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ANTICORPOS ANTI-NEUTRÓFILOS EM PACIENTES COM CÂNCER DE MAMA E SUA CORRELAÇÃO COM PARÂMETROS CLINICOPATOLÓGICOS

FRANCISCO BELTRÃO – PR
(FEVEREIRO/2019)

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

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DEDICATÓRIA

Aos meus pais.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

DCs – Células Dendríticas

NK – Natural Killer

IL-1β – Interleucina 1 beta

IL-6 – Interleucina 6

TNF-α – Fator de Necrose Tumoral Alfa

MPO – Mieloperoxidase

H_2O_2 – Peróxido de Hidrogênio

$HOCl^{\bullet}$ – Ácido Hipocloroso

$HOBr^{\bullet}$ – Ácido Hipobromoso

$HOSCN^{\bullet}$– Ácido Hipotiocianoso

ROS – Espécies reativas de oxigênio

DNA - Ácido Desoxirribonucleico

RNA – Ácido Ribonucleico

ANCA – Anticorpos anti-neutrófilos

c-ANCA – ANCA citoplasmático

PR3 – Proteinase 3

p-ANCA – ANCA perinuclear

NETs – Armadilhas Extracelulares de Neutrófilos

VAA – Vasculites associadas ao ANCA

CEONC – Hospital do Câncer

PRRs – Receptores de Reconhecimento de Padrões

PAMPs – Padrões Moleculares Associados a Patógenos

DAMPs – Padrões Moleculares Associados a Danos

TLRs – Receptores Tipo *Toll/Toll-Like*

RLRs – Receptores Tipo RIG-I/RIG-I-*Like*

NLRs – Receptores do Tipo NOD

ATP – Adenosina Trifosfato

HAMPs – *Homeostasis-altering Molecular Processes* (sem tradução oficial para o português)

MEFV – Gene da Febre Mediterrânea

IFN-γ – Interferon Gamma

IL-10 – Interleucina 10

NF-κB – Fator de Transcrição Nuclear Kappa B

AP1 – Proteína Ativadora 1

IRFs – Fatores Reguladores de Interferon

IL-8 – Interleucina 8

IL-22 – Interleucina 22

Th17 – Células T helper 17

T_{reg} – Célula T reguladora

IL-37 – Interleucina 37

TGF-β – Fator de Transformação do Crescimento Beta

kDA - Quilodalton

NADPH - Nicotinamida Adenina Dinucleótido Fosfato

NOX2 – NADPH Oxidase 2

O_2^{-} •- ânion Superóxido

MPO-Fe III – Forma férrica da MPO

Cl^- – ânion Cloreto

Br – Bromo

CD11b – Cluster de Diferenciação 11b

PI3K – Fosfoinositídeo 3-quinase

Ca^{2+} - Íon de Cálcio

IgG – Imunoglobulina G

CD86 – Cluster de Diferenciação 86

IL-12 – Interleucina 12

TCD4+ - Linfócito T CD4 positivo

Th1 – Célula T helper 1

H3K27me3 – Histona Trimetilada H3 na Lisina 27

RUNX3 – Fator de Transcrição runt-relacionado 3

GEPA - Granulomatose Eosinofílica com Poliangeíte

PAM – Poliangeíte Microscópica

IL-17A – Interleucina 17A

IL-21 – Interleucina 21

C5a – Componente complemento C5a

p38 MAPK – Via de sinalização celular em respostas à citocinas e estresse

ERK – Quinase regulada pela sinalização extracelular

PKC – Proteína Quinase C

MIF – Fator Inibitório de Migração dos Macrófagos

LLC – Leucemia Linfocítica Crônica

LMC – Leucemia Mielóide Crônica

MN – Nefropatia Membranosa

CAAE - Certificado de Apresentação para Apreciação Ética

TCLE – Termo de Consentimento Livre e Esclarecido

EDTA - Ácido Etilenodiamino Tetra-Acético

rpm – Rotações por minuto

CDIs – Carcinoma Ductal in situ

CDI – Carcinoma Ductal Invasivo

HER2 – Gene do Receptor-2 do Fator de Crescimento Epidérmico Humano

µl – Microlitro

ELISA – Imunoensaio Enzimático (*Enzyme Linked ImmunonoSorbent Assay*)

nm – Nanômetro

ANOVA – Análise de Variância

UI – Unidades Internacionais

mL – Mililitro

Anticorpos Anti-neutrófilos em Pacientes com Câncer de Mama e sua Correlação com Parâmetros Clinicopatológicos

Resumo

Introdução: Os anticorpos anti-citoplasma de neutrófilos (ANCA) são capazes de ativar neutrófilos através da ligação com seus antígenos, como a mieloperoxidase (p-ANCA), induzindo a degranulação de neutrófilos e burst respiratório mesmo em situações estéreis. Isto corrobora para dano celular e tecidual, podendo estar envolvido no desenvolvimento e na progressão do câncer de mama. **Objetivo:** Neste trabalho realizou-se a dosagem dos níveis de p-ANCA no plasma e no tecido mamário de mulheres (n=150) diagnosticadas com lesões benignas da mama (n=58) e carcinoma mamário (n=92), correlacionando-os com aspectos clinicopatológicos. **Metodologia**: Amostras de sangue periférico e de tecido mamário foram coletadas de mulheres atendidas no Hospital do Câncer de Francisco Beltrão – CEONC, Paraná, Brasil, no período de maio de 2015 à Dezembro de 2017. As amostras de sangue foram centrifugadas para obtenção de plasma, e os fragmentos de tecido homogeneizados e centrifugados para posterior análise dos sobrenadantes. As análises foram conduzidas em pools, agrupadas de acordo com diferentes características clinicopatológicas. A dosagem de p-ANCA foi realizada através de imunoensaio enzimático (kit IBL International Myeloperoxidase-Ab ELISA, RE75601). Os resultados foram avaliados pelo programa estatístico GraphPad Prism 6.0, considerando-se $p \leq 0,05$ como significante. **Resultados**: Níveis de p-ANCA foram encontrados elevados em pools relacionados com aspectos de pior prognóstico como alto grau histológico (plasma $p=0.0465$, tecido $p<0.0001$) status linfonodal positivo (plasma $p<0.001$, tecido $p=0.034$) e fatores de risco para câncer, como índice de massa corporal (tecido $p<0.001$), status menopausal (tecido $p<0.001$) e <45 anos (plasmap=0.0006, tecido $p<0.0001$) ao diagnóstico. **Conclusões**: Maiores níveis de p-ANCA demonstram uma associação com parâmetros clinicopatológicos relacionados ao pior prognostico do câncer de mama, podendo inferir que ocorra maior ativação de

neutrófilos e, consequentemente, maior degranulação e formação de NETs nestas condições patológicas.

