorter overall survival (31). To the best of our knowledge, this study is the first report highlighting this information on survival according to stratification by circulating TGF-β1 levels. A continuous follow-up of such patients is necessary to understand the long-term meaning of these findings.

Finally, we found a significant positive correlation between plasmatic TGF-β and erythrocytic GSH content. The occurrence of oxidative stress in women with breast cancer is well known (19, 33) and this can contribute to some pathological processes mediated by TGF-β. The antioxidant system has been described as a protective mechanism against latent TGF-β activation in pathological processes (32), indicating that the positive correlation observed here between this cytokine and GSH levels may reflect TGF-β redox-sensor activity (3).

In conclusion, our data adds new information that contributes to the better comprehension of the clinical meaning of circulating TGF-β1 in women with breast cancer. Furthermore, reduced circulating TGF-β1 seems to be a determinant of poor disease prognosis.

Conflict of Interest

The Authors declare no conflict of interest.

Acknowledgements

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Pró-Reitoria de Pesquisa e Pós-Graduação
Diretoria de Pesquisa
Divisão de Projetos de Pesquisa

OF. CIRC. DP/DPP. 043/2010 Londrina, 02 de julho de 2010

Prezado(a) Professor(a),

É com satisfação que comunicamos a Vossa Senhoria que de acordo com os critérios aprovados pelo **Conselho de Ensino, Pesquisa e Extensão** em reunião realizada no dia 26-11-98, o projeto de pesquisa de sua autoria/coordenação, protocolado sob o nº **6081/2010 (6967)**, foi aprovado em **10/06/2010** com duração inicial de **20 meses**, de acordo com a **Resolução 274/05** de 01/11/05.

Aproveitamos para informar que **registramos o início do projeto a partir de 10/06/2010**

Em anexo seguem:
- Cópia do cadastro;
- Formulário de Orientações;

Na oportunidade, reiteramos protestos de elevada estima e distinta consideração e colocamo-nos desde já ao seu inteiro dispor para esclarecimentos necessários.

Atenciosamente,

Profª. Drª. Carmen Silvia Vieira Janeiro Neves
Diretora de Pesquisa

Égle Maria de Souza
Chefe da Divisão de Projetos de Pesquisa

OF.CIRC. DP/DPP.043/2010

Ilmo(a). Sr(a).
Prof(a). Rubens Cecchini
Departamento de Ciências Patológicas
CCB

UNIVERSIDADE
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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

A – Informações sobre a pesquisa:

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 – Procedimentos do Estudo:

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 expectativa 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 – Confidencialidade da Pesquisa

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. A participação no estudo não acarretará custos para você e não haverá nehuma compemsação 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 33714521.Você poderá entrar em contato com o Comitê de Ética em Pesquisa com seres humanos da Universidade Estadual de Londrina pelo telefone 43 33712490.

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

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarefcido 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.

Londrina, ____ de _____, 200 ___.

_____
Assinatura do doador

_____
Dra Ana Cristina da Silva do Amaral Herrera
Oncologista responsável pela coleta de material
CRM 15214

_____
Dr Rubens Cecchini
Pesquisador Responsável