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RODRIGO KERN

ANÁLISE DA EXPRESSÃO DA PROTEÍNA DE CHECKPOINT IMUNOLÓGICO CTLA-4 EM BIÓPSIAS DE PACIENTES COM CÂNCER DE MAMA E SEU IMPACTO NA PRODUÇÃO SISTÊMICA DE MEDIADORES DA RESPOSTA IMUNE

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RODRIGO KERN

ANÁLISE DA EXPRESSÃO DA PROTEÍNA DE CHECKPOINT IMUNOLÓGICO CTLA-4 EM BIÓPSIAS DE PACIENTES COM CÂNCER DE MAMA E SEU IMPACTO NA PRODUÇÃO SISTÊMICA DE MEDIADORES DA RESPOSTA IMUNE

Essa dissertação foi julgada adequada para obtenção do título de Mestre em Ciências Aplicadas à Saúde e aprovada em sua forma final pela Orientadora e pela Banca Examinadora.

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DEDICATÓRIA

Aos pacientes do Hospital do Câncer de Francisco Beltrão.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS

CM: câncer de mama

CMTN: câncer de mama triplo negativo

RE: receptor hormonal para estrógeno

RP: receptor hormonal para progesterona

HER2: receptor do fator de crescimento epidérmico humano tipo 2

Ki-67: índice de proliferação celular

LIT: linfócitos infiltrantes de tumor

MHC: complexo principal de histocompatibilidade

CTLA-4: antígeno de linfócito T citotóxico 4

PD-1: proteína de morte celular programada 1

PD-L1: ligante de proteína de morte celular programada 1

IHQ: imuno-histoquímica

IC: inibidores de checkpoint

pCR: resposta patológica completa

ACS: American Cancer Society

NCCN: National Comprehensive Cancer Network

ANÁLISE DA EXPRESSÃO DA PROTEÍNA DE CHECKPOINT IMUNOLÓGICO CTLA-4 EM BIÓPSIAS DE PACIENTES COM CÂNCER DE MAMA E SEU IMPACTO NA PRODUÇÃO SISTÊMICA DE MEDIADORES DA RESPOSTA IMUNE

RESUMO

O câncer de mama (CM), além de apresentar alta incidência populacional, é caracterizado por heterogêneos subtipos moleculares, cada qual com uma biologia tumoral própria. Isso resulta em carcinomas distintos quanto à imunogenicidade e à capacidade de evasão tumoral ao sistema imune. Assim, diversos estudos investigam o significado clínico dos receptores proteicos PD-1 e CTLA-4 (também conhecidos como checkpoints imunológicos), cuja função fisiológica é a modulação negativa da resposta imune celular. Entretanto, o papel do CTLA-4 na biologia tumoral do CM ainda não é completamente compreendido. Portanto, este trabalho tem por objetivo estudar a expressão tecidual de CTLA-4 em amostras de pacientes portadoras de CM, seu impacto na produção de mediadores sistêmicos da resposta imune e sua correlação clínico-patológica. Para isso, pacientes atendidas no Hospital do Câncer de Francisco Beltrão/PR – Brasil no período de 2016 a 2019 foram convidadas a participar do estudo, aprovado pelo Comitê de Ética em Pesquisa Institucional sob o número CAAE N° 35524814.4.0000.0107. Foram incluídas 117 pacientes com diagnóstico anatomopatológico de Carcinoma Ductal Invasivo (CDI). Amostras de sangue periférico heparinizado foram coletadas para avaliação do perfil de mediadores imunológicos circulantes (IL-4 e IL-12 por ELISA; níveis de lipoperóxidos por quimioluminescência; e estimativa de óxido nítrico (NO) pela medida de nitrito via método cádmio-cobre). Lâminas de tecido da ressecção cirúrgica da biópsia foram obtidas para análise da expressão de CTLA-4 por imunofluorescência. Dados clínico-patológicos foram obtidos através de consulta aos prontuários médicos. Foi realizada a análise dos dados no software GraphPad Prism 7.0 (USA) e SPSS 25.0.0 (IBM, USA). Os resultados apresentaram expressão de CTLA-4 significativamente correlacionada ao subtipo molecular triplo negativo. Além disso, pacientes com expressão positiva de CTLA-4 em células tumorais apresentaram níveis reduzidos de NO. Pacientes que apresentaram expressão de CTLA-4 apenas em linfócitos infiltrantes de tumor (LIT) revelaram reduzidos níveis de IL-12 no plasma. Não foram identificadas correlações referentes à análise de IL-4 e peroxidação lipídica. Portanto, os achados do presente trabalho sugerem que a expressão de CTLA-4 em microambiente tumoral é capaz de interferir na geração de inflamação sistêmica no câncer de mama, afetando diretamente os níveis de IL-12 e NO, e correlacionando-se com um perfil mais agressivo de doença.

Descritores: câncer de mama; checkpoint imunológico CTLA-4; linfócitos infiltrantes de tumor; imunidade tumoral; estresse oxidativo; lipoperoxidação; óxido nítrico; inflamação; IL-4; IL-12.

EVALUATION OF CTLA-4 EXPRESSION AND ITS INFLUENCE IN SYSTEMIC INFLAMMATORY RESPONSE OF BREAST CANCER PATIENTS

ABSTRACT

Purpose: Breast cancer is the leading cause of women's death among all cancers. It is a heterogeneous disease consisting of subgroups with different molecular features and clinical outcomes. Cancer cells can evade antitumor T-cell responses by expressing some immune inhibitory molecules as the programmed cell death protein 1 (PD-1) and/or the cytotoxic T-lymphocyte associated protein 4 (CTLA-4), known as immune checkpoints, which have the function of reducing or limiting the cellular immune responses in a physiological scenario. Recent evidences have pointed out that the expression of the CTLA-4 in tumors is a poor prognosis factor, but the systemic impact of its expression in tumor or infiltrating leukocytes is not clear for breast cancer. In this context, it is important to investigate CTLA-4 expression in BC microenvironment and its potential role in systemic inflammation modulation. Based on this, we analyzed CTLA-4 expression by immunofluorescence, and according to its expression in tumor or TILs, evaluated the circulating levels of interleukins and oxidative stress mediators known as players of the inflammatory response. This study was approved by the Ethics Committee on Research of State University of Western Paraná under the number CAAE 35524814.4.0000.0107. **Methods**: Paraffin-embedded breast tumors and whole blood samples were collected from 117 women diagnosed with breast cancer. Clinicopathological data were obtained through medical records. In blood samples, oxidative stress parameters were evaluated by measuring its plasmatic lipoperoxidation by high-sensitivity chemiluminescence and estimating nitric oxide metabolites (NO) by the cadmium-copper system coupled to Griess reaction. Interleukins 12 (IL-12) and 4 (IL-4) were measured in plasma samples by ELISA kits. CTLA-4 expression was determined by immunofluorescence and evaluated by its labeling in tumor-infiltrating leukocytes (TILs) or breast tumors. Statistical analyses were performed using the GraphPad Prism 7.0 software package (GraphPad Software, San Diego, CA, USA). Also, SPSS 22.0 software (IBM, USA) was used to obtain the clinicopathological data frequencies and Spearman's correlations. **Results**: CTLA-4 expression in TILs significantly correlated to triple negative breast tumors, Patients carrying CTLA-4 positive tumors exhibited lower plasmatic NO levels, while those with CTLA-4 expression only in TILs exhibited reduced levels of IL-12 in plasma. No variations were observed in IL-4 or lipid peroxidation profiles in any CTLA4 status. **Conclusion:** CTLA-4 expression in BC is a putative marker of clinical significance as well as a rationale therapeutic target in the emerging field of immunotherapy. Moreover, our findings suggest that CTLA-4 expression in both tumor and TILs can affect the systemic inflammatory status of breast cancer patients, affecting directly the levels of antitumor molecules as IL-12 and NO, and correlating to most aggressive disease.

Keywords: breast cancer; CTLA-4 antigen/antagonists & inhibitors; lymphocytes, tumor- Infiltrating/immunology"; oxidative stress; lipoperoxidation; nitric oxide; inflammation; IL-4; IL-12.

SUMÁRIO

1 INTRODUÇÃO

O câncer de mama (CM) é a neoplasia mais comum entre as mulheres (MCGUIRE, 2016), sendo a quinta mais letal entre todos os tipos de câncer (SIEGEL; MILLER; JEMAL, 2019). Trata-se de uma doença heterogênea, cujas apresentações clínicas e moleculares são variadas. O crescente conhecimento desses subtipos moleculares possibilitou um melhor entendimento da biologia tumoral em cada paciente, fato que vem determinando protocolos de tratamento personalizados para cada paciente (SØRLIE et al., 2001).

Atualmente, cinco subtipos moleculares são adotados na prática clínica: Luminal A – alta expressão de receptores hormonais para estrógeno (RE) e/ou progesterona (RP) e baixo índice de proliferação (Ki-67); Luminal B – alta expressão de RE e alto Ki-67; Luminal-HER2 – expressão de receptores hormonais e receptor HER 2; Superexpressão de HER2 – alta expressão de receptores HER2 RE e alto Ki-67; e Triplo Negativo (TN) – nenhuma expressão de receptores hormonais nem de receptores HER2 e alto Ki-67**.** Considerando-se tais subtipos moleculares, pacientes triplo negativas apresentam pior desfecho do que as subtipos luminais (SØRLIE et al., 2001; VODUC et al., 2010). Os casos de CM em estadiamento metastático e casos de CM resistente a terapia representam os maiores desafios dos vigentes protocolos terapêuticos.(VALIENTE et al., 2018) Como exemplo de apresentação clínica, casos metastáticos de CM luminal e superexpressão de HER2 apresentam tropismo por ossos e linfonodos, ao passo que CMTN apresenta tropismo por pulmão e cérebro (BUONOMO, et al., 2017). Essas distintas apresentações clínicas suportam hipóteses de diferentes mecanismos de metástase para cada subtipo molecular da doença.

Na última década, houve ampliação do acesso às terapias-alvo dirigidas, especialmente do Trastuzumab que têm como alvo o receptor do fator de crescimento epidérmico humano do tipo 2 (HER2). Isso, somado a hormonioterapia e a quimioterapia, tem aumentado o sucesso dos tratamentos de CM. Entretanto, o CMTN, que representa 15-20% dos CM, ainda carece de opções terapêuticas com melhores taxas de resposta (BIANCHINI et al., 2016; KHOSRAVI-SHAHI; CABEZÓN-GUTIÉRREZ; CUSTODIO-CABELLO, 2018). Nesse contexto, ensaios clínicos investigam promissoras estratégias de

tratamento para os casos agressivos de CMTN: os medicamentos Inibidores de Checkpoints (IC) (DE LA CRUZ-MERINO et al., 2019; RIBAS; WOLCHOK, 2018; VIKAS; BORCHERDING; ZHANG, 2018). Esses medicamentos têm como alvo terapêutico os receptores PD-1 e CTLA-4 (checkpoints imunológicos), os quais têm função fisiológica de modulação negativa da resposta imune celular (BRUNET et al., 1988).

Entretanto, a interação desses checkpoints imunológicos com mediadores de resposta imune, como citocinas pro-inflamatórias, ainda não está clara. Por exemplo, poucos estudos avaliaram a potencial relação da expressão de CTLA-4 em microambiente tumoral com o perfil de mediadores imunológicos circulantes. Assim, estudos sobre potencial influência do microambiente tumoral sobre mediadores sistêmicos da resposta imune são fundamentais para compreensão da dinâmica antitumoral em pacientes com CM. Somente de tal modo é possível promover imunoterapias direcionadas a checkpoints imunológicos de forma personalizada e, assim, otimizar custos e ganhos clínicos.(SANTA-MARIA; NANDA, 2018) Portanto, o objetivo do presente trabalho foi avaliar se existe algum perfil clínico associado à expressão tecidual de CTLA-4 e o impacto dessa expressão na produção de mediadores da resposta imunológica em pacientes com CM.

1.1 Mediadores sistêmicos da resposta imune e estresse oxidativo

A natureza pro-inflamatória dos carcinomas humanos induz uma série de respostas imunes em seu microambiente tumoral, evidenciada pelos linfócitos infiltrantes de tumor (LIT) (BURNET, 1967; HANAHAN; WEINBERG, 2011). Entretanto, os tumores podem transpor as imunidade anti-tumoral através da promoção de desregulação imune. Assim um microambiente tumoral imuno deficiente é formado, o qual conta com diversas citocinas e checkpoints imunológicos, cuja inter-relação ainda não está totalmente elucidada (EMENS, 2012; KOCH et al., 2012; YU et al., 2014).

Considerando esse controle da resposta imune por tais moléculas, a Interleucina 12 (IL-12) é reportada na literatura como um dos principais mediadores da resposta imune antitumoral e essencial para ativação da capacidade tumoricida das células natural killer (NK) (CLANCY et al., 2011; DENARDO; COUSSENS, 2007; GOMES; CORREIA; SILVA-SANTOS, 2007;

STEWART; SMYTH, 2011). A IL-12 é produzida principalmente por células apresentadoras de antígenos, como: células dendríticas, macrófagos e linfócitos B (TRINCHIERI et al., 1993).

Paralelamente, o processo inflamatório em pacientes com CM está associado uma gama de modificações plasmáticas oxidativas. Estudos sugerem que as espécies reativas de oxigênio estão envolvidas na etiologia e progressão do CM, uma vez que marcadores de estresse oxidativo, como produtos de peroxidação lipídica, estão frequentemente presentes em pacientes com CM (GÖNENÇ et al., 2006; RAJNEESH et al., 2008). Ademais, apesar de diversas pesquisas discutirem as múltiplas funções do óxido nítrico na imunidade tumoral de pacientes com carcinoma (NAKAMURA et al., 2006; PORRO et al., 2010; SALIMIAN RIZI et al., 2015; WINK et al., 1998), poucos estudos avaliaram uma potencial relação dessa molécula com a expressão de checkpoints, seja em células tumorais, seja em linfócitos infiltrantes de tumor.