Palavras-chave: Câncer de mama, anticorpos anti-neutrófilos, mieloperoxidase

Anti-neutrophil antibodies in Patients with Breast Cancer and Its Correlation with Clinicopathological Parameters

Abstract

Introduction: Anti-neutrophil cytoplasmic antibodies (ANCA) are able to activate neutrophils through binding to their antigens, such as myeloperoxidase (p-ANCA), inducing neutrophil degranulation and respiratory burst even in sterile situations. This corroborates for cellular and tissue damage, and may be involved in the development and progression of breast cancer. **Objective:** This study measured p-ANCA levels in serum and breast tissue of women (n= 150) diagnosed with benign breast lesions (n= 58) and mammary carcinoma (n= 92), correlating these levels with clinicopathological aspects. **Methods:** Peripheral blood and breast tissue samples were collected from women attended at Francisco Beltrão Cancer Hospital - CEONC, Paraná, Brazil, from May 2015 to December 2017. Blood samples were centrifuged to obtain serum, and the tissue fragments were homogenized and centrifuged for further analysis of the supernatants. The analyses were conducted in pools, grouped according to different clinicopathological characteristics. The p-ANCA dosage was performed by enzyme immunoassay (IBL International Myeloperoxidase-Ab ELISA Kit, RE75601). The results were evaluated by the statistical program GraphPad Prism 6.0, considering $p \leq 0,05$ as significant. **Results:** p-ANCA levels were found to be elevated in pools related to worse prognostic aspects such as high histological grade (serum $p=0.0465$, tissue $p<0.0001$) lymph nodal status (serum $p<0.001$, tissue $p=0.034$) and risk factors for breast cancer, as body mass index (tissue $p<0.001$), menopausal status (tissue $p<0.001$) and <45 years (serum $p=0.0006$, tissue $p<0.0001$) at diagnosis. **Conclusion:** Higher levels of p-ANCA demonstrate an association with clinicopathological parameters related to worst prognosis of breast cancer. It can be inferred that greater neutrophil activation might occurs in these conditions, and, consequently, greater degranulation and formation of NETs.

Keywords: Antineutrophil antibodies, Myeloperoxidase, Breast Neoplasms, Prognosis

SUMÁRIO

1. INTRODUÇÃO GERAL

As respostas imunológicas são parte de um grande e complexo sistema de processos bioquímicos que auxiliam na manutenção da homeostase do organismo através do reconhecimento de infecções, regulação imunológica, capacidade de gerar memória, cicatrização e reparo celular. O sistema imunológico é subdividido em imunidade inata e a adquirida. A imunidade inata correspondente à primeira linha de defesa do organismo, atuando sob qualquer perturbação à homeostase tecidual através de células dendríticas (DCs), natural killers (NK), neutrófilos e macrófagos (células fagocíticas), mastócitos, eosinófilos e basófilos (DE VISSER; EICHTEN; COUSSENS, 2006. Já a resposta adquirida garante a memória imunológica, permitindo com que o organismo, em um segundo contato com um imunógeno já conhecido, possa apresentar maior eficácia, especificidade e rapidez na resposta, atuando através dos linfócitos B e T (MARSHALL et al., 2018).

Neste contexto, o processo inflamatório primário se inicia na imunidade inata. Esta resposta é ativada por uma amplitude de antígenos, de origem celular ou infecciosos, (MEDZHITOV, 2007), e induz uma resposta rápida, porém sem especificidade. Este processo pode resultar não somente em eliminação do antígeno, mas também em dano tecidual, através de produtos gerados por neutrófilos e macrófagos ativados (PARKIN; COHEN, 2001). Dentre as substâncias produzidas pela ativação da resposta imune inata, destacam-se as citocinas, proteínas produzidas por células ativadas intrinsicamente envolvidas no aumento da atividade pró-inflamatória, como interleucina 1 beta (IL-1β), interleucina 6 (IL-6) e fator de necrose tumoral alfa (TNF-α) (ZHANG; AN, 2007). Estes sinalizadores, em conjunto com as quimiocinas (MANTOVANI et al., 2004), são responsáveis pelo homing leucocitário, atraindo neutrófilos da corrente sanguínea até o local onde estão presentes os antígenos que desencadearam tal resposta, liberando enzimas proteolíticas pelo processo de degranulação (PARKIN; COHEN, 2001).

Dentre as enzimas liberadas pelos grânulos azurofílicos dos neutrófilos, destaca-se a mieloperoxidase (MPO). A MPO é uma peroxidase com grupamento heme, que lhe confere coloração esverdeada, presente em grande quantidade nos neutrófilos (ODOBASIC; KITCHING; HOLDSWORTH, 2016). Essa enzima atua

como um potente antimicrobiano e tem capacidade de produzir substâncias oxidativas na presença de peróxido de hidrogênio (H2O2), como HOCl•, ácido hipobromoso (HOBr•), ácido hipotiocianoso (HOSCN•), radicais de tirosil e espécies reativas intermediárias de nitrogênio (ARATANI, 2018; NAUSEEF, 2014). Estas moléculas são precursoras de espécies reativas de oxigênio (ROS), reagem com inúmeras moléculas do organismo e possuem efeito citotóxico, sendo capazes de causar danos ao organismo (NAUSEEF, 2014). Um exemplo é a capacidade da MPO em catalisar a formação de produtos oxidativos através de seus subprodutos como o HOCl•, capaz de causar danos nas moléculas de DNA e RNA através da quebra de fitas simples e duplas (VANHAMME et al., 2018).