1.2 Imunidade tumoral e linfócitos infiltrantes de tumor

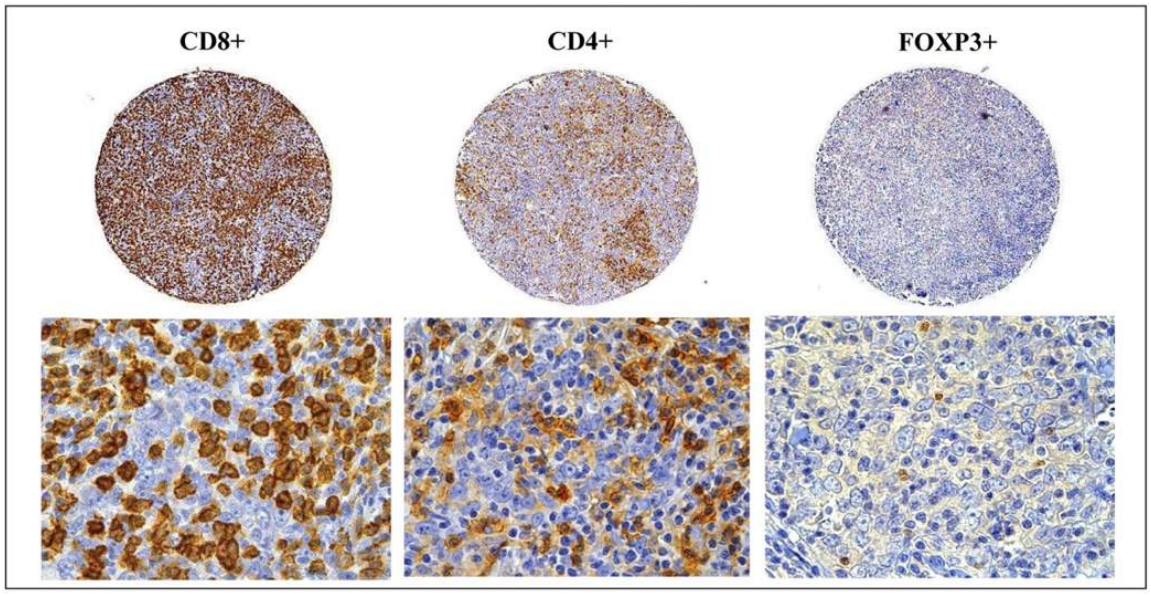
O conceito "*immune surveillance*" já é estudado há décadas (BURNET, 1967), sendo consenso que os carcinomas induzem uma resposta imunológica em seu microambiente tecidual (SCHREIBER; OLD; SMYTH, 2011). Essa resposta imune tem papel crucial em limitar a progressão do câncer, existindo amplas evidências que confirmam que a imunidade antitumoral é determinante no desfecho e progressão do câncer (HANAHAN; WEINBERG, 2011).

Nesse sentido, a avaliação da presença de linfócitos infiltrantes de tumor (LIT) tem sido relatada em estudos oncológicos como um fator preditor de sobrevida (LUEN et al., 2017). Lu e colaboradores (2017) apresentam que a taxa de LIT é diretamente relacionada a maior taxa de pCR (*patological complete response*). Assim sendo, além de melhor prognóstico, esses pacientes com maior taxa de LIT poderiam teoricamente ter maior benefício à imunoterapia (LU; BAI; WANG, 2017).

O CM de maneira geral, apesar da presença de LIT (**figura 1**), não é considerado um carcinoma fortemente imunogênico como melanoma ou carcinomas renais. Entretanto, os subtipos moleculares CMTN e HER2 são caracterizados pela maior presença LIT do que os demais subtipos moleculares (LOI et al., 2013; STANTON; ADAMS; DISIS, 2016), ou seja, são CM

especificamente mais imunogênicos. Assim, essa dinâmica imune coloca em questão se a avaliação anatomopatológica e imuno-histoquímica (IHQ) dos LIT deva ser parte dos protocolos de assistência à paciente com CM. Até o momento, os LIT não apresentam clara utilidade clínica no CM e ainda não podem ser utilizados isoladamente como biomarcadores para ajustes de terapêutica em CM (LUEN et al., 2017).

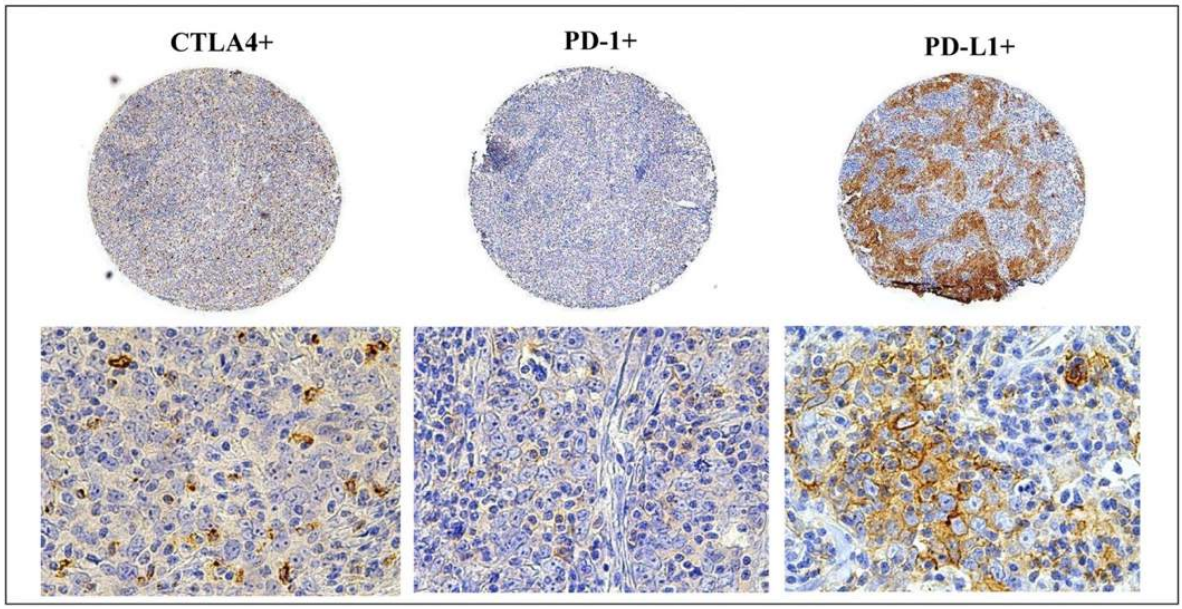
Figura 1 – Linfócitos infiltrantes de tumor em câncer de mama



(CATACCHIO et al., 2019)

Além de considerar o grau de LIT, também é preciso considerar se os linfócitos estão ativos ou em anergia. Sendo assim, a ativação dos linfócitos é influenciada pelo balanço entre ligações inibitórias ou ligações estimulantes com a família CD28 (ABBAS; LICHTMAN; PILLAI, 2015). O processo de ativação dos LIT ocorre com a interação do receptor CD28 como receptor B7 da célula apresentadora de antígeno. Por outro lado, os LIT também expressam receptores inibitórios da família CD28, como CTLA-4 (antígeno de linfócito T citotóxico 4) e PD-1 (proteína de morte celular programada 1), como. exemplificado na **figura 2**.

Figura 2 - Expressão de PD-L1, PD-1, CTLA-4 em IHQ de câncer de mama



Magnificação em 50× e 630× (CATACCHIO et al., 2019)

Ambos receptores promovem a redução da atividade dos LIT, limitando assim a efetividade da imunidade tumoral em promover apoptose das células cancerosas (EGEN; ALLISON, 2002). Especificamente, o receptor CTLA-4 atua como uma receptor inibitório competitivo ao receptor CD28 uma vez que tem ligação química mais ávida com as moléculas do receptor B7 (ABBAS; LICHTMAN; PILLAI, 2015).

Portanto, esses receptores funcionam como checkpoints imunológicos e seu grau de expressão pode ser responsável pela efetividade da resposta imune antitumoral (KHAJA et al., 2017). Além disso, interessante estudo laboratorial com culturas celulares de CM, positivas para CTLA-4, identificou que a função das células dendríticas estava reduzida nesse microambiente (CHEN et al., 2017). Considerando isso, a integralidade do processo de resposta imune antitumoral ainda não está completamente esclarecido, mas pesquisas sugerem que seja esse o processo de imunoevasão estabelecido pelos tumores, inclusive no CM (LUEN et al., 2017).

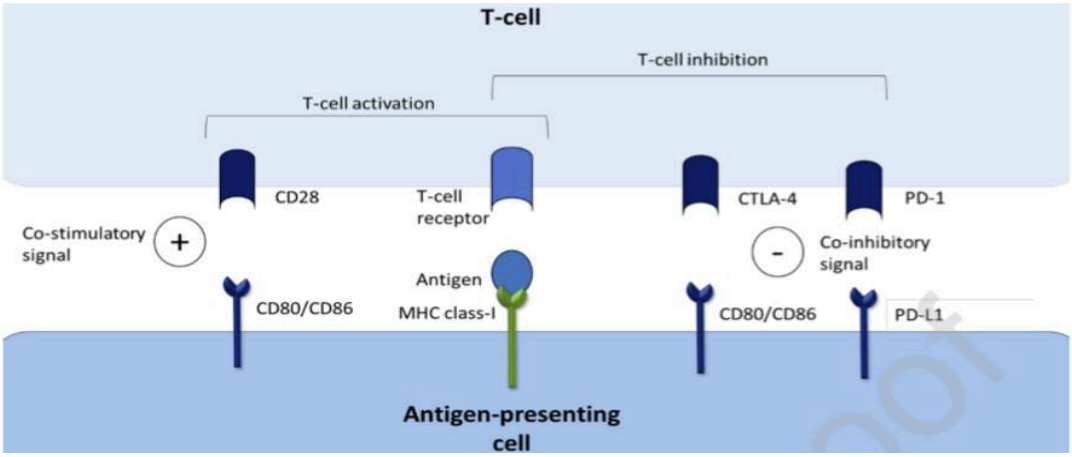
1.3 Checkpoints Imunológicos

Todo processo de carcinogênese em humanos induz uma resposta imune em seu microambiente tumoral (BUHLMANN; ELKIN; SHARPE, 2003), sendo

esse processo descrito como crucial para reduzir a progressão do câncer. Por outro lado, um dos "*Hallmarks"* do câncer é a capacidade do tumor utilizar mecanismos para evadir as respostas imunológicas (HANAHAN; WEINBERG, 2011; SCHREIBER; OLD; SMYTH, 2011). Assim, compreender a interação entre o microambiente tumoral e o sistema imune é essencial para formulação de estratégias de imunoterapias que sejam capazes de sobrepor os mecanismos de evasão tumoral (BINNEWIES et al., 2018).

Os tumores evadem a ação dos LIT através do engajamento de moléculas como CTLA-4 e/ou PD-1 (**figura 3**), as quais são conhecidas como Checkpoints imunológicos, que funcionam fisiologicamente para prevenir reações autoimunes e regular as respostas imunes a microrganismos (PARDOLL, 2012). Em situação de homeostasia, a ligação PD-1:B7 inibe a ação do linfócito em tecidos. De maneira similar, a interação CTLA-4:B7 promove a inibição do linfócito em estágios iniciais, ou seja, em órgãos linfoides secundários (BUCHBINDER; DESAI, 2016; BUHLMANN; ELKIN; SHARPE, 2003).

Figura 3 - Checkpoints Imunológicos



(GAYNOR; CROWN; COLLINS, 2020)

Os tumores são capazes de apropriarem-se desses mecanismos imunoregulatórios a fim de reduzir a função efetora dos linfócitos (EMENS; MIDDLETON, 2015; MAKKOUK; WEINER, 2015). Dessa maneira, as células tumorais conseguem escapar da vigilância imunológica, entrar em hiperplasia e promover a progressão da doença (SHARMA; ALLISON, 2015).

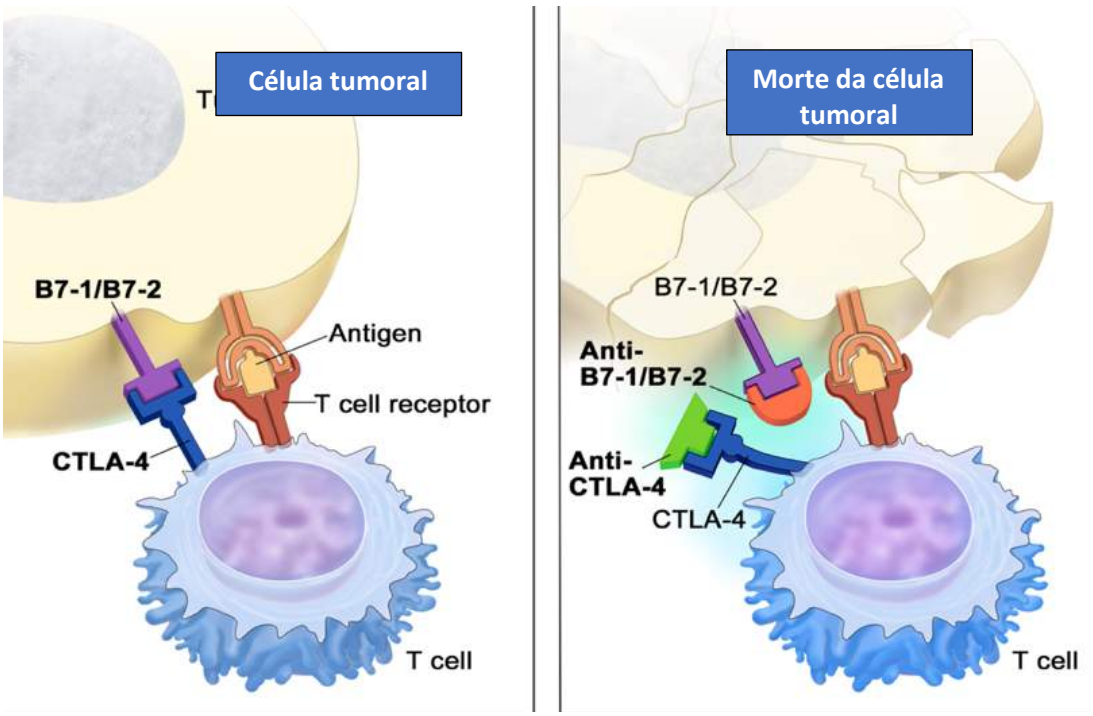
Nesse contexto, por exemplo, estudos confirmam que o PD-L1 está frequentemente superexpresso em diversos tipos de câncer (DONG et al., 2002),

levando os LIT a um estado de anergia (BARBER et al., 2006). Por outro lado, o papel da expressão de CTLA-4 ainda não é totalmente compreendido, particularmente em CM. Apesar da interação inibitória CTLA-4:B7 ser relatada principalmente em órgãos linfoides secundários (MARENGÈRE et al., 1996), é importante investigar a potencial função desse checkpoint imunológico em nível intratumoral. Nesse sentido, Khaja et al. (2017) complementam que a progressão tumoral do CM ocorra justamente através das células Treg, as quais utilizam variados mecanismos imunossupressores, entre estes os checkpoint imunológico CTLA-4 e PD-1 (KHAJA et al., 2017).

1.4 Inibidores de Checkpoint (IC)

Considerando a importante expressão dos checkpoints imunológicos na biologia do carcinoma mamário, o tratamento com IC tem por premissa utilizar esses mecanismos para reestabelecer a resposta imunológica antitumoral endógena (BARBER et al., 2006; BUCHBINDER; DESAI, 2016). Esse medicamentos já revolucionaram as perspectivas de tratamento para tumores como melanoma e câncer de pulmão, sendo hoje considerados primeira linha de tratamento para essas patologias (PARDOLL, 2012; RECK et al., 2016) (figura 4).