Há pouco mais de 30 anos, foi descoberta a presença de anticorpos contra alguns constituintes de neutrófilos e monócitos, chamados de anticorpos contra o citoplasma de neutrófilos (ANCA) ou anticorpos anti-neutrófilos, associados à fisiopatologia de doenças sistêmicas idiopáticas autoimunes como vasculite e gromerulonefrite (DAVIES et al., 1982; FALK; JENNETTE, 1988). Há dois tipos de marcações possíveis de serem identificadas por imunofluorescência indireta para o ANCA: a marcação citoplasmática (c-ANCA) específica para Proteinase 3 (PR3), e a marcação perinuclear (p-ANCA), específica majoritariamente para MPO (GROSS; SCHMITT; CSERNOK, 2008)

Os ANCAs são capazes de se ligar nos neutrófilos, gerando um aumento na sua ativação, ocasionando danos vasculares, epiteliais e teciduais através da formação de ROS, armadilhas extracelulares de neutrófilos (NETs, Neutrophil extracellular traps), necrose e apoptose (JENNETTE; FALK, 2014). Essa ativação se dá através da ligação com seu antígeno exposto na superfície celular, geralmente presente em neutrófilos sensibilizados ou expostos nos filamentos das NETs (REUMAUX et al., 2003).

As NETs são estruturas feitas de filamentos de cromatina descondensada, histonas, proteases e proteínas granulares como a MPO. A formação de NETs é bem estabelecida na defesa inata contra patógenos extracelulares como bactérias, fungos e vírus. Recentemente, tem sido associadas à doenças não infecciosas como artrite reumatoide, vasculite, aterosclerose, diabetes, trombose e câncer (JORCH; KUBES, 2017; KAPLAN; RADIC, 2012).

Em pacientes portadores de tumores sólidos, a literatura demonstra a existência de associação entre o risco de desenvolvimento de câncer com doenças

derivadas de vasculites associadas ao ANCA (VAA), como observado em pacientes portadores de granulomatose poliangeíte (antiga Wegener's), síndrome de Churg-Strauss e glomerulonefrite idiopática (CRUZ, 2007; HEAF; HANSEN; LAIER, 2018; VAN DAALEN et al., 2017; WESTER TREJO; BAJEMA; VAN DAALEN, 2018). Até onde se sabe, não há relatos sobre o perfil de produção de p-ANCA em pacientes portadoras de câncer de mama, bem como se desconhece sua associação com o prognóstico da doença.

1.2 REVISÃO BIBLIOGRÁFICA

1.2.1 Visão geral da resposta imune e inflamação

O sistema imune inato corresponde à primeira linha de defesa do organismo contra moléculas estranhas (LIU; CAO, 2016; RANKIN; ARTIS, 2018). As células do sistema imune inato apresentam em sua superfície receptores de reconhecimento de padrões (PRRs), os quais reconhecem moléculas denominadas padrões moleculares associados à patógenos (PAMPs) através de um mecanismo altamente conservado durante a evolução (LISTON; MASTERS, 2017). Os PRRs podem, ainda, reconhecer moléculas e subprodutos derivados de danos teciduais ou células necrosadas, chamados de padrões moleculares associados à danos (DAMPs) (CAO, 2016). Assim, o reconhecimento de ambos PAMPs e DAMPs é crucial na manutenção da homeostase imunológica.

Os PRRs estão distribuídos em grandes famílias de receptores: receptores tipo *Toll* (TLRs), receptores tipo RIG-I (RLRs) e os receptores tipo NOD (NLRs). Todos são fundamentais no reconhecimento de proteínas, ácidos nucleicos e outras moléculas derivadas de patógenos (CAO, 2016; KUMAR; KAWAI; AKIRA, 2011). Os DAMPs também são reconhecidos pelos PRRs em situações onde são detectados danos celulares quando há morte celular não programada, com liberação de moléculas como ácido úrico, adenosina trifosfato (ATP) e outras proteínas (TANG et al., 2012).

Recentemente foi proposto uma outra via de sinalização do sistema imune inato – a via de processos de alteração molecular da homeostase (HAMPs, do inglês *homeostasis-altering molecular processes*). Esta via seria capaz de

reconhecer distúrbios homeostáticos sem a necessidade de receptores específicos, detectando alterações nos processos celulares (LISTON; MASTERS, 2017). A pirina, uma proteína codificada pelo gene da febre mediterrânea (MEFV) localizado no cromossomo 16, é encontrada majoritariamente em células do sistema imunológico como neutrófilos e monócitos e atuaria como um desses sensores da imunidade inata, sendo ativada por citocinas como TNF-α, interferon gama IFN-γ e interleucina 10 (IL-10) e, posteriormente, recrutando e ativando complexos proteicos chamados de inflamassomas que contém moléculas como a caspase-1, promotoras da inflamação (HEILIG; BROZ, 2018). Entretanto, os autores sugerem que tal via seja mais propensa a ativação do sistema imune inato por distúrbios homeostáticos estéreis, como a produção de ROS, desencadeando ativação de inflamassomas e do processo inflamatório, contribuindo para o desenvolvimento e/ou progressão de doenças crônicas como o Alzheimer (LISTON; MASTERS, 2017).

O reconhecimento de PAMPs e/ou DAMPs ativa uma série de cascatas de sinalizações que culminam na resposta inflamatória, com a ativação de fatores transcricionais como fator nuclear κB (NF-κB), proteína ativadora-1 (AP1) e fatores reguladores de interferon (IRFs), que são mediadores pró-inflamatórios (KUMAR; KAWAI; AKIRA, 2011; LAMKANFI; DIXIT, 2014; PASPARAKIS, 2009).