Figura 4 – Medicamentos Inibidores de Checkpoints Imunológicos



https://www.teresewinslow.com/breast-related

Contudo, o uso de IC em CM ainda é recente e diversos ensaios clínicos estão em andamento para elucidar as possibilidades dessa modalidade terapêutica. Esses estudos avaliam desde IC em monoterapia até terapias combinadas de medicamentos, seja no cenário adjuvante e/ou neoadjuvante,

1.4.1 Principais ensaios clínicos em CM

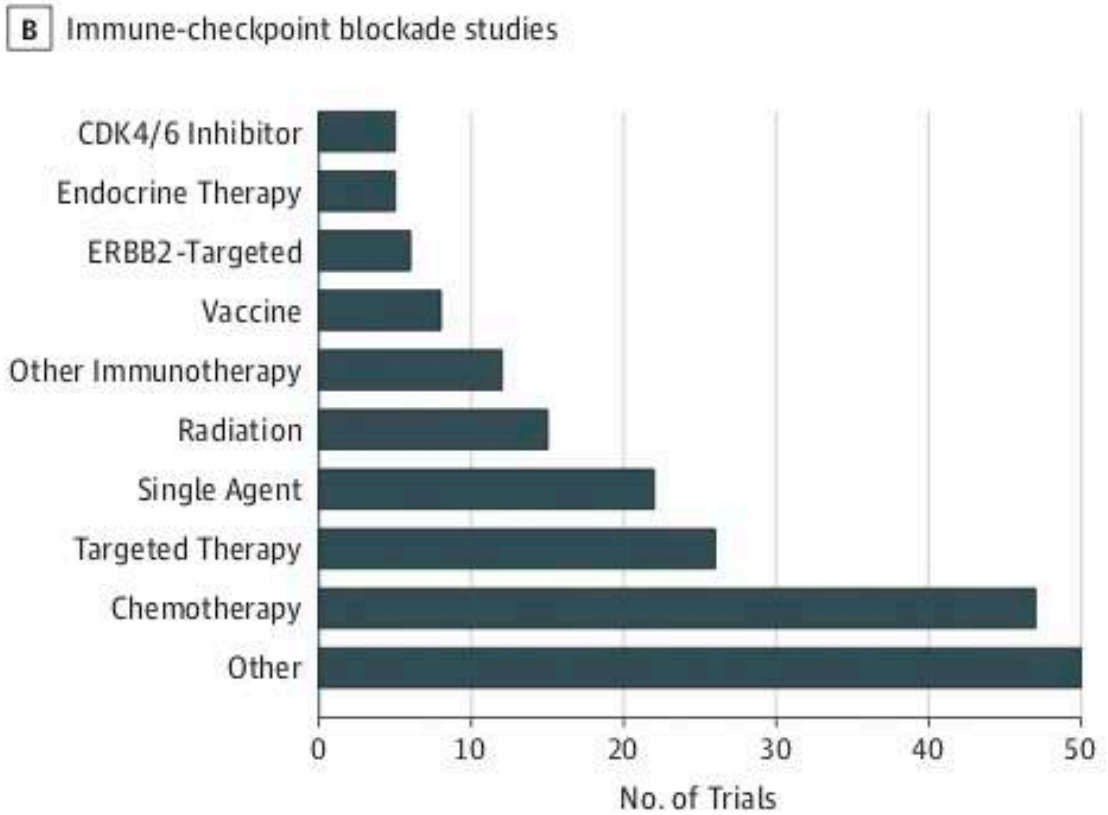
Emens et al. (2019) demonstrou que o Atezolizumab em monoterapia, um IC anti-PD-L1, é bem tolerado e promove benefícios clínicos duráveis em pacientes com CMTN metastático. Os autores identificaram melhores taxa de resposta ao tratamento em pacientes que receberam o IC mais precocemente, assim suportando a hipótese de uso do Atezolizumab como primeira linha de tratamento para CM metastático (EMENS et al., 2019). Entretanto, apesar de alguns resultados animadores, o uso de IC em monoterapia para CM apresenta benefícios para menos de 10% dos pacientes com doença metastática (ADAMS et al., 2017; EMENS et al., 2019). Portanto, ensaios clínicos em andamento com IC para CM têm focado em regimes de tratamento com combinação de medicamentos a fim de desfazer a tolerância imune ao tumor.

Outros estudos têm avaliado regimes combinados de IC com quimioterapia, um vez que a quimioterapia pode aumentar a imunogenicidade do tumor e deixá-lo mais suscetível à ação das células imunológicas (ZITVOGEL et al., 2013). Ou seja, a adição de agentes citotóxicos pode agir de maneira sinérgica com os IC (ADAMS et al., 2019a). Considerando isso, o estudo IMpassion130, com 902 pacientes de 41 países, demonstrou aumento na sobrevida livre de doença das pacientes CMTN metastático que receberam Atezolizumab associado a Nab-Paclitaxel em comparação às pacientes que receberam apenas Nab-Paclitaxel (7.2 vs 5.5 months, p = 0.002) (SCHMID et al., 2018). Os pesquisadores identificaram ainda ganho de sobrevida global, sendo esta de 21.0 meses no grupo Atezolizumab associado a Nab-Paclitaxel versus 18.7 meses no grupo placebo associado a Nab-Paclitaxel (HR: 0.86; 95% CI: 0.73–1.02) (SCHMID et al., 2020). Desde então, os *guidelines* de *American Cancer Society* (ACS) e do *National Comprehensive Cancer Network* (NCCN) recomendam Nab-Paclitaxel combinado a Atezolizumab como novo tratamento

padrão para pacientes CMTN metastático, cujo tumor seja PD-L1 positivo em IHQ com Ventana SP142.(NCCN, 2020)

Apesar dos resultados iniciais animadores para a combinação terapêuticas supracitadas, o impacto clínico desse protocolo ainda é modesto. Assim, demais terapias combinadas em CM estão sendo investigadas.(LIU et al., 2018) De maneira geral, as estratégias visam aumentar a imunogenicidade do microambiente tumoral, transformando um "*cold tumor*" em um "*hot tumor".* Entre as terapias combinadas pesquisadas estão o uso de crioablação (MCARTHUR et al., 2016), radioterapia (HU; MCARTHUR; HO, 2017), viroterapia oncolítica (BROWN et al., 2017), dupla terapia com IC (CTLA-4 e PD-1/PD-L1) (SANTA-MARIA et al., 2018) dentre outras. (**figura 5)**

Figura 5 - Pesquisas com Inibidores de Checkpoint e terapias combinadas



(ADAMS et al., 2019b)

1.4.2 Experiência com anti-CTLA-4 em câncer de mama

Em modelos animais de estudos, o tratamento com radiação combinado ao uso de IC anti-CTLA-4 induziu uma significativa resposta imune antitumoral e promoveu ganho de sobrevida (DEMARIA et al., 2005). Por conseguinte, McArthur et al. (2016) avaliaram a combinação de crioablação com o IC Ipilimumab (anti-CTLA-4) e constataram segurança e boa tolerância das pacientes a esse IC (MCARTHUR et al., 2016). Já Santa-Maria et al. (2018) avaliaram o benefício do uso combinado/duplo de IC, Tremelimumab e Durvalumab, em pacientes com CM metastático, tendo observado taxa de resposta de 43%, apenas para o grupo de pacientes do subtipo molecular CMTN (SANTA-MARIA et al., 2018).

Atualmente, os IC anti-PD1 têm sido mais amplamente investigados em comparação aos IC anti-CTL-4 devido a menores efeitos colaterais e maior número de autorizações pela agência americana FDA. Entretanto, considerando as limitações da terapêutica anti-PD1, há grande valor em aumentar o entendimento acerca do CTLA-4 na biologia do CM.

Portanto, essa dissertação tem por objetivo estudar a expressão tecidual de CTLA-4 em CM, a fim de identificar sua potencial contribuição nos mecanismos tumorais de evasão da resposta imune.

2 OBJETIVOS

2.1 Geral

- Avaliar a expressão tecidual de CTLA-4 em biópsias de CM e seu impacto na produção de mediadores sistêmicos da resposta imune.

2.2 Específicos

- Revisar sistematicamente a literatura a respeito do tema;
  Investigar associações da expressão de CTLA-4 em biópsias de pacientes com CM com parâmetros clínico-patológicos;
- Interpretar a expressão tecidual de CTLA-4 quanto a:
    - negativa *versus* positiva;
    - tipo celular;
- Avaliar a co-expressão plasmática de demais mediadores pró ou anti-inflamatórios (IL-4; IL-12);
- Avaliar o perfil de dano oxidativo das pacientes.

3 METODOLOGIA

3.1 Revisão sistemática do tema

A fim de identificar os estudos sobre o padrão de expressão de CTLA-4 em CM, foram consultados no *Pubmed* os descritores MeSH para revisão desta temática, sendo definido: "breast cancer", "CTLA-4 Antigen/antagonists & inhibitors" e “Lymphocytes, Tumor-Infiltrating/immunology”.

Procedeu-se então com revisão sistemática na Biblioteca Virtual em Saúde. Foram obtidos 62 artigos e após a leitura dos resumos desses trabalhos, e selecionados 33 artigos para análise textual na íntegra. Por fim, 12 artigos foram utilizados para revisão do tema proposto. Não foram incluídos outros artigos tipo revisões de literatura, sendo adicionados apenas artigos originais publicados nos últimos 10 anos.

3.2 Desenho do Estudo

O presente trabalho é um estudo observacional transversal realizado no Hospital do Câncer de Francisco Beltrão (CEONC), com informações coletadas do período de 2016 a 2019. Foram incluídas 105 pacientes operadas para neoplasia mamária no período do estudo, cujo diagnóstico anatomopatológico fora Carcinoma Ductal Invasivo (CDI). O bloco de parafina com tecido amostral da ressecção cirúrgica foi devidamente processado e armazenado.(ROLLS; FARMER, 2008) Foram excluídas pacientes que não apresentaram diagnóstico anatomopatológico de CDI.

Amostras de 10 mL de sangue foram obtidas por punção venosa periférica e armazenadas em tubo heparizinado até subsequente processamento para obtenção de plasma. Para isso, foi realizada centrifugação à 4000 rpm por 5 minutos, com posterior armazenamento à -20°C até o momento das análises.

Dados clínico-patológicos e sócio-demográficos foram obtidos através de consulta aos prontuários médicos. Assim, foram coletadas informações de variáveis clínico-patológicas importantes para determinar o prognóstico da paciente, como idade ao diagnóstico, subtipo molecular do CM e status dos receptores de estrógeno (ER), progesterona (PR), índice de proliferação ki67 e do receptor do fator de crescimento epidermal humano 2 (HER2). A combinação destes dados resulta na categorização dos tumores em melhor prognóstico

(Luminal A: ER e/ou PR positivos, com ki67 abaixo de 14%, ou HER-2 amplificados: qualquer status de ER e PR, e HER2 positivos, com qualquer ki67) ou de pior prognóstico (Luminal B: ER e/ou PR positivos com ki 67 acima de 14%, ou Triplo negativos: ER, PR e HER2 negativos com qualquer valor de ki67). Além disso foram coletadas informações sobre tamanho do tumor, grau histológico, presença de êmbolos, invasão linfonodal, menopausa ao diagnóstico e índice de massa corporal.

Esta pesquisa foi aprovada pelo Comitê de Ética em Pesquisa com Seres Humanos Institucional (Parecer CAAE número 35524814.4.0000.0107). Todas as participantes assinaram termos de consentimento livre e esclarecido (TCLE). (Anexos I e II).

3.3 Investigação da expressão tecidual de CTLA-4 por imunofluorescência

Para a realização dos testes de imunofluorescência, foram obtidos cortes de 3µm de espessura utilizando-se micrótomo Leica RM 2045 (Leica Biosystems, Wetzlar, Alemanha), os quais foram dispostos em lâminas de vidro previamente tratadas com 3-aminopropil-trietoxisilano. Para iniciar o preparo do corte histológico, as lâminas foram mantidas por 24 horas em estufa a 70°C e então realizou-se desparafinização química dos cortes histológicos com banho de solução de xileno em temperatura ambiente por 5 minutos. Em seguida, as amostras receberam banho de 5 minutos em etanol absoluto e banho de 5 minutos em água. A recuperação antigênica foi realizada em calor úmido com imersão das lâminas em tampão de citrato de sódio (1Mm) pH 6.0, durante 15 minutos a 100 °C. Após a recuperação antigênica, foi realizado bloqueio da peroxidase endógena por 15 minutos com solução de peróxido de hidrogênio 3%. Lavagens dos cortes histológicos foram realizadas entre cada etapa com solução salina (Cloreto de Sódio – NaCl 150mM, pH 7,6). Em seguida foi realizado o bloqueio de sítio inespecífico com solução de leite em pó 5% e triton X 0,3% por 15 minutos em temperatura ambiente. Imediatamente após, procedeu-se a incubação do anticorpo primário (Anti-CTLA-4, Thermo Fischer Scientific®), na diluição de 1:1000, *overnight*, a 4^0C e em câmara úmida fechada. A procedência dos anticorpos utilizados e sua titulação estão descritos na tabela 2. Terminada a incubação, foram realizados dois banhos de 5 minutos

em solução salina, seguida da incubação do anticorpo secundário em solução com isotiocianato de fluoresceína (FITC) (Alexa Fluor 488 goat anti-mouse IgG, Thermo Fischer Scientific) na titulação 1:1000. As lâminas foram então novamente lavadas em dois banhos de 5 minutos em solução salina. Para coloração de núcleo, os cortes histológicos foram incubados em 4´,6 diamidinofenilindol (DAPI) (Sigma Aldrich®), solução 5 mg/mL, por 30 minutos a temperatura ambiente. Terminada a incubação, as lâminas foram recobertas com glicerol e lamínulas e armazenadas no escuro. Para o controle negativo não foi feita a incubação do anticorpo primário nos cortes histológicos das lesões avaliadas.

Para a observação das lâminas e registro de imagens utilizou-se Microscópio de Fluorescência Motic BA410E, contendo câmera MOTICAM ProS5 Plus e software de aquisição e processamento da imagem Motic Images Plus 3.0ML

Tabela 1 – Anticorpos utilizados e metodologia de incubação

Anticorpo	Clone	Reatividade	Titulação	Incubação
Anti-CTLA-4	BNI3 monoclonal antibody	mouse anti-human CD152	1:1000	*overnight*, escuro, câmara úmida, 4 ^{0}C
Alexa Fluor 488	Superclonal Recombinant Secondary Antibody	goat anti-mouse IgG	1:1000	1 hora, escuro, câmara úmida, temperatura ambiente

A leitura das lâminas foi realizada utilizando-se filtro de excitação DAPI e filtro FITC em aumento 20X e 40X. A marcação foi considerada positiva para CTLA-4 quando as células apresentaram fluorescência verde de membrana e/ou citoplasma em filtro FITC. O procedimento de digitalização foi realizado com o Software Motic Images Plus 3.0ML. Para todas digitalizações, foram predefinidos os seguintes ajustes: exposição *auto*, ganho +20 e offset *zero*. As imagens obtidas foram armazenadas com formato BMP, resolução 2048 x 1536 pixels. Foram obtidas imagens de 4 campos diferentes de cada corte histógico em aumento 40X. Para cada campo foi salva uma imagem com filtro de excitação DAPI e uma com filtro FICT, totalizando 8 imagens por paciente. Como critério de seleção do campo a ser registrado, buscou-se contemplar áreas com células

ductais anaplásicas, dispostas em ninhos e cordões. Também foram consideras alta celularidade, crescimento infiltrativo, além de figuras mitóticas e pleomorfismos.

As marcações positivas para CTLA-4 foram classificadas em três grupos: CTLA-4(+) apenas em infiltrado inflamatório; CTLA-4(+) apenas em células tumorais, CTLA-4(+) em ambos. Esse padrão de marcação tecidual foi relatado para CTLA-4 através de outras metodologias. (ÁCS et al., 2017; YU et al., 2015), e foi escolhido para criação dos grupos de estudo das pacientes.

3.4 Obtenção do perfil de mediadores sistêmicos da resposta imune

A análise foi obtida a partir da determinação dos níveis de lipoperóxidos através da técnica de quimioluminescência induzida por tert-butil, após padronização do teste no laboratório para ajuste das condições técnicas analíticas. Para esta avaliação da lipoperoxidação plasmática adicionou-se 125 µL de amostra em 865 µL de tampão fosfato monobásico 10 mM e pH 7,4 em NaCl 0,9% com incubação a 37 ºC por 5 minutos. Para disparo da reação adicionou-se uma alíquota de 10 µL da solução de t-butil. A leitura da reação foi realizada em luminômetro GloMax® 20/20 (Promega, USA) no protocolo de uma leitura por segundo, durante 60 minutos, onde foi avaliada a curva de emissão de fótons qualitativa, mensurada em unidades relativas de luz (URL). O composto tert-butil hidroperóxido é um potente formador de radicais peroxil. Nas membranas biológicas, estes radicais atacam os lipídeos gerando lipoperóxidos que podem reagir com outros lipídeos, oxidando-os. Assim, o tert-butil inicia uma reação de lipoperoxidação em cadeia com outros lipídeos que pode ser detectada através da emissão de fótons ocorrida durante a formação dos lipoperóxidos.(GONZALEZ FLECHA; LLESUY; BOVERIS, 1991)

Em seguida, realizou-se dosagem de nitrito plasmático para estimar os níveis de **óxido nítrico** (NO). O método avalia a redução de nitrato a nitrito mediada por reações de oxirredução ocorridas entre o nitrato presente na amostra e o sistema cádmio-cobre dos reagentes, com posterior diazotação e detecção colorimétrica do azocomposto formado pela adição do reagente de Griess a 550 nm. Para uso no ensaio, 60uL de amostra foram adicionados a 50uL de ZnSO4 75mM, centrifugadas por 2 minutos a 10000 rpm a 4ºC. e 70uL de 55mmol/L NaOH, novamente centrifugadas por 5 minutos a 10000 rpm a 4ºC,

com posterior recuperação de 150 uL do sobrenadante, adicionando-se 50 uL de tampão glicina-NaOH 45g/L pH 9,7. Grânulos de cádmio foram ativados em CuSO4 5mM por 5 minutos e adicionados ao sobrenadante coletado das amostras desproteinizadas durante 10 minutos, sob agitação. Em microplaca, adicionou-se 50 uL de amostra a 50 uL de Reagente de Griess (Sulfanilamida: 0,4 g sulfanilamida em 20 mL ácido Fosfórico 5% - protegido da luz; NEED: 40mg em 20mL H2O destilada). Por fim, a placa foi incubada à temperatura ambiente durante 10 minutos e medida sua absorbância a 550 nm em leitor de microplaca. Os resultados foram expressos em uM de nitrito.(PANIS et al., 2011) Níveis plasmáticos de **IL-4** e **IL-12** foram determinados com Kit comercial ELISA Cytokines Levels (eBiosciences®, USA).

3.5 Análises estatísticas

Foram utilizadas frequências relativas e absolutas para análise estatística. A normalidade da distribuição dos dados foi testada pelo teste de Kolmogorov-Smirnov. As diferenças nas características gerais foram verificadas pelo Teste – T ou Mann-Whitney. Todas as análises foram realizadas no programa estatístico GraphPad Prism 7.0 (USA) com nível de significância de $p<0,05$. As correlações e associações foram feitas no programa SPSS 25.0.0 (IBM< USA).

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5 RESULTADOS

5.1 ARTIGO CIENTÍFICO 1

Archivum Immunologiae et Therapiae Experimentalis

CTLA-4 expression and its clinical significance in breast cancer.

--Manuscript Draft--

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Full Title:	CTLA-4 expression and its clinical significance in breast cancer.
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Abstract:	Purpose Breast cancer is the leading cause of women's death among all cancers. The main reason associated with this is the development of metastasis and therapy-resistant breast carcinoma (BC), which pose the main challenge of oncology nowadays. Shreds of evidence suggest that these tumors seem to have inhibitory mechanisms that may favor their progression and surveillance. Cancer cells can evade antitumor T-cell responses by expressing some immune inhibitory molecules as the cytotoxic T-lymphocyte associated protein 4 (CTLA-4), which clinical meaning has emerged in the last few years and is poorly understood in the BC context. Material and methods A literature search was performed in Pubmed and LILACS databases, using the mesh terms "breast cancer"; "CTLA-4 Antigen/antagonists & inhibitors"; "Lymphocytes, Tumor- Infiltrating/immunology", published in the last 10 years. A total of 12 studies were included in this review. Systematic review used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Results Despite the modest number of eligible studies, the literature reports some associations between CTLA-4 expression in the tumor microenvironment and worse BC outcomes, regardless of its molecular subtype. Conclusion CTLA-4 expression in BC is a putative marker of clinical significance as well as a rationale therapeutic target in the emerging field of immunotherapy.
Suggested Reviewers:	L Puztai Breast Medical Oncology, Yale Cancer Center, Yale University, New Haven, USA.

MINI REVIEW

CTLA-4 expression and its clinical significance in breast cancer.

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ABBREVIATIONS
ACS: American Cancer Society
BC: breast cancer
CTLA-4: cytotoxic T-lymphocyte antigen-4
CD: cluster of differentiation
DFS: disease-free survival
ER: estrogen receptors
HER2: human epidermal growth factor receptor 2
HR: hormone receptors
IHC: immunohistochemistry
TIL: tumor infiltrating leukocytes
MeSH: medical subject headings
MHC: major histocompatibility complex
NAC: neoadjuvant chemotherapy
NCCN: National Comprehensive Cancer Network
OS: overall survival
PD-1: programmed cell death-1
PD-L1: programmed cell death ligand-1
pCR: pathological complete response
PR: progesterone receptors
RT-PCR: reverse transcription polymerase chain reaction
TNBC: triple negative breast cancer
Treg: regulatory T lymphocytes

Conflict of interest disclosure: The authors have no conflict to declare.

ABSTRACT

Purpose: Breast cancer is the leading cause of women's death among all cancers. The main reason associated with this is the development of metastasis and therapy-resistant breast carcinoma (BC), which pose the main challenge of oncology nowadays. Shreds of evidence suggest that these tumors seem to have inhibitory mechanisms that may favor their progression and surveillance. Cancer cells can evade antitumor T-cell responses by expressing some immune inhibitory molecules as the cytotoxic T-lymphocyte associated protein 4 (CTLA-4), which clinical meaning has emerged in the last few years and is poorly understood in the BC context. **Material and methods**: A literature search was performed in Pubmed and LILACS databases, using the mesh terms "breast cancer"; "CTLA-4 Antigen/antagonists & inhibitors"; "Lymphocytes, Tumor-Infiltrating/immunology", published in the last 10 years. A total of 12 studies were included in this review. Systematic review used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). **Results:** Despite the modest number of eligible studies, the literature reports some associations between CTLA-4 expression in the tumor microenvironment and worse BC outcomes, regardless of its molecular subtype**. Conclusion:** CTLA-4 expression in BC is a putative marker of clinical significance as well as a rationale therapeutic target in the emerging field of immunotherapy.

Keywords: "breast cancer"; "CTLA-4 Antigen/antagonists & inhibitors"; "Lymphocytes, Tumor- Infiltrating/immunology".

Introduction

Breast cancer (BC) is the most common female cancer worldwide and has the fifth-highest mortality rate of all cancers.[1, 2] It is a heterogeneous disease consisting of subgroups with different molecular features and clinical outcomes, and the discovery of intrinsic subtypes improved the understanding of BC biology, which has influenced the treatment conducts.[3, 4]

The current treatments consider the molecular differences among the BC subtypes; however, they also need to consider specific treatments for each metastatic subtype since they have distinct features.[5] Despite BC treatment has increased its success in recent years, more aggressive phenotypes as the triple negative BC (TNBC) still lacks targeted treatment options.[6, 7] Further, it is estimated that a significant percent of BC patients will not respond to the available treatment, reinforcing the need to understand the molecular mechanisms implicated in treatment failure.

Translational studies have suggested that BC infiltration by tumor-infiltrating lymphocytes (TIL) and regulatory T (Treg) cells might have a great significance in the final clinical outcomes.[8] In this sense, the emerging field of immunotherapy rises with promising drugs for aggressive BC, such as checkpoint inhibitors.[9–11] These drugs target the molecules programmed cell death protein 1 (PD-1) and/or the cytotoxic T-lymphocyte associated protein 4 (CTLA-4), known as immune checkpoints, which have the function of reducing or limiting the cellular immune responses in a physiological scenario.[12]

Evidence has emerged demonstrating the importance of TIL in the clinical evolution of many cancer types.[13] In spite of BC has not previously been considered particularly immunogenic, molecular subtypes as TNBC have generally higher TIL levels than estrogen-positive BC.[14, 15] At the moment, TILs have not reached a clear clinical utility in any BC molecular subtype, and thus should not be used alone as a biomarker to adapt clinical therapies in daily practice. Further studies are required to document the subtype-specific immune dynamics in advanced disease.[13]

Through these molecular mechanisms, tumor cells can circumvent immune surveillance and promote their growth and progression.[16] So, checkpoint inhibitor-based treatments aim to stimulate and/or magnify a patient's endogenous antitumor immune response by blocking these checkpoints.[17, 18]

These treatments have changed the outlook for many aggressive tumors, particularly melanoma and lung carcinoma, where it has rapidly become a standard-of-care therapy for patients.[19, 20] Nonetheless, the use of checkpoint inhibitors in BC still require additional clinical trials and discussion. In BC, single-agent immune checkpoint experience has shown modest but interesting responses through BC subtypes. [21–23]

Despite encouraging initial results of checkpoint inhibitor-based treatments, the clinical impact of CTLA-4 expression on BC treatment is still not clear. Therefore, this systematic literature review aims to identify studies about CTLA-4 expression in BC, and address what is known about its clinical meaning. It may support the rationale for new therapeutic associations and may identify clinical indications for assessing CTLA-4 expression in BC.

DESIGN OF THE STUDY

Search Strategy

We conducted the literature search using Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).[24] On May 2020, we were able to identify 48 articles in PubMed database, and 13 publications in the Latin American and Caribbean Health Sciences Literature database (LILACS) using the following Medical Subject Headings (MeSH): "breast cancer"; "CTLA-4 Antigen/antagonists & inhibitors"; “Lymphocytes, Tumor-Infiltrating/immunology”.

Study Selection and Data Extraction

Eligibility criteria considered the following study characteristics: CTLA-4 expression evaluation on BC patients; publication year 2010 or later; English language. Data extraction from reports was done in duplicate. Two reviewers evaluated all abstracts independently to identify applicable studies. Subsequent and separately, two authors reviewed the full text of all selected studies to determine the eligibility for inclusion. Data extraction from all the included articles were performed independently. In case of any discrepancies, by a third author was consulted. Studies were excluded when there was a lack of information about CTLA-4 evaluation method, or not concerning BC participants, or if patients were not differentiated between treated and untreated subjects. Moreover, review

articles were excluded. So, after evaluated title and summary, 29 publications were excluded. The 21 publications selected were read in full and at this stage 9 articles were excluded. 12 publications remained in the study. (**Figure 1**)

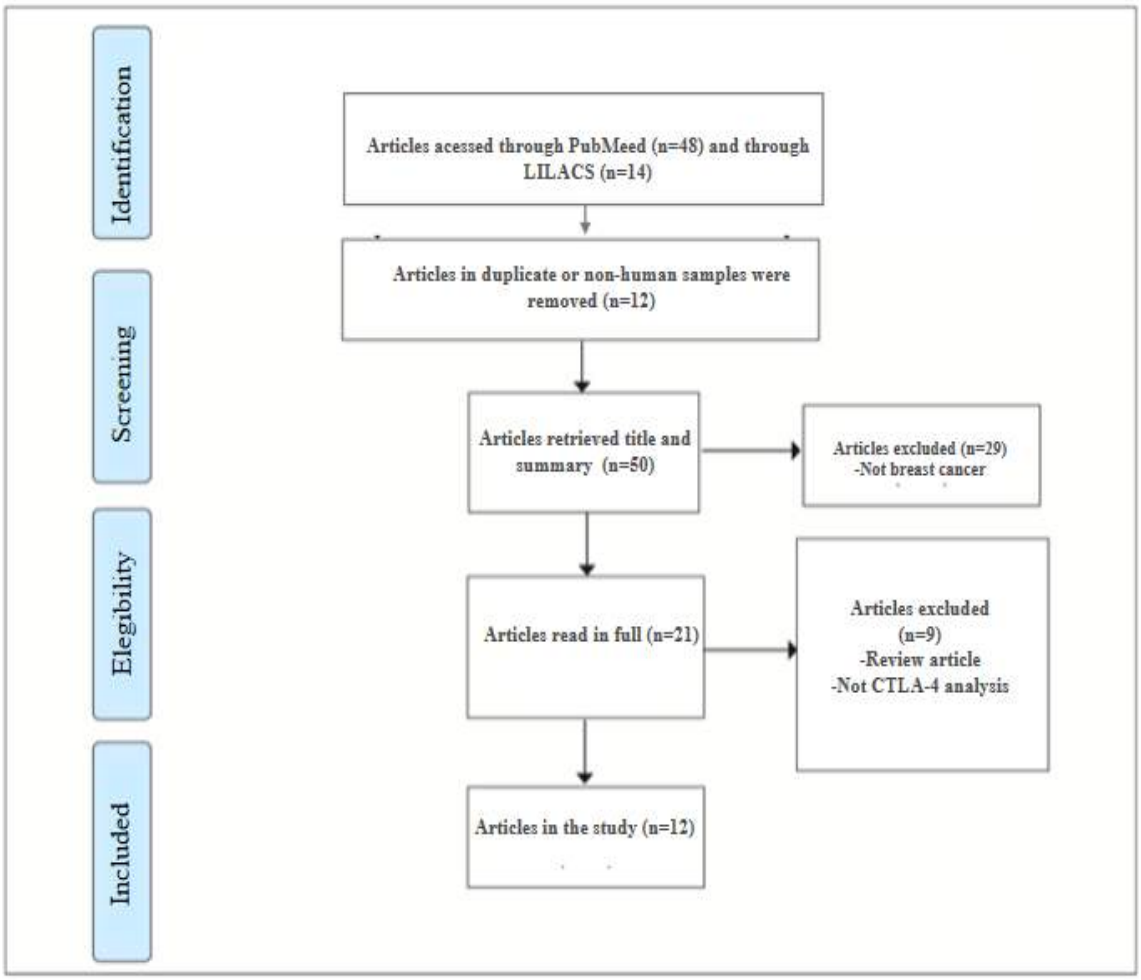

Figure 1- Systematic review flow diagram

RESULTS

CTLA-4 expression and clinicopathologic factors were evaluated in BC patients. **Table 1** presents the characteristics (population, intervention, comparisons, study design, and outcomes) for each of the 12 selected studies**.** The design of the studies was mostly reported as cross-sectional studies (n = 8). Several methodologies were applied to evaluate CTLA-4, especially flow cytology and immunohistochemistry techniques, alone or combined. The great majority of studies included a modest number of patients (close to 100 or less – n=9) and a half considered the molecular status of breast tumors (n=7) to evaluated CTLA-4 meaning. Concerning the clinical significance of CTLA-4 expression, the

articles reported associations on disease-free survival, overall survival, and BC clinical stage. Moreover, articles included in this review reported CTLA-4 expression might be useful to predict the benefits of CTLA-4 blockade or to evaluate immune changes induced by neoadjuvant chemotherapy (NAC), suggesting that its blockade might be considered as an effective additional method in BC treatment.