A resposta inflamatória se divide em três fases –inicial, inflamatória e de restauração (NETEA et al., 2017). A fase inicial se daria com o reconhecimento de DAMPs/PAMPs usualmente por DCs, monócitos, macrófagos ou neutrófilos, que liberam moléculas pró-inflamatórias como citocinas e quimiocinas, aumentando a permeabilidade vascular e atraindo células do sistema imunológico para o tecido danificado (LIU; CAO, 2016). Em situações não-inflamatórias, os neutrófilos permanecem circulando livremente na corrente sanguínea, não aderem ao endotélio e possuem morfologia arredondada. Porém, induzido por mediadores inflamatórios, o epitélio passa a expressar moléculas de aderência e os neutrófilos passam a exibir receptores para que ocorra a adesão. Essa ligação provoca o rolamento destas células pelo endotélio vascular, mediado pela L-selectina presente em leucócitos e pela E-selectina e P-selectina no endotélio, fazendo com que o neutrófilo siga até o local da inflamação, atraído pela interleucina 8 (IL-8) e outras quimiocinas. Este processo resulta na diapedese ou transmigração celular, onde o neutrófilo altera sua morfologia para facilitar a passagem do endotélio para

dentro do tecido lesado (PHILLIPSON; KUBES, 2011; WITKO-SARSAT et al., 2000).

A segunda fase é responsável pelos efeitos clínicos da inflamação – dor, calor, rubor, tumor e perda de função – e ocorre devido aos mediadores vasoativos e citocinas liberadas, como o TNF-α, IL-1β e IL-6 (JONES, Simon A.; JENKINS, 2018; KONO; ROCK, 2008; MARSHALL et al., 2018). Também ocorre a ativação da função microbicida e pró-inflamatória dos neutrófilos, através dos processos de fagocitose, degranulação e *burst* oxidativo (PHILLIPSON; KUBES, 2011; WITKO-SARSAT et al., 2000). A ativação induzida pelo IFN-γ, em conjunto com a interleucina 22 (IL-22), induzida pelas células T helper 17 (Th17), faz com que ocorra a degranulação e o *burst* oxidativo, liberando para o meio extracelular moléculas pró-inflamatórias como a MPO e ROS (ARATANI, 2018; ARTIS; SPITS, 2015; CHEN; NUÑEZ, 2010; NETEA et al., 2017).

Por fim, para retomar a homeostase, o processo inflamatório deve cessar. Os mediadores inflamatórios devem ser antagonizados e catabolizados, assim como o número de células do sistema imune inato no local da inflamação deve diminuir para que o tecido possa ser restaurado. Este processo ocorre através da produção de mediadores e citocinas pelas células T reguladoras (T_{reg}), monócitos/macrófagos e plaquetas, como as prostaglandinas D2, resolvina E1, protectina D1, IL-10, interleucina 37 (IL-37) e fator de transformação do crescimento beta (TGF-β), diminuindo o recrutamento de neutrófilos para o local da inflamação e a liberação de citocinas pró-inflamatórias. Além disso, é necessário a retirada dos neutrófilos apoptóticos através de um processo semelhante à fagocitose, chamado de eferocitose, realizado pelos macrófagos. Caso este retorno à homeostase falhe, há o surgimento de doenças derivadas de processos inflamatórios crônicos como artrite, asma, doenças neurodegenerativas e autoimunes, câncer, entre outras (EL-KENAWI; RUFFELL, 2017; FRIDMAN et al., 2017; NETEA et al., 2017; SCHETT; NEURATH, 2018).

1.2.2 Neutrófilos e Mieloperoxidase

Os neutrófilos, também conhecidos como leucócitos polimorfonucleados devido ao seu núcleo lobulado característico, são de vital importância para a defesa

do organismo contra agentes patogênicos, em especial bactérias e fungos (ROSALES, 2018). São células de vida-curta e constituem a maior parte dos leucócitos circulantes no organismo humano, chegando a 70% do total. São continuamente produzidos na medula óssea através de células tronco hematopoiéticas (linhagem mieloide) e passam por diversos estágios durante sua maturação: mieloblasto, promielócito, mielócito, metamielócito, bastões e, por fim, polimorfonucleado/segmentado (KOLACZKOWSKA; KUBES, 2013).

Durante a maturação, três tipos de grânulos se desenvolvem em conjunto com a célula, todos com propriedades pró-inflamatórias: grânulos primários (azurofílicos), detectados já nos promielócitos, grânulos secundários (específicos), detectados em mielócitos e metamielócitos, e grânulos terciários (gelatinase), detectados em bastões. Há também o desenvolvimento de vesículas secretórias, presentes apenas nos neutrófilos maduros. Estes grânulos estocam inúmeras moléculas que constituem o arsenal dos neutrófilos contra patógenos e DAMPs, como a enzima MPO presente nos grânulos azurofílicos, a lactoferrina dos grânulos específicos e a gelatinase dos grânulos terciários (ARATANI, 2018; BORREGAARD; SØRENSEN; THEILGAARD-MO, 2007; COWLAND; BORREGAARD; HA, 2010; KOLACZKOWSKA; KUBES, 2013; ROSALES, 2018).

O gene da MPO está localizado no braço longo do cromossomo 17, loci q22, sendo expresso entre o desenvolvimento do mieloblasto à promielócito (HANSSON; OLSSON; NAUSEEF, 2006; NAUSEEF, 2018). Foi assim denominada por ter inicialmente sido identificada nas células da linhagem mieloide, embora estudos recentes tenham encontrado a enzima em células da linhagem linfoide (OKADA et al., 2016).

Nos neutrófilos, a MPO existe como uma proteína de peso molecular de 150kDA e consiste em dois dímeros idênticos ligados por uma ponte de bissulfeto, sendo cada dímero composto de uma subunidade de cadeia leve e uma pesada, com grupos heme funcionalmente idênticos (HANSSON; OLSSON; NAUSEEF, 2006; NAUSEEF, 2018). Além disso, a MPO é a molécula mais abundante dos neutrófilos, constituindo 5% de seu peso seco e 25% das proteínas nos grânulos azurofílicos (SEGAL, 2005).

A MPO é armazenada na sua forma inativa, e não se sabe de que forma a MPO é liberada durante a degranulação dos neutrófilos. A ativação dos neutrófilos ativa a enzima NADPH oxidase (NOX2) de forma excessiva, o que resulta em

grande produção de superóxido (O_2^-), uma ROS rapidamente convertida pelo sistema antioxidante em H_2O_2, molécula necessária para a ativação e funcionamento da MPO (KHAN; ALSAHLI; RAHMANI, 2018; VANHAMME et al., 2018).