DISCUSSION

Understanding the interaction between the immune system and BC microenvironment is crucial for identifying patients most likely to respond to treatment and for optimizing immunotherapy-based treatment strategies.[25] The immune system requires the CTLA-4 checkpoint function to avoid uncontrolled immune responses and also prevent autoimmune reactions.[19] The CTLA-4:B7 interactions inhibit the early activation of T lymphocytes in secondary lymphoid organs.[17, 26] However, tumors may co-opt these immunoregulatory pathways to decrease T-cell function in antitumor immune surveillance.[27, 28] In this sense, it might be critical to determine CTLA-4 expression on BC patients, to investigate its role in initiating and maintaining the neoplastic pathogenesis.

The small number of studies found in literature reinforces the need to discuss the meaning of CTLA-4 expression in breast cancer. Verma et al (2013) [50] RT-PCR analysis found CTLA-4+ Tregs expression in the blood of BC patients was 7 fold higher when compared with HFDs (healthy, age-matched female donors). Mao et al (2010) [31] identified spontaneous expression of $CD3^{+}CTLA\text{-}4^{+}$ on peripheral blood mononuclear cells of tumor patients was also significantly higher than controls CTLA-4 expression.

Under different methods, Yu et al (2016), Plitas et al (2016), Lu et al (2017), Khaja et al (2017) and Lan et al (2018) [32, 51, 53, 54, 58] investigated CTLA-4 expression on BC tissue. Plitas et al (2016) and Khaja et al (2017) [51, 54] found CTLA-4 was more highly expressed in tumor-resident Treg cells. Yu et al (2016) and Lu et al (2017) [32, 53] identified high CTLA-4 levels were associated with poor overall survival. Moreover, Lan et al (2018) [58] described disease-free survival (DFS) of the tumor CTLA-4 positive group as significantly shorter compared with patients with tumor CTLA-4 (mean, 89.070 vs. 39.022

months; (p<0.05). Finally, Solis-Castillo et al. (2020) recent study also found high expression of CTLA-4 on TILs CD4 and CD8 when compared to blood T lymphocytes. [29] In the same way, Gu-Trantien, et al (2017) [10] reported intracellular CTLA-4+ (iCTLA-4+) CD4+ TIL subpopulation are associated with extensive infiltration in BC. Therefore, TILs have their effector capacity or state of anergy modulated in part by immune checkpoints in BC patients.

In the same way, regarding the cellular type, Chen et al (2017) have demonstrated that there is a significant increase of CTLA-4 expression not only by TIL but also by BC cells themselves. CTLA-4 expression on BC cell lines was detectable through Fluorescence-activated Cell Sorting (FACS) flow cytometry and the intracellular expression was generally higher than the surface expression.[30] Still**,** Mao et al (2010) [31] and other recent studies have also demonstrated that CTLA-4 is overexpressed in BC cells.[31–33]

On another hand, leave out case-control comparations and cell type, Kaewkangsadan et al (2016) and Catacchio et al (2019) [52, 59] analyzed CTLA-4 expression according to lymphocytes tissue location, whether stromal or within the tumor (TILs). Catacchio et al (2019) [59] found no association between T cells CTLA-4 expression and T cells location. Nonetheless, Kaewkangsadan et al (2016) [52] reported high levels of CTLA-4+ (stromal) T cells were associated with a pathological complete response (pCR).(p=0.041), but no association was seen with CTLA-4+ levels (intratumoral) T cells and pCR.(p = 0.060). Additionally, Mao et al (2010) [31] reported elevated expression of CTLA-4 in BC tissues was related to obvious axillary lymph nodes metastases and higher clinical stage.

Regarding BC molecular subtypes, Gu-Trantien et al (2017) [57] found CTLA-4 expression, principally on CD4+ T cells, was heterogeneous among BC subtypes, with high expression CTLA-4CD4+ TIL most frequently identified in TNBC (71%) and HER2+ (60%) BC. Also, the presence of in situ carcinoma was associated with iCTLA-4 low/intCD4+ TIL. However, Mao et al (2010) [31] did not identify a correlation between CTLA-4 levels and BC molecular subtypes. Plitas et al (2016), Lu et al (2017), Khaja et al (2017), Ács et al (2017), Solinas et al (2017) and Catacchio et al (2019) [51, 53, 54, 55, 56, 59] did not identify the association between tissue expression of CTLA-4 and the BC molecular subtypes. Lastly, a recent literature review on TNBC also did not identify distinct CTLA-4 expressions for this molecular subtype of BC.[34] Accordingly, Yu et al

(2015) found no differences in CTLA-4 expression in subtypes of BC.[32] Nevertheless, CTLA-4 is an independent predictor of shorter DFS (hazard ratio (HR) 2.176, and OS (HR 2.820) in 130 BC patients.[34]

In addition to the 12 studies embraced in the present systematic review, more studies bring collaborations regarding the BC immune evasion process. Gil Del Alcazar et al (2017) reported CTLA-4 signaling pathways were upregulated in IDC (invasive ductal carcinomas) than in DCIS (ductal carcinoma *in situ)*.[35] It supports the notion of a suppressive immune microenvironment during invasive progression. Finally, a recent in vitro study has shown that mononuclear blood cells increase CTLA-4 expression when exposed to a TNBC cell culture.[36]

Concerning methodological approaches, 8 studies applied IHQ to evaluate CTLA-4 expression in BC tissue and 3 studies performed RT-PCR. Flow cytometry on tissue homogenates and plasma was concomitantly implemented in 7 studies. These studies disclose the exhaustion or anergy in antigen-specific T cells in BC patients. Hence, tumor biology research remains crucial for understanding the interaction between the immune system and BC microenvironment. Studies in the literature often describe and discuss immune checkpoints by IHC.[37] However, although the IHQ technique is well described, studies results are controversial due to the use of different primary antibodies, different staining platforms, and/or varied cut-off values for positivity.[38, 39] On another hand, assessment of tumor samples by flow cytometry is very difficult to carry out, mainly due to the limitations of sample volume. Flow cytometry, however, may prove to be most informative. It can provide functional data on specific immune cell types, rather than on the tumor as a whole, and allow more accurate quantification than IHC and gene expression profiling. Besides that, technological developments using multicolor tissue immunofluorescence can be incorporated to maximize the information obtained from BC biopsy specimens. Further technical methods of TIL characterization remain important in the research setting.

The use of PD-L1 inhibitors appears to have limited, or only temporary, therapeutic response due to exhaustion of immune cells or due to development of resistance by the tumor. Dual checkpoint blockade therapy is moving forward in BC.[40, 41] It seems intuitive: CTLA-4 carries out its function at the sites of priming whereas PD-1 is responsible for maintaining tolerance by dampening the

activation of T-lymphocytes in the periphery.[42] However, combinatory approaches have shown improved efficacy at the cost of increased toxicity.[43] Moreover, the revolution of cancer immunotherapy has brought attention to its financial costs, and the combination of nivolumab and ipilimumab, albeit efficient, is not cost-effective.[44]

It is crucial identifying patients most likely to respond to treatments because some patients may not derive any benefit from immunotherapies.[13] This highlights the importance of the development of predictive biomarkers. Yu et al (2016) and Lu et al (2017) [32, 53] propose CTLA-4 expression profiles may be an additional biomarker of response to immunotherapies. In this perspective, for melanoma, Van Allen et al. (2015) reported CTLA-4 greater expression in good responders patients to checkpoint inhibitors treatments (P=0.033).[45] However, in BC the literature is controversial. Differently**,** Fang et al (2020) found correlations between immune checkpoint CTLA-4 gene expression, better OS, and relapse-free survival in BC patients.[46] Currently, the main biomarkers to predict response to checkpoint inhibitors under investigation are TILs [47], PD-L1 tissue expression [21], and tumor mutational burden.[48]

Thus, the studies found in this review support the feasibility of clinical trials on dual checkpoint blockade therapy or anti-CTLA-4 as second-line therapy for BC. Accordingly, clinical trials are now investigating the dual checkpoint blockade in BC. (NCT03546686, NCT03818685, NCT02536794). Finally, Sun et al (2020) translational research highlights that the combined immunotherapy with these immune checkpoint inhibitors can inhibit breast tumor growth, prevent postsurgical cancer relapse and increase survival of experimental breast tumor.[49]

CONCLUSIONS

Although a growing number of clinical trials in immunotherapy, yet relatively few studies have assessed CTLA-4 expression on TILs and tumor cells in large series of BC tissue biopsies or surgical excision specimens. Abnormal expression and dysregulation of CTLA-4 could partly explain the mechanism of evasion of anti-tumor immune responses in BC patients. Some studies have reported its increased levels being associated with advanced disease clinical stage. It emphasizes CTLA-4 importance in the development and progression of

BC. Thus, CTLA-4 expression in BC is a potential prognostic biomarker as well as a rationale therapeutic target in the emerging field of BC immunotherapy.

BC inflammatory milieu is characterized by the different phenotype of lymphocytes, so the expression of this additional immune checkpoint should be considered for the development of new therapeutic approaches. Useful synergies between existing therapies for BC and new immunotherapies remain to be discovered. In this sense, novel immunotherapy combinations may have the potential to reduce conventional chemotherapy and also may provide long-term immunity, which could translate into increased cure rates.

Finally, the evaluation of immunogenic status in BC setting have to be considered to support novel treatments with checkpoint inhibitors. This way oncologists may predict which patients will best respond to which checkpoint blockade therapy. For these reasons, accurate predictive biomarkers are needed, and CTLA-4 study seems appropriate to be further explored in the emerging field of BC immunotherapy.

CONFLICTS OF INTEREST/COMPETING INTERESTS

The authors report no conflicts of interest in this work.

AVAILABILITY OF DATA AND MATERIAL

All data are provided in the manuscript.

AUTHORS' CONTRIBUTIONS

The authors equally contributed to the study design, written and discussion.

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TABLE 1 – Systematic Review Results - Population, Intervention, Comparisons and Outcomes

	Autor/Year/ Location	CTLA-4 Evaluation Method	Design of the study	Number of patients	BC Molecular Subtypes	Outcomes: CTLA-4 Potential Clinical significance in BC
1	Mao, et al., 2010 China [31]	IHC RT-PCR Flow Cytometry (blood T lymphocytes)	Case-control study	60 BC patients 30 healthy	Ø	# Patients with higher mRNA level of CTLA-4 had obvious axillary lymph nodes metastases and higher clinical stage. # Appeared to be no correlation between CTLA-4 level and BC molecular subtypes or age of onset.
2	Verma, et al., 2013 England [50]	Flow Cytometry (blood T lymphocytes) RT-PCR	Case-control study	16 BC patients 8 healthy	Ø	#RT-PCR analysis: CTLA-4+ *Tregs* expression in the blood of BC patients was 7 fold increased compared with healthy age-matched females donors.
3	Yu, et al., 2016 China [32]	IHC	Retrospective study / cross-sectional study	130 BC patients	Ø	# Associated higher density of CTLA-4+ *tumor cells* expression with shorter overall survival (OS). #Elevated *soluble* CTLA-4 in tumor microenvironment associated with poor prognosis. #Patients' CTLA-4 profiles might be used to predict the benefits of CTLA-4 blockade.
4	Plitas, et al., 2016 USA [51]	Flow Cytometry (tissue homogenates)	Cross-sectional study	105 treatment-naive BC patients	33 ER+, 18 TNBC, 6 HER2+	# CTLA-4 was more highly expressed in TIL *Treg* cells in comparison to normal tissue resident *Treg*.
5	Kaewkangsadan, et al., 2016 England [52]	IHC Flow Cytometry (blood T lymphocytes)	Cross-sectional study	16 BC patients undergoing NAC	Ø	#There was a reduction of CTLA-4+ *T cells* in the blood (% [p=0.017] and breast cancers (stromal [p = 0.029]) following 8 cycles of NAC. Immune changes induced by NAC suggests that immune-mediated cell death may be a crucial component of NAC-associated tumor cell destruction. #IHC techniques used, could be clinically useful to further define women who may benefit from NAC.
6	Lu, et al., 2017 USA [53]	RNA-seq dataset	Cross-sectional study	1087 BC patients	ER: 77.0% positive, PR: 67.0% positive. HER2 22.4% positive. TNBC: 22.4% HER2-enriched: 7.1%	# High *T cell* CTLA-4 levels was associated with a poor OS. # CTLA-4 profiles might reflect *T cell* activation score and might serve as a marker of personalized immunotherapy in BC patients.