A MPO possui dois ciclos de reações, demonstrados na figura 1, o ciclo da peroxidase e o ciclo do halogênio. Em seu estado inativo, a MPO conta com um grupamento heme férrico (MPO-Fe(III)), que reage com o H_2O_2 e é oxidado à um intermediário de vida curta chamado de Composto I. O Composto I utiliza haletos como Cl^- e Br para formar subprodutos extremamente oxidantes, principalmente HOCl. Esta produção de oxidantes é realizada no ciclo do halogênio. Na falta de haletos, é iniciado o ciclo da peroxidase, onde o Composto I é oxidado à Composto II e forma espécies de ROS como radicais tirosil (HUANG et al., 2016; SOUBHYE et al., 2016; VANHAMME et al., 2018).

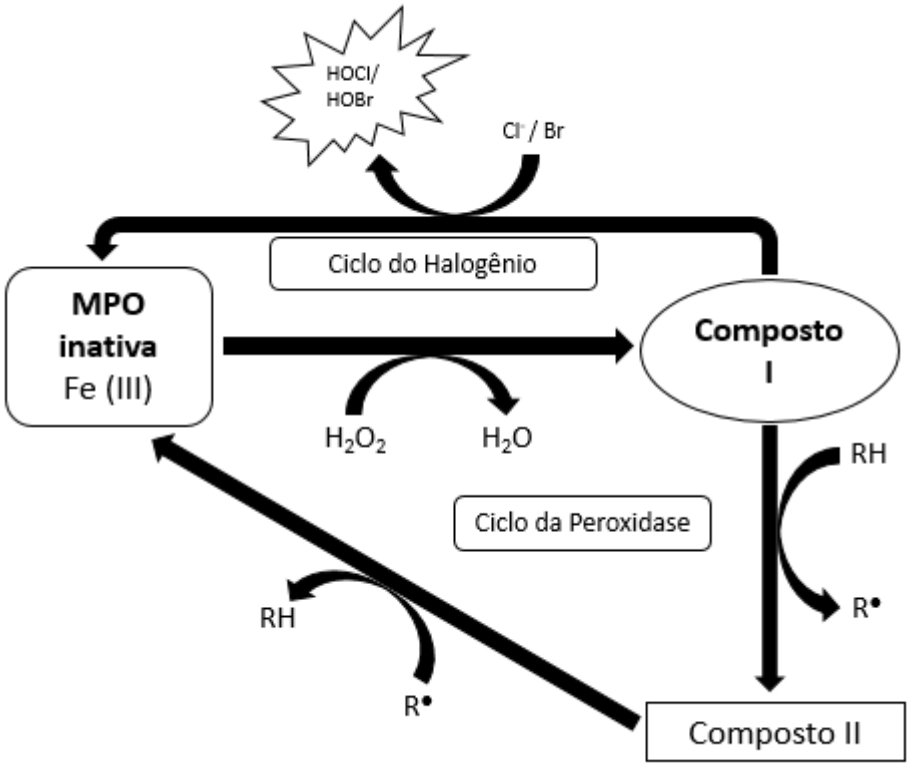

Figura 1 - Ciclo da Mieloperoxidase

A liberação da MPO usualmente ocorre na presença de algum patógeno; entretanto, em processos inflamatórios crônicos, o excesso de produção dessa enzima e de seus subprodutos acarreta em danos para o tecido, proteínas e DNA , o que favorece a manutenção do ambiente inflamatório (ANAND; ANAND, 2012;

ARATANI, 2018). Além disso, a MPO é capaz de atuar como um regulador autócrino dos neutrófilos, induzindo a degranulação no meio extracelular através da ligação com o antígeno de superfície CD11b, ativação de enzimas tirosina quinases, fosfoinositídeo 3-quinase (PI3K) e do aumento de íons de cálcio (Ca^{2+}) intracelular, resultando em um processo de retroalimentação (GRIGORIEVA et al., 2016).

Além do processo de fagocitose, os neutrófilos apresentam uma estratégia adicional, recentemente identificada, que seria a formação de NETs, conforme mostra a figura 2 (ERPENBECK; SCHÖN, 2017). As NETs são estruturas compostas por cromatina descondensada, proteínas citoplasmáticas e granulares que ocasionam a morte celular através do processo chamado de netose, bastante distinto da apoptose, que seria a morte celular autônoma, programada e controlada, ou da necrose, morte celular acidental derivada de perturbações do microambiente onde ela está presente (ERPENBECK; SCHÖN, 2017; FINK; COOKSON, 2005).