7	Khaja, et al., 2017 England [54]	IHC Flow Cytometry (tissue homogenates)	Case-control studies	11 BC patients 9 healthy	ER (8) PR (7) TNBC (2)	# *Tregs* are preferential accumulated in tumor microenvironment compared to normal tissue. These *Tregs* express high levels of CTLA-4 and PD-L1. This preferential accumulation of *Treg* subset with inhibitory molecules have a negative effect on BC prognosis.
8	Ács, et al., 2017 Hungary [55]	IHC	Case-control study	42 BC patients	Luminal A (3), Luminal B (19), HER2 (5), TNBC (15)	# CTLA-4 experssion did not show significant association with any of the clinicopathologic factors.
9	Solinas, et al., 2017 Belgium [56]	Flow Cytometry (tissue homogenates) IHC	Cross-sectional study	95 BC patients	34% Luminal A (N = 32), 33% Luminal B (N = 31), 20% HER2+ (N = 19), 13% TNBC (N = 13)	# The presence of in situ carcinoma was associated with CTLA-4 low *CD4+ TIL* # CTLA-4 hi *CD4+ TIL* most frequently identified in TNBC (71%) and HER2+ (60%) BC.
10	Gu-Trantien, et al., 2017 Belgium [57]	Flow Cytometry (tissue homogenates)	Cross-sectional study	42 BC patients 5 healthy	Ø	# intracellular CTLA-4+ *CD4+ TIL* subpopulation are associated with extensive infiltration in BC. # High proliferation rates of specific lymphocytes subpopulations could reflect an active response to the tumor.
11	Lan, et al., 2018 China [58]	IHC	Cross-sectional study	102 BC patients	Luminal B HER2-negative	# DFS of the *tumor cell* CTLA-4+ group was significantly shorter compared with patients with tumor *cell* CTLA-4- (mean, 89.070 vs. 39.022 months; P<0.0001). # Additionally, the DFS of *interstitial* CTLA-4+ group was shorter compared with the *interstitial* CTLA-4- group (mean, 85.526 vs. 46.574 months; P<0.0001).
12	Catacchio, et al., 2019 Italy [59]	IHC (on tissue microarrays)	Cross-sectional study	180 BC patients	ER positive status (82%) PR positive status (64%) HER2/positive(15.3%) TNBC 8.3%	# Negative CTLA-4 expression was associated with the histological grade 2. (P=0,0103) # No association between CTLA-4 expression and TIL location (stromal CD8+ or intratumoral CD8+)

BC, breast cancer**; CTLA-4,** cytotoxic T-lymphocyte antigen-4**; DFS,** disease-free survival**; ER,** estrogen receptors**; HER2,** human epidermal growth factor receptor 2; **IHC, i**mmunohistochemistry**; TIL**, tumor infiltrating leukocytes**; mRNA**, messenger ribonucleic acid**; NAC,** neoadjuvant chemotherapy**; OS,** overall survival**; PD-L1,** programmed cell death ligand-1**; pCR,** pathological complete response**; PR,** progesterone receptors**; RT-PCR**, reverse transcription polymerase chain reaction; **TNBC,** triple negative breast cancer**; Treg,** regulatory T lymphocytes.

5.2 ARTIGO CIENTÍFICO 2

Cancer Immunology, Immunotherapy

CTLA-4 expression in tumors or infiltrating leukocytes affects distinctly the systemic inflammatory response of breast cancer patients.

--Manuscript Draft--

Manuscript Number:	
Full Title:	CTLA-4 expression in tumors or infiltrating leukocytes affects distinctly the systemic inflammatory response of breast cancer patients.
Article Type:	Original Article
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Abstract:	Background : Tumor-derived immune dysregulation is a key feature of breast cancer. Patients carrying breast tumors present a variety of oxidative plasmatic modifications and proinflammatory cytokines changes, but few is known about its relation with tumor features. Recent evidences have pointed out that the expression of the cytotoxic T lymphocyte associated antigen 4 (CTLA-4) in tumors is a poor prognosis factor, but the systemic impact of its expression in tumor or infiltrating leukocytes is not clear for breast cancer. Methods and Results : Paraffin-embedded breast tumors and whole blood samples were collected from 117 women diagnosed with breast cancer. In blood samples, oxidative stress parameters were evaluated by measuring its plasmatic lipoperoxidation by high-sensitivity chemiluminescence and estimating nitric oxide metabolites (NO) by the cadmium-copper system coupled to Griess reaction. Interleukins 12 (IL-12) and 4 (IL-4) were measured in plasma samples by ELISA kits. CTLA-4 expression was determined by immunofluorescence, and evaluated by its labeling in tumor-infiltrating leukocytes (TILs) or breast tumors. CTLA4 expression in TILs significantly correlated to triple negative breast tumors, Patients carrying CTLA-4 positive tumors exhibited lower plasmatic NO levels, while those with CTLA-4 expression only in TILs exhibited reduced levels of IL-12 in plasma. No variations were observed in IL-4 or lipid peroxidation profiles in any CTLA4 status. Conclusion: Our findings suggest that CTLA-4 expression in both tumor and TILs can affect the systemic inflammatory status of breast cancer patients, affecting directly the levels of antitumor molecules as IL-12 and NO, and correlating to most aggressive disease.
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Author Comments:	February 11th, 2021 To Cancer Immunology Immunotherapy Dear Editor We are glad for submitting to your consideration our brief report article titled: "CTLA-4 expression in tumors or infiltrating leukocytes affects distinctly the systemic inflammatory response of breast cancer patients". All authors have seen a draft copy of the manuscript and agree with its publication. In this manuscript, we show that the expression of the immune checkpoint molecule CTLA-4 in tumor-infiltrating leukocytes and breast tumor cells from women diagnosed with breast cancer can impact on their systemic inflammatory status, by affecting IL-12 and nitric oxide metabolites circulating levels, as well as correlates to the diagnosis of triple negative breast tumors. We sincerely believe that our manuscript deserves your attention in your journal. This implies that the data contained therein have not previously been published nor are being considered for publication elsewhere, and that they have been tacitly or explicitly approved by the responsible authorities in the laboratories where the work was carried out. The authors hereby confirm that neither the manuscript nor any part of it has been published or is being considered for publication elsewhere. By signing this letter each of us acknowledges that he or she participated sufficiently in the work to take public responsibility for its content. Please contact me if you have any further question. Thank you. Prof. Carolina Panis, Ph.D. Head of the Laboratory of Tumor Biology, State University of West Paraná, Brazil Collaborating Professor at State University of Londrina, Paraná, Brazil
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Question	**Response**
Please provide one or two short sentences highlighting what makes your manuscript especially interesting. This précis is meant for our table of contents and must not exceed 250 characters, including spaces. Please include an identical précis in your manuscript text, following the abstract	The manuscript highlights the impact of CTLA-4 expression in tumor-infiltrating leukocytes and breast tumor cells on the systemic inflammatory status of breast cancer patients.

BRIEF RESEARCH REPORT

CTLA-4 expression in tumors or infiltrating leukocytes affects distinctly the systemic inflammatory response of breast cancer patients.

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Précis: The manuscript highlights the impact of CTLA-4 expression in tumor-infiltrating leukocytes and breast tumor cells on the systemic inflammatory status of breast cancer patients.

ABSTRACT

Background: Tumor-derived immune dysregulation is a key feature of breast cancer. Patients carrying breast tumors present a variety of oxidative plasmatic modifications and proinflammatory cytokines changes, but few is known about its relation with tumor features. Recent evidences have pointed out that the expression of the cytotoxic T lymphocyte associated antigen 4 (CTLA-4) in tumors is a poor prognosis factor, but the systemic impact of its expression in tumor or infiltrating leukocytes is not clear for breast cancer.

Methods and Results: Paraffin-embedded breast tumors and whole blood samples were collected from 117 women diagnosed with breast cancer. In blood samples, oxidative stress parameters were evaluated by measuring its plasmatic lipoperoxidation by high-sensitivity chemiluminescence and estimating nitric oxide metabolites (NO) by the cadmium-copper system coupled to Griess reaction. Interleukins 12 (IL-12) and 4 (IL-4) were measured in plasma samples by ELISA kits. CTLA-4 expression was determined by immunofluorescence and evaluated by its labeling in tumor-infiltrating leukocytes (TILs) or breast tumors. CTLA4 expression in TILs significantly correlated to triple negative breast tumors. Patients carrying CTLA-4 positive tumors exhibited lower plasmatic NO levels, while those with CTLA-4 expression only in TILs exhibited reduced levels of IL-12 in plasma. No variations were observed in IL-4 or lipid peroxidation profiles in any CTLA4 status. **Conclusion:** Our findings suggest that CTLA-4 expression in both tumor and TILs can affect the systemic inflammatory status of breast cancer patients, affecting directly the levels of antitumor molecules as IL-12 and NO, and correlating to most aggressive disease.

Keywords: breast cancer**;** Cytotoxic T lymphocyte associated antigen 4; CTLA-4; inflammation.

INTRODUCTION

Global cancer statistics estimates about 2 million new cases of female breast cancer (BC) each year.[1] This heterogeneous and complex disease results from a combination of genetic and environmental factors, which contribute to sustained chronic inflammatory and genetic instability.[2]

Several studies support that reactive oxygen species are involved in the etiology and progression of BC. Circulating markers of oxidative stress, such lipid peroxidation products, are frequently identified in the blood of BC patients, suggesting that tumors may have an important systemic impact.[3]

Other reactive species as nitric oxide (NO) itself and its metabolites are also reported, and constitutes pivotal mediators for tumor invasiveness and progression.[4, 5] Such proinflammatory nature of human carcinomas characterizes and inflammatory microenvironment, rich in immune response-related components.[6] Nonetheless, tumor may overcome immunosurveillance through tumor-derived immune dysregulation. Thus, this is a key feature of BC: an immunosuppressive microenvironment consisted of cytokines and immune checkpoint molecules that can block anti-tumor immunity.

Recent studies describe the expression of immune checkpoint molecules, such as the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). CTLA-4 is a protein expressed in the surface of T cells or cancer cells that function as immunological brakes against uncontrolled immune responses.[7] This homodimer glycoprotein belongs to the human immunoglobulin gene superfamily, and was originally described on the surface of T cells.[8] But, it was found as also expressed on the surface of nonlymphoid cells, including breast cancer.[9]

Plasma soluble CTLA-4 and its expression in peripheral mononuclear cells of BC patients are reported to be higher than in healthy controls [10], suggesting that this molecule might be involved in controlling functions other than the well-known T-cell response inactivation. Studies have suggested that CTLA-4 inhibitory function is related to the impairment of IL-2, IFN-γ and IL-4 production in both normal and cancer cells.[11, 12] However, its clinical meaning is far to be understood.

In this context, it is important to investigate CTLA-4 expression in BC microenvironment and its potential role in systemic inflammation modulation. Based on this, the present study investigated the impact of CTLA-4 expression in breast tumor cells and tumor-infiltrating leukocytes (TILs) on the systemic inflammatory profile of breast cancer patients. To reach this goal, we analyzed CTLA-4 expression by immunofluorescence, and according to its expression in tumor or TILs, evaluated the circulating levels of interleukins and oxidative stress mediators known as players of the inflammatory response.

MATERIALS & METHODS

Design of the study and sample obtention

Figure 1 provides a general overview about the design of the study. This study was approved by the Ethics Committee on Research of State University of Western Paraná under the number CAAE 35524814.4.0000.0107. All volunteers signed an informed consent form prior to biological material collection (201 women, from May 2015 to May 2018). Patients did not receive any treatment prior to sample collection. Only those diagnosed with invasive ductal BC were included in the study (n=117). Heparinized whole blood samples (10 mL) were obtained by peripheral venipuncture, centrifuged for 5 min at 1400×g at 4 ºC, and the plasma stored until the moment of analyses. Clinicopathological data were obtained through medical records and are detailed in Table 1.

CTLA-4 immunofluorescence

Paraffin-embedded tissue sections (3 µm) were processed for immunofluorescence labeling investigation against CTLA-4 epitope, as detailed in the Supplementary File 1 and Figure 1. The intensity and localization of the immunoreactivity were analyzed with the Motic BA 410E fluorescence microscope and MOTICAM ProS5 Plus**.** BC slides were evaluated by two independent authors who were unaware of patients' clinical and prognostic information. Two variables - density of interstitial CTLA- 4^+ lymphocytes and density of tumor CTLA-4^+ expression - were evaluated as follows: CTLA-4^+ cells

in interstitial areas adjacent to tumor nests were counted in 8 fields adjacent to tumor nests (400× magnitude) that were randomly selected from the entire section. Density of was calculated by dividing positive cells by the area of the fields. Percentages of immunofluorescence positive cells were categorized from zero to five (negative=0; <1%=1; 1-10%=2; 11-33%=3; 34%-66%=4; >60%=5). Samples exhibiting both labeling (TILs and breast tumor) were not included in the study.

Inflammatory status determination

Plasma lipoperoxidation and nitrite levels (NO estimative) were measured as previously described [13–15] to evaluate the oxidative stress profile. For NO, plasma aliquots were deproteinized, centrifuged, the supernatant recovered and diluted in glycine buffer, with further incubation with activated cadmium granules. After, aliquots of the recovered supernatant were mixed with the same volume of Griess reagent. The absorbance was measured at 550nm on a standard microplate reader, and the results are expressed as μM nitrite. For determination of lipid peroxidation, plasma samples were diluted in phosphate buffer mixed with tert-butyl hydroperoxide. The real-time high sensitivity chemiluminescent reaction was monitored in a Glomax 20/20 luminometer (Promega, USA) for 60 minutes, and the results got from the total curve integration, expressed as relative light unities (RLU). Interleukins 4 (IL-4) and 12 (IL-12) were determined in plasma samples by using a commercial antibody-specific ELISA kit (Invitrogen, USA) following the analysis protocol recommended by the manufacturer. The kits have a sensitivity of 4pg/mL.

Data analysis

All variables were analyzed for statistical assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). All analyses were performed in duplicate datasets, and represented in box plots (min-max). + indicates the average value of each group. The results were assessed with Grubbs test for the detection of outliers. No outliers were detected in the study. The results were compared by Student's t test or the Mann-Whitney test, in accordance to the distribution of variances, considering $p \leq 0.05$ as statistically significant. All statistical analyses were performed using the GraphPad Prism 7.0

software package (GraphPad Software, San Diego, CA, USA). Also, SPSS 22.0 software (IBM, USA) was used to obtain the clinicopathological data frequencies and Spearman's correlations. A cross-sectional study was performed searching for a correlation between plasmatic levels of IL-4, IL-12, lipid peroxidation and NO levels according to CTLA-4 expression in breast tumor cells or breast TILs. Further, all data was analyzed regarding the following clinicopathological variables: age at diagnosis, menopausal status, body mass index, expression of hormonal receptors, overexpression of human epidermal growth factor 2, ki67 proliferation index, histological grade, molecular subtype of BC, tumor size, presence of metastases in lymph nodes, and the presence of TILs.

RESULTS

Table 1 shows the clinicopathological characterization of patients. The mean age at diagnosis was 55.66 years, and most were menopausal at the time of diagnosis (61.8%), while 73.5% were overweight. The most common subtype was Luminal A (42.9%), while the triple-negative subtype had a prevalence of 23.6%. Histopathological grade II was present in 42.7%, and lymph node metastasis occurred in 48% of patients.

Representative images of CTLA-4 immunofluorescence labeling are shown in Figure 2. It was found CTLA-4 labeling in the cell membrane of TILs and in the cytoplasm and membranes from breast tumor cells. Samples exhibiting both CTLA-4 labeling (TILs and breast tumor) were not included in the study.

The systemic inflammatory status of patients carrying CTLA-4 expression in TILs or breast tissue are presented in Figure 2.

Concerning TILs labeling, IL-4 levels ranged from 18.24-71.08 pg/mL in CTLA-4 negative samples and 6.46-82.00 pg/mL in the positive ones. For IL-12, levels varied from 5.14-108.00 pg/mL in CTLA-4 negative samples and 10.88-56.51 pg/mL in the positives. A significant difference was found when comparing IL-12 levels between CTLA-4 positive versus negative samples ($p=0.0502$) but not to IL-4 ($p=0.9207$). NO metabolites levels and lipid peroxidation profiles did not change regarding CTLA-4 labeling in TILs (data for NO: 25.46-160.90 μM for CTLA-4 negative TILs, 7.20-125.70 μM for CTLA-4 positive TILs, $p=0.7234$ and

for lipid peroxidation: 291608- 5621944 RLU for CTLA-4 negative TILs and 382282- 18865527 RLU for CLTA-4 positive TILs, p=0.4951).