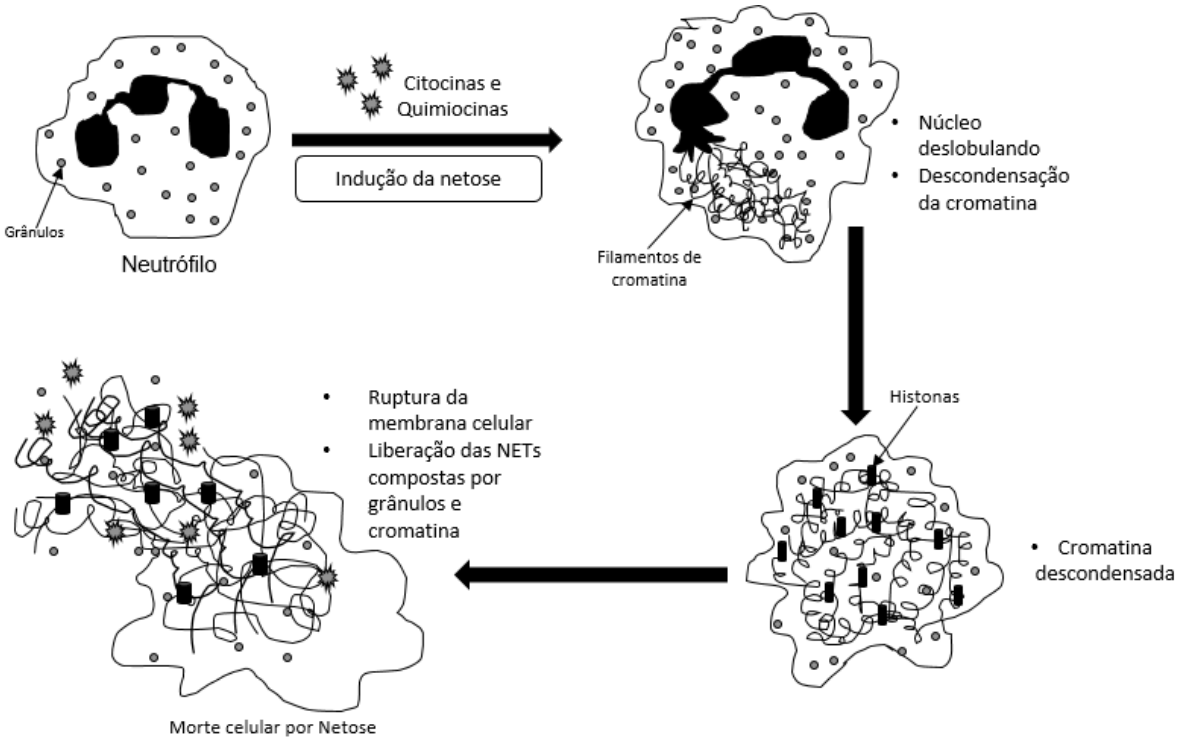

Figura 2 - Esquema da formação de NETs

Estudos mostram que a MPO é requisito-chave para que este processo ocorra (BJÖRNSDOTTIR et al., 2015; METZLER et al., 2014). A netose pode ser induzida por fatores como ROS, presença de MPO e elastase, IL-8, TGF-β,

plaquetas ativadas e ANCAs. Apesar do seu papel na imunidade, sabe-se que a produção exagerada de NETs pode favorecer a progressão de patologias crônicas (ERPENBECK; SCHÖN, 2017).

Alguns estudos tem evidenciado a participação dos neutrófilos e da MPO em inflamações crônicas. Fonseca e colaboradores (2014) encontraram associação positiva entre marcadores da síndrome metabólica como glicose e insulina em jejum com níveis de MPO presentes no plasma de pacientes, indicando um envolvimento da dislipidemia com os processos inflamatórios através da manutenção do desbalanço redox em pacientes com síndrome metabólica. Outro estudo buscou correlacionar sobrepeso/obesidade com a gravidade do processo inflamatório na mucosa colorretal através de infiltrados de neutrófilos/macrófagos contendo MPO, e se isto acarretaria risco para o desenvolvimento de adenomas. Como resultado, encontraram que indivíduos obesos, do gênero feminino ou masculino, possuíam um número significativamente elevado de infiltrados celulares MPO positivos quando comparados à indivíduos não obesos. Além disso, verificaram que o grau de inflamação está associado ao desenvolvimento de adenomas em indivíduos com sobrepeso/obesidade. Ainda, observaram que mulheres com sobrepeso possuem uma maior presença de células MPO positivas na mucosa colorretal (MARIANI et al., 2017). Da mesma forma, mulheres obesas e mulheres com pré-eclâmpsia possuem maior número de infiltrados de neutrófilos, e consequentemente MPO, no endotélio vascular em comparação à mulheres em condições normais, e isto está correlacionado ao aumento da pressão arterial, inflamação vascular e maior risco de mulheres obesas desenvolverem pré-eclâmpsia ao engravidar (SHUKLA; WALSH, 2015).

Outros trabalhos correlacionaram a presença da MPO como um fator negativo em diversas outras patologias como glomerulonefrite (ARIMURA et al., 2013), esteatose não-alcóolica (sugerindo um possível papel na síndrome metabólica) (RENSEN et al., 2012), doença de Alzheimer e outras doenças neurodegenerativas (GREEN et al., 2004; RAY; KATYAL, 2016; TZIKAS et al., 2014), doenças cardiovasculares (ANATOLIOTAKIS; DEFTEREOS, 2013), câncer de ovário (CASTILLO-TONG et al., 2014; FLETCHER et al., 2012), obesidade e síndrome metabólica, artrite reumatoide, esclerose múltipla e inflamações pulmonares (ARATANI, 2018; KHAN; ALSAHLI; RAHMANI, 2018).

1.2.3 p-ANCA e sua relação com doenças

A primeira descrição de anticorpos contra neutrófilos data-se de 1982, quando Davies e colaboradores (1982) reportaram marcações ANCA em pacientes com glomerulonefrite pauci-imune, e que essa marcação em neutrófilos desaparecia após o início do tratamento. Van der Woude e colegas (1985) encontraram a presença de ANCA-IgG no soro de pacientes com granulomatose de Wegener, atualmente chamada granulomatose com poliangeíte (FALK et al., 2011), significantemente correlacionada com a doença e sugerida como um marcador de diagnóstico. Na década de 90, pesquisadores identificaram um alvo para ANCA nos grânulos azurofílicos de neutrófilos e monócitos, chamado de p-ANCA, sendo a molécula alvo majoritariamente a MPO (CALAFAT et al., 1990; FALK, 1990; FALK; JENNETTE, 1988).

Naquela época, Falk (1990) levantava a suspeita de que o p-ANCA poderia induzir o *burst* oxidativo dos neutrófilos e inativar o *feedback* negativo que a MPO produzia ao ser liberada, potencializando os efeitos danosos do processo inflamatório. Algum tempo depois, demonstraram que os anticorpos p-ANCA estavam associados com a ativação de neutrófilos e danos à células endoteliais, acarretando em processos inflamatórios como a vasculite (EWERT; JENNETTE; FALK, 1992).

Em estudos *in vitro* e *in vivo*, a MPO foi capaz de inibir a migração de DCs aos linfonodos e sua posterior ativação, através de um mecanismo pelo qual o acúmulo de MPO promove a supressão da expressão de CD86 positivas e de citocinas como interleucina 12 (IL-12) e interleucina 23 (IL-23), inibindo a ativação de TCD4+ e respostas Th1 e Th17 da imunidade adaptativa (ODOBASIC; KITCHING; HOLDSWORTH, 2016). Como a IL-12 é responsável pela ativação das células NK, a diminuição da produção de IL-12 pelas DCs acarreta na supressão da sua atividade (ZUNDLER; NEURATH, 2015). Além disso, a MPO é capaz de induzir a produção de anticorpos contra si própria (SUWANCHOTE et al., 2018), gerando auto anticorpos anti-MPO.