In relation to breast tissue labeling, IL-4 and IL-12 levels did not vary, spite of any CTLA-4 status (for IL-4: 6.46-71.08 pg/mL in CTLA-4 negative breast tissue samples and 11.40-82.00 pg/mL in CTLA-4 positive breast tissue samples, p=0.6603, and for IL-12: 10.88-108.00 pg/mL for CTLA-4 negative breast tissue samples and 5.00-56.51 pg/mL in CTLA-4 positive breast tissue samples, p=0.3457). NO metabolites levels significantly reduced in breast tissue samples who were positive to CTLA-4 labeling (7.41-159.00 μM in CTLA-4 negative samples and 6.99-76.11 μM to CTLA-4 positive samples, p=0.0431). Lipid peroxidation levels were not different between groups (307457- 6220347 RLU for CTLA-4 negative breast tissue and 278574- 18982231 RLU for CTLA-4 positive breast tissue, p=0.2224).

Spearman's correlations were performed (Table 2), crossing all plasma measurements and clinicopathological data considering CTLA-4 status for TILs or breast tissue. A positive significant correlation concerning CTLA-4 positivity in TILs and the molecular subtype of cancer was found, associating its labeling in TILs of triple negative breast tumors (R=0.1801 and p=0.051). None of the other evaluated parameters correlated were significant.

DISCUSSION

The investigation of CTLA-4 expression in several cell types, including tumors, has emerged as a point of interest in recent years. This study evaluated CTLA-4 expression in TILs and breast tumors, and analyzed its impact on the systemic inflammatory status of breast cancer patients. We observed that CTLA-4 affects the systemic levels of IL-12 and NO metabolites, showing association with triple negative breast tumors depending on this checkpoint be expressed on TILs or breast tumor cells. As far as we know, this information is novelty and adds to the literature regarding the meaning of immune checkpoints in the context of BC.

First, our results shown that CTLA-4 is expressed in TILs and breast tumor samples. CTLA-4 has been measured in BC patients and other cells by several

methods, as immunohistochemistry, immunofluorescence, ELISA and western blot.[9, 10] We chose to analyze its expression by immunofluorescence, since we previously tested immunohistochemistry and western blot in such patients tissue samples, but the results were not satisfactory majorly due to weak signal (data not shown). Recent studies have described CTLA-4 as a highly expressed molecule in BC, reaching more than 50% of cases, on contrary of other commonly investigated immune checkpoints, such PD-L1 that is expressed in less than 4% of breast tumors.[16] This information highlights that CTLA-4 expression may have a clinical impact on tumor microenvironment and, consequently, on systemic mediators for disease resolution – especially those from the inflammatory response - since BC is well established as a systemic disease.[4, 5]

Cytokines are among the chief players that coordinate immune responses against tumors. In our study, patients exhibiting CTLA-4 positivity in breast TILs had a reduction in circulating IL-12. This information is new, and may probably have a profound impact on disease outcome. IL-12 is a key mediator of antitumor response, necessary for natural killer cells (NK) activation to promote tumor cells elimination.[17] Thus, its depletion might strongly impact the immune response against BC, especially when considering that NK cells infiltrating breast tumor-draining lymph nodes also express checkpoint inhibitors as PD-1 [18], which could further potentiate the immune response impairment of such patients.

Beyond, we also investigated the impact of CTLA-4 expression on the systemic oxidative stress profile, which is a pivotal arm of inflammation in breast cancer induced by cytokines.[4] The presence of tumor mass is determinant for the sustained proinflammatory systemic status in BC patients, which includes high circulating levels of NO metabolites and the presence of sustained oxidative stress as measured by its metabolites as lipid peroxides.[19] Therefore, we investigated whether CTLA-4 status in TILs and breast tumors could affect the systemic redox profile of BC patients. NO is a pleiotropic molecule with multiple functions in redox and immune responses, well-established as a powerful tumoricidal molecule.[20] We found that patients expressing CTLA-4 in breast tumors presented lower NO levels when compared to those that did not express this checkpoint. So, CTLA-4 expression in TILs and breast tissue influences the systemic levels of pivotal tumoricidal mediators. This hypothesis could help to

understand the significant correlation found in our study concerning between CTLA-4 labeling in TILs and the occurrence of triple negative breast tumors.

CONCLUSION

The current study points out that CTLA-4 expression in TILs and breast tumors can induce systemic changes on essential mediators of inflammation that have tumoricidal functions, and also correlates with the diagnosis of most aggressive subtypes. These preliminary findings indicate that CTLA-4 might downregulate immune responses and also could putatively promote peripheral immune tolerance to BC.

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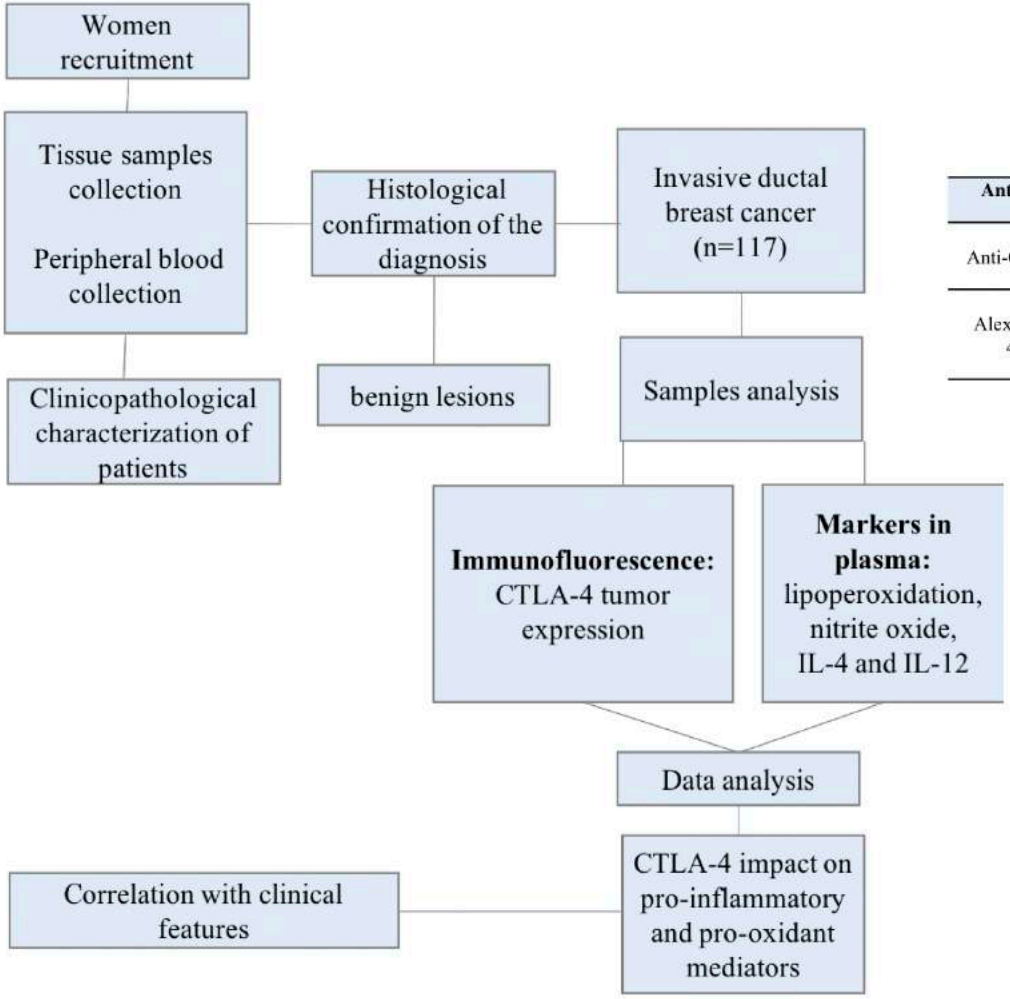
Click I

Table 1 – Clinicopathological variables from breast cancer patients included in the study.

Parameter		Patients
Age (years	<40	7.6%
	40-60	46.1%
	>60	46.1%
Menopause at diagnosis	Negative	38.1%
	Positive	61.8%
Body Mass Index (kg/m^2)	<18.5	1.7%
	18.5 - 24.9	24.7%
	>24.9	73.5%
Molecular subtype of breast cancer	Luminal A	42.9%
	Luminal B	27.1%
	HER2-amplified	6.1%
	Triple negative	23.6%
Histopathological Grade	I	32.4%
	II	42.7%
	III	24.7%
Lymph Node Metastasis	Absence	52%
	Presence	48%

Legend: HER2 = human epidermal growth receptor 2.



CTLA4 immunofluorescence conditions

Antibody	Clone	Reactivity	Dilution	Conditions
Anti-CTLA-4	BNI3 monoclonal antibody	mouse anti-human CD152	1:1000	*overnight*, 4^{0}C
Alexa Fluor 488	Superclonal Recombinant Secondary Antibody	goat anti-mouse IgG	1:1000	1 hour, 25^0 C

Figure 1 – Design of the study. In the period of the study, 201 women were recruited in the hospital to investigate suspicious breast cancer lesions. A total of 117 women had positive biopses for breast cancer and were included in the study. The expression of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) was evaluated in both tumor cells and tumor-infiltrating leukocytes (TILs) by immunofluorescence. Blood samples collected in the same moment of biopsy were analyzed to measure interleukins 4 and 12 (IL-4 and Il-12, respectively) levels, and oxidative stress status by measuring lipid peroxidation and nitric oxide metabolites (NO). All measruments were evaluated according to specific clinicoatological features.

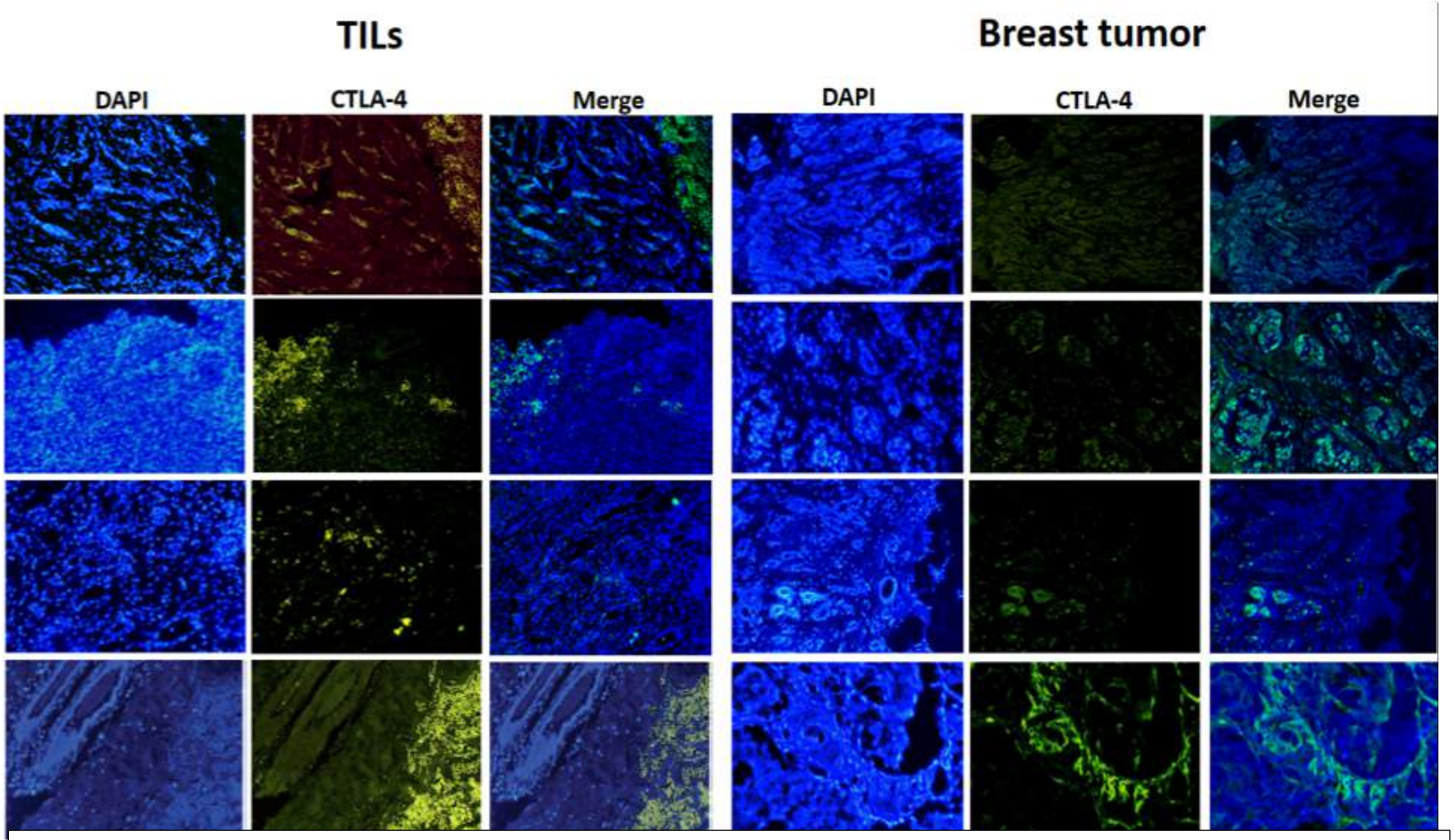


Figure 2 – Immunofluorescence labeling of CTLA4 in breast tumor cells and tumor infiltrating leukocytes. 3 µm formalin-fixed paraffin-embedded sections of breast carcinoma were dewaxed, rehydrated and subject to antigen retrieval. Sections were incubated with primary antibody mouse antihuman CTLA-4 IgG (BNI3 monoclonal antibody, Thermo Fischer Scientific) and secondary antibody against mouse immunoglobulins (Alexa Fluor 488 goat anti- mouse IgG). Nuclei were counterstained with DAPI and slides were mounted with glicerol. Immunoreactivity were analyzed with the Motic BA410E fluorescence microscope, MOTICAM ProS5 Plus and software Motic Images Plus 3.0ML. Sequential scanning was taken wih DAPI filter and FITC filter (exposure auto, gain +20 and offset zero. 2048 x 1536 pixels). Two pictures of the same field were taken, one for each filter.(400× magnitude) The resulting images were merged in ImageJ to generate the final images. In some images, the contrast was enhanced by turning down DAPI (gray) intensity. For all images: CTLA-4 IgG (green) and DAPI (blue). Samples exhibiting both labeling (TILs and breast tumor) were not included in the study.

Table 2 – Spearman correlations concerning CTLA4 expression in tumor-infiltrating leukocytes (TILs) and breast tumor cells with clinicopathological variables from breast cancer patients.

CTLA4 expression in TILs versus:	Spearman R value	p value
CTLA4 expression in tumor cells	0.067	0.469
Percent of tumor labeled area	0.2272	0.014
Estrogen receptor expression in tumor cells	-0.2337	0.0119
Progesterone receptor expression in tumor cells	-0.1337	0.1544
HER2 expression in tumor cells	0.006	0.944
Ki 67 proliferation index	0.1063	0.2605
Molecular subtype of cancer	0.1801	0.051*
Tumor size	-0.1323	0.1606
Lymphnodal invasion	-0.1214	0.2385
Age at diagnosis	0.1143	0.2237
Menopausal status	0.052	0.5882
Body mass index	-0.1141	0.2509

** Indicates statistical significance ($p \leq 0.05$)*

Table 2 – cont.

CTLA4 expression in breast tumor cells vs	Spearman R value	p value
CTLA4 expression in tumor infiltrating leukocytes	0.067	0.4698
Presence of infiltrating leukocytes	0.0939	0.3171
Estrogen receptor expression in tumor cells	0.011	0.9002
Progesterone receptor expression in tumor cells	0.0439	0.6413
HER2 expression in tumor cells	-0.042	0.6552
Ki 67 proliferation index	-0.081	0.3905
Molecular subtype of cancer	-0.035	0.7049
Tumor size	0.017	0.8510
Lymphnodal invasion	0.098	0.3413
Age at diagnosis	0.1264	0.1781
Menopausal status	0.1597	0.095
Body mass index	-0.045	0.6465

** Indicates statistical significance ($p \leq 0.05$)*

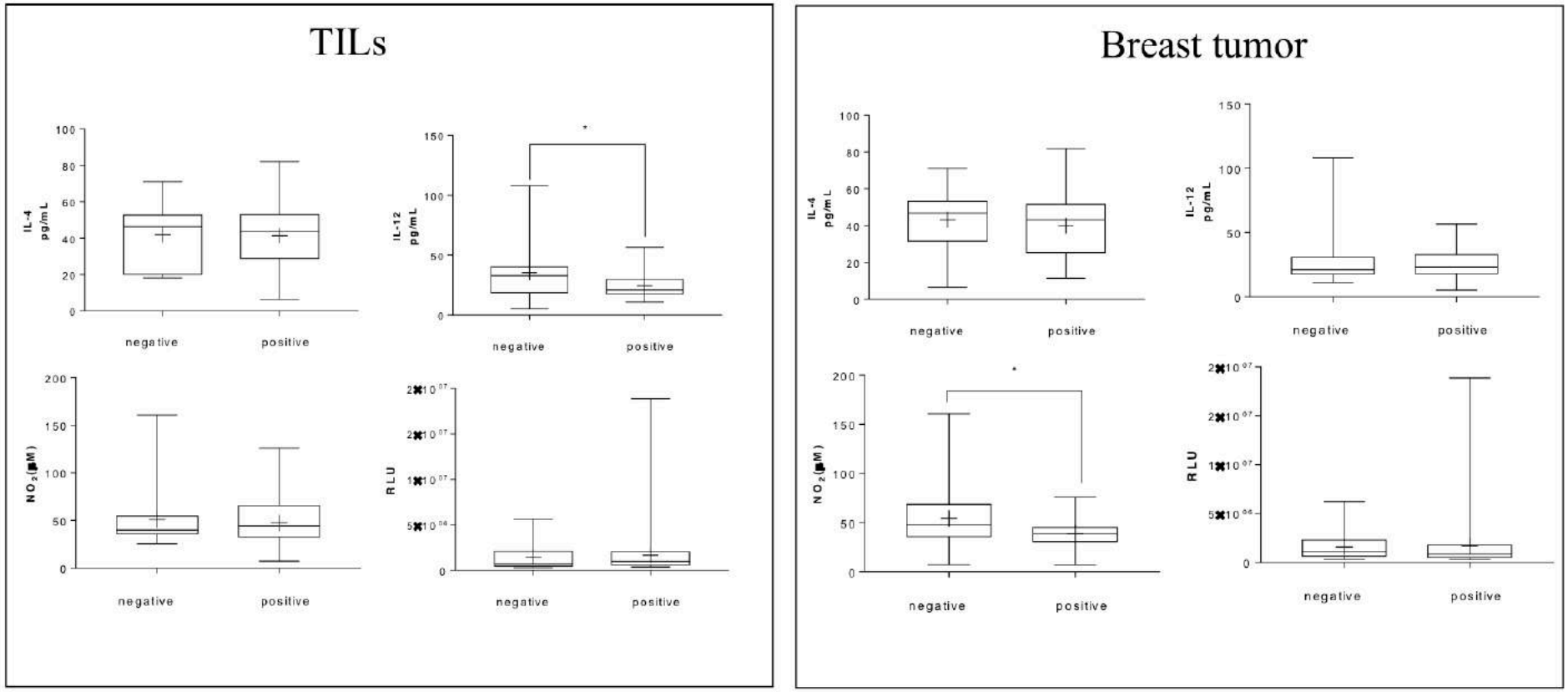


Figure 3 – Systemic inflammatory status of breast cancer patients according to the expression of CTLA-4 in tumor infiltrating leukocytes (TILs) or breast tumor. Interleukins 4 (IL-4) and 12 (IL-12) were measured in plasma samples by enzymeimmunoassay (ELISA). Lipid peroxidation levels and nitric oxide metabolites (NO_2) were evaluated as oxidative stress parameters. RLU = relative light unities. The data are represented in box plots (min-max). + indicates the average value of each group. * indicates $p \leq 0.05$. CTLA-4 expression. status was assesssed by microscope immunofluorescence.

6 ANEXOS

ANEXO I – Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - TCLE

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

Pesquisador responsável: Profª Drª CAROLINA PANIS – Telefones (43)99165316 e (46) 30553026

Equipe do projeto: Ms. Aedra Bufalo – Professora Adjunta do Curso de Medicina da Unioeste, Campus de Francisco Beltrão.Dra Rosebel Prates – Professora Adjunta do Curso de Medicina da Unioeste, Campus de Francisco Beltrão.Dra Claudicéia Risso Pascotto - Professora Adjunta do Curso de Medicina da Unioeste, Campus de Francisco Beltrão.Dra Léia Carolina Lúcio - Professora Adjunta do Curso de Medicina da Unioeste, Campus de Francisco Beltrão. Ms. Geraldo Vicentini – Professor do Curso de Medicina da Unioeste, Campus de Francisco Beltrão.

Convidamos você a participar de nossa pesquisa que tem o objetivo de identificar os casos de câncer de mama em mulheres que tem história da doença na família, que moram na região Sudoeste do Paraná. Para isso será realizada a coleta de um tubo de sangue (10 mL) para fazer os exames necessários para identificar porque alguns tumores de mama levam à doenças tão agressivas.

Durante a execução do projeto também vamos precisar de uma parte do tecido tumoral que o médico irá remover durante a sua cirurgia ou que foi coletado para o diagnóstico da doença (na biópsia). Também precisaremos consultar o prontuário médico, para saber informações sobre sua saúde e sua ocupação de trabalho. Para algum questionamento, dúvida ou relato de algum acontecimento os pesquisadores poderão ser contatados a qualquer momento, pelos telefones (43)99165316 e (46) 30553026. Estamos disponíveis para esclarecer quaisquer dúvidas, a qualquer momento.

Desta forma, você está contribuindo para a identificação de fatores que levam à alta incidência de cânceres agressivos na nossa região.

Este termo será entregue em duas vias, sendo que uma ficará com você. Você não pagará nem receberá para participar do estudo. Seus dados serão mantidos em sigilo, ou seja, ninguém além dos pesquisadores terá acesso ao material ou informações coletadas. Estes dados serão utilizados somente para fins científicos. Você poderá cancelar sua participação a qualquer momento. Se necessitar de maiores informações, o telefone do comitê de ética é 3220-3272 e da pesquisadora responsável é 46 30553026. A coleta de material será feita dentro do Ceonc, portanto qualquer imprevisto será resolvido imediatamente no local. Ao término do projeto, se a pesquisa identificar que a sua doença se classifica como câncer familial, você será chamado ao Ceonc para receber esclarecimentos sobre como proceder no acompanhamento da doença nos próximos anos.

Declaro estar ciente do exposto e desejo participar do projeto.

Nome do sujeito de pesquisa ou responsável:

Assinatura:

Eu, **Carolina Panis**, declaro que forneci todas as informações do projeto ao participante e/ou responsável.

Data:

ANEXO II – Comprovante do Comitê de Ética

UNIOESTE - CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE DA UNIVERSIDADE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Mapeamento do câncer de mama e estudo de associação de risco com a exposição ocupacional aos agrotóxicos no Paraná: mecanismos moleculares e implicações clínicas

Pesquisador: CAROLINA PANIS

Área Temática:

Versão: 4

CAAE: 35524814.4.0000.0107

Instituição Proponente: UNIVERSIDADE ESTADUAL DO OESTE DO PARANA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.309.144

Apresentação do Projeto:

Trata-se de uma emenda ao projeto. Essa emenda solicita modificações quanto a extensão do prazo de conclusão, readequação do título do projeto, inclusão de uma nova atividade na metodologia, inclusão de instrumento de coleta de dados para investigar a exposição a agrotoxicos e inclusão de análise de material de biópsias.

Objetivo da Pesquisa:

Descritos adequadamente e detalhadamente

Avaliação dos Riscos e Benefícios:

Descritos os riscos e benefícios adequadamente.

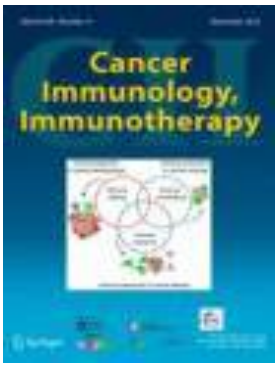
Comentários e Considerações sobre a Pesquisa:

Projeto bem apresentado além de ser muito pertinente a área da saúde em geral e da medicina em particular. A pesquisadora solicita algumas modificações que estão detalhadas em documento anexo e transcritas abaixo com a respectiva análise do CEP:

1. Extensão do prazo de conclusão para dezembro de 2021 - ACATADO

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

ANEXO III – Normas das Revistas



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o Clinical Trial Reports – please review the guidelines of Original research Articles and include relevant immunological data.

o Editorials- it is a forum for exchanging ideas, innovations, new updates in a fast-moving field or topic. Word limitation 1200 with up to 10 references and one figure or table.

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Review Procedures

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Subject matter that is of general interest to the readership of the Journal and will contribute to the advancement of the field.
Originality.
Appropriate study design and methodology.
Conclusions which are supported by the data.
Focussed and concise writing style.

Manuscripts that do not meet these criteria will be returned to the author without review. Manuscripts that meet these criteria will be further evaluated by a minimum of two expert reviewers who will anonymously provide unbiased, critical and independent assessment of the submission. The Editor-in-Chief will make a final decision on the disposition of the manuscript based on the recommendations of these reviewers. Authors are encouraged to provide the full names and contact information of potential reviewers who have expertise in the subject of the manuscript. Authors may request the exclusion of specific reviewers and should provide justification for the request. The corresponding author will be notified of the editorial decision by E-mail which may include some or all of the reviewers' comments.

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Nomenclature: Insofar as possible, authors should use systematic names similar to those used by Chemical Abstract Service or IUPAC.
Genus and species names should be in italics.
Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples: 1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list

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

The entries in the list should be numbered consecutively. Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731- 738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

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

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086
Book
South J, Blass B (2001) The future of modern genomics. Blackwell, London
Book chapter
Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
Online documen
thttps://www.springer.com/journal/262/submission-guidelines#Instructions for Authors 8/32
Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
Dissertation
Trent JW (1975) Experimental acute renal failure. Dissertation, University of California
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Tables should always be cited in text in consecutive numerical order.
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Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

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Vector graphics containing fonts must have the fonts embedded in the files.
Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Archivum Immunologiae et Therapiae Experimentalis

Submission guidelines

General information

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Please make sure your title page contains the following information.

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The title should be concise and informative.

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- The name(s) of the author(s)
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Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

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Code availability (software application or custom code)
Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)
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- Do not use field functions.
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3. Results: These should be presented concisely, possibly in the form of Tables and Figures which should not be described in detail in the text. Each experiment should be illustrated only once, either by a Table or by a Figure.

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5. Acknowledgments (if any): These should be placed directly after the discussion and include the dedication (if any), thanks for financial support etc.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

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

Reference list entries should be alphabetized by the last names of the first author of each work. Please alphabetize according to the following rules: 1) For one author, by name of author, then chronologically; 2) For two authors, by name of author, then name of coauthor, then chronologically; 3) For more than two authors, by name of first author, then chronologically.
If available, please always include DOI numbers or full DOI links in your reference list.

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

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- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

ANEXO IV – Comprovante de Submissão

Gmail Rodrigo Kern <rodrigokern1@gmail.com>

AITE-D-21-00011 - Submission Notification to co-author - [EMID:76661d67ecde69f1]
1 mensagem

Archivum Immunologiae et Therapiae Experimentalis (AITE) <em@editorialmanager.com> 11 de fevereiro de 2021 20:44
Responder a: "Archivum Immunologiae et Therapiae Experimentalis (AITE)" <stec@iitd.pan.wroc.pl>
Para: Rodrigo Kern <rodrigokern1@gmail.com>

Re: "CTLA-4 expression and its clinical significance in breast cancer."
Full author list: Rodrigo Kern; Carolina Panis
The submission id is: AITE-D-21-00011

Dear Mr Kern,

We have received the submission entitled: "CTLA-4 expression and its clinical significance in breast cancer." for possible publication in Archivum Immunologiae et Therapiae Experimentalis, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr. Carolina Panis who will be able to track the status of the paper through his/her login.

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Thank you very much.

With kind regards,

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Archivum Immunologiae et Therapiae Experimentalis

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Gmail

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CIIM-D-21-00110 - Submission Notification to co-author - [EMID:5a1583434b626a25]

1 mensagem

Cancer Immunology, Immunotherapy (CIIM) <em@editorialmanager.com> 12 de fevereiro de 2021 06:23
Responder a: "Cancer Immunology, Immunotherapy (CIIM)" <karthiga.anbalagan@springernature.com>
Para: Rodrigo Kern <rodrigokern1@gmail.com>

Re:"CTLA-4 expression in tumors or infiltrating leukocytes affects distinctly the systemic inflammatory response of breast cancer patients."
Full author list: Rodrigo Kern; Janaina Carla da Silva; Fabio Negretti; Vitor Teixeira Maito; Daniel Rech; Mariane Okamoto Ferreira; Pâmella F Fagotti; Stefania Tagliari Oliveira; CAROLINA PANIS
The submission id is: CIIM-D-21-00110

Dear Mr Kern,

We have received the submission entitled: "CTLA-4 expression in tumors or infiltrating leukocytes affects distinctly the systemic inflammatory response of breast cancer patients." for possible publication in Cancer Immunology, Immunotherapy, and you are listed as one of the co-authors.

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Thank you very much.

With kind regards,
Springer Journals Editorial Office
Cancer Immunology, Immunotherapy

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