Ciavatta et al. (2010) demonstrou que existem mecanismos de silenciamento epigenéticos associados à maior expressão da MPO e PR3 em pacientes que produzem ANCA quando comparados à indivíduos saudáveis, como a depleção da histona trimetilada H3 na lisina 27 (*trimethylated histone H3 at lysine 27* -

H3K27me3) em pacientes ANCA positivos, sugerindo que a H3K27me3 possa estar associada ao silenciamento de MPO e PR3 em neutrófilos de pacientes saudáveis. Além disso, observaram que em pacientes ANCA positivos há diminuição da expressão do fator de transcrição runt-relacionado 3 (RUNX3), regulador gênico que age inclusive como supressor tumoral, em contraste com o aumento da expressão MPO. Recentemente foi publicada uma revisão sobre a patogênese da VAA, sugerindo que sua patogenia seja multifatorial, envolvendo aspectos genéticos, epigenéticos e desregulação imunológica (LAMPRECHT et al., 2018), enfatizando, a importância de mecanismos epigenéticos no surgimento de anticorpos p-ANCA.

VAA são doenças sistêmicas inflamatórias que afetam vasos de pequeno calibre como arteríolas, capilares e vênulas, raramente atingindo vasos maiores. Caracteriza-se por infiltração transmural de neutrófilos, ruptura de células endoteliais das paredes do vaso necrose associada a edema e necrose. A degranulação dos neutrófilos acarreta na liberação de citocinas e quimiocinas proinflamatórias, ativação de vias alternativas do sistema complemento, além da geração de NETs, avançando para infiltrados de macrófagos e linfócitos T e B, tornando o processo danoso crônico (LAMPRECHT et al., 2018). Apesar da maior parte dos estudos serem direcionados para a MPO dos neutrófilos, os monócitos também são produtores dessa enzima (porém em menor escala), e a ligação do p-ANCA aos monócitos acarreta em aumento da produção de IL-1β, IL-6 e IL-8, todas citocinas pró-inflamatórias (O'BRIEN et al., 2015). A IL-8, extensamente estudada e associada ao câncer, possui efeito quimiotáxico em neutrófilos, regulando infiltração destes no microambiente tumoral, além de estar associada a inúmeros processos como angiogênese, mutagênese e progressão metastática (WAUGH; WILSON, 2008).

Curiosamente, nem todos indivíduos ANCA positivos desenvolvem alguma patologia, sugerindo que estes possam fazer parte do conjunto de anticorpos naturalmente produzidos pelo organismo. Assim, sugere-se que ligações em epítopos específicos sejam necessárias para o potencial patogênico do ANCA ser observado clinicamente (SPECKS, 2009). Um relato de caso demonstrou que anticorpos anti-MPO podem ser passados via transplacentária, acarretando em problemas renais e hemorragia pulmonar ao neonato (BANSAL; TOBIN, 2004),

embora outro estudo tenha observado outro caso de transferência de anti-MPO sem sequelas clínicas (SILVA et al., 2009).

Um estudo realizado com anticorpos p-ANCA do isotipo IgG em pacientes com VAA demostrou variações significativas de glicosilação na região variável da imunoglobulina, aumentando a avidade do anticorpo e a capacidade de induzir o *burst* respiratório dos neutrófilos (XU et al., 2012). Em uma análise retrospectiva acerca da prevalência de ANCA na granulomatose eosinofílica com poliangeíte (GEPA) e poliangeíte microscópica (PAM), doenças já associadas com a presença desses anticorpos, discutiu-se a hipótese de que tais pacientes produzem p-ANCA contra diferentes epítopos da MPO, o que resultaria em diferentes desfechos clínicos (BREMER et al., 2013).

Estudos apontam que existe formação de NETs em mais de 50% dos pacientes com glomerulonefrite p-ANCA positivos, além de MPO aderida e internalizada nas células endoteliais glomerulares (O'SULLIVAN et al., 2015). Sangaletti et al. (SANGALETTI et al., 2012) demonstrou o papel patogênico das NETs no reconhecimento da MPO como um antígeno pelo p-ANCA através da exposição extracelular da mesma, e sugeriu uma interação entre NETs e DCs, estas últimas conhecidas responsáveis pela ativação da imunidade adaptativa, e, consequentemente, autoimunidade.

As respostas derivadas de células Th17, produtoras de interleucina 17A (IL-17A), interleucina 21 (IL-21) e IL-22, são fatores causais em doenças derivadas de distúrbios no sistema imunológico como artrite, encefalite e outras inflamações. Estudos em ratos demonstraram que as respostas Th17 também podem estar envolvidas no desenvolvimento doenças associadas à p-ANCA, como VAA, através de mecanismos como ativação de neutrófilos e recrutamento de macrófagos pela IL-17A para locais onde há MPO reconhecida como antígeno (GAN et al., 2010). Além disso, observou-se que o componente 5a (C5a) do sistema complemento é capaz de tornar os neutrófilos hiper-responsivos ao *burst* respiratório mediado por p-ANCA, e que o bloqueio de vias como a p38 proteíno-quinase ativadas por mitógenos (p38MAPK), quinases reguladas por sinal extracelular (ERK), PI3K e proteína quinase C (PKC) foi capaz de diminuir consideravelmente essa degranulação através da inibição do C5a (HAO et al., 2012; HAO; CHEN; ZHAO, 2013). Da mesma forma, foi identificado que a presença do fator de inibição de migração de macrófagos (MIF) também atua aumentando a sensibilidade dos

neutrófilos, translocando o antígeno do p-ANCA para a superfície da membrana, auxiliando na sua subsequente ativação e degranulação em pacientes com vasculite (HAO et al., 2016). Portanto, é possível visualizar que inúmeras vias e processos estão associados a ativação e manutenção da produção de p-ANCA, resultando em diversos processos patofisiológicos nas doenças relacionadas à produção desordenada destes anticorpos (KETTRITZ, 2012; SCHULTE-PELKUM et al., 2014).

1.2.4 p-ANCA no câncer

Poucos estudos correlacionam a presença de ANCAs com o desenvolvimento e progressão do câncer. Entretanto, a semelhança entre o surgimento de um tumor com processos inflamatórios derivados de danos teciduais já é reconhecida há alguns anos, ambas as situações apresentando características similares como a infiltração por células do sistema imunológico, particularmente macrófagos e neutrófilos (DVORAK, 1986). A formação de NETs resultando em netose está associada à inúmeras doenças descritas no tópico anterior, e parece estar envolvida no câncer através da interação com células tumorais que permitem sua aderência e facilitam a formação de metástases (COOLS-LARTIGUE et al., 2013).

A análise de amostras de sangue de pacientes com leucemia linfocítica crônica (LLC) demonstrou que as NETs são capazes de induzir a produção de IL-8 e ativação de DCs (PODAZA et al., 2017). Em outro estudo, verificou-se que não apenas os neutrófilos de ratos com leucemia mielóide crônica (LMC) eram mais predispostos a formar NETs, mas também as formavam de forma mais eficaz do que os ratos sem a doença. Foi demonstrado ainda que neutrófilos de tumores sólidos como mama e pulmão também possuíam essa predisposição em formar NETs, sugerindo que o microambiente tumoral torna os neutrófilos hipersensitivos à indução de netose e formação de trombos (DEMERS et al., 2012). Embora tais estudos não tenham realizado nenhuma análise referente aos ANCAs, é possível observar que os processos induzidos pelo microambiente tumoral são muito semelhantes àqueles derivados da indução por ANCA, como a formação/indução

de NETs, contribuintes tanto quanto fonte de antígenos como sinalizadores co-estimulatórios do processo inflamatório.

Navarro et al. (1994) publicou um relato de caso sobre um paciente diagnosticado e tratado inicialmente com glomerulonefrite necrosante p-ANCA positivo. Poucos meses depois o paciente retornou ao hospital com sintomas semelhantes (anorexia, perda de peso, fraqueza), porém p-ANCA negativo, e, através de uma tomografia computadorizada, foi identificado um adenocarcinoma no mediastino, evoluindo à óbito rapidamente. Os pesquisadores não descartaram a ideia do “acaso” e de que o tratamento imunossupressor possa ter acelerado a evolução tumoral, entretanto, reiteraram que associações entre vasculites e tumores possam existir graças a formação de complexos imunológicos (antígeno e anticorpo) que depositam-se e provocam inflamações crônicas, além da formação de complexos in situ nos vasos sanguíneos.

Complexos imunológicos estão associados com a patogênese de inúmeras doenças autoimunes, corroborando para infiltração e ativação de células imunes como os neutrófilos, induzidos pelo complemento C5a e aumentando a inflamação local (MIYABE et al., 2017). Como já descrito, C5a é capaz de tornar o neutrófilo mais apto à ativação por p-ANCA, portanto a formação de complexos imunológicos pode auxiliar na produção de anticorpos contra neutrófilos. Mais recentemente, um grupo de pesquisadores relatou que um paciente pós-cirúrgico com adenocarcinoma de cólon retornou ao atendimento após alguns meses, obtendo diagnóstico de glomerulonefrite p-ANCA positivo associada à nefropatia membranosa (MN). A análise do caso levantou a suspeita de que MN poderia estar associada à malignidade, e que esta, de certa forma, precedeu a glomerulonefrite p-ANCA positivo (SHIMADA et al., 2013).

Em outro estudo, Jones et al. (2003) relatou paciente com massa pélvica, p-ANCA positivo, com a presença de vasculite. Inicialmente, os pesquisadores imaginaram que a vasculite seria derivada de alguma malignidade, entretanto, após tratamento com imunossupressores e identificação histológica foi possível confirmar que a massa pélvica era, na verdade, devido ao processo inflamatório sistêmico induzido pela vasculite. Já Yorita et al. (2018) relatou o primeiro caso de paciente diagnosticado com carcinoma escamoso acantolítico pulmonar que possuía níveis elevados de p-ANCA presentes no soro, enquanto que c-ANCA foi encontrado em níveis considerados normais.

Outro estudo mostrou comparativamente a prevalência de p-ANCA e doenças reumáticas em pacientes com linfoma de Hodgkin e não-Hodgkin, encontrando positividade em oito pacientes com linfoma de Hodgkin. Destes, seis eram p-ANCA positivos, e nenhum cumpria os critérios de diagnóstico para vasculite. Os autores sugeriram que, para pacientes p-ANCA positivos e sem sintomatologia para vasculite ou outras doenças reumáticas, deve-se buscar diagnóstico diferencial para linfoma de Hodgkin (CIL et al., 2009).

Estudos demonstram que p-ANCAs não sejam apenas ativadores de neutrófilos, mas também indutores da formação de NETs, criando um ciclo de retroalimentação onde quanto maior a formação de NETs, maior a exposição dos antígenos MPO e PR3, perpetuando a geração de ANCAs. Além disso, há uma tendência de as NETs estarem associadas a um pior prognóstico de cânceres como sarcoma de Ewing, adenocarcinoma ductal pancreático, melanoma cutâneo e câncer de pequenas células do intestino (BONAVENTURA et al., 2018).

A proposta deste trabalho surgiu através da suspeita de que mecanismos desencadeadores de processos inflamatórios idiopáticos estão associados com a evolução do câncer e sua agressividade. Como descrito acima, o p-ANCA está associado à progressão e manutenção de inúmeros processos inflamatórios crônicos através da ativação desregulada de neutrófilos e sua degranulação, além do estímulo à formação de trombos e NETs. Embora o p-ANCA ainda estar majoritariamente associado à doenças inflamatórias autoimunes, é possível observar que o mecanismo de ação e as respostas que ele desencadeia sugerem uma possível correlação com processos inflamatórios descontrolados em geral, o que leva a hipótese de que estes anticorpos possam, também, estar associados ao câncer de mama.

2. OBJETIVOS

2.1 Geral

Quantificar os níveis de p-ANCA em amostras plasmáticas e tecidual de pacientes portadoras de câncer de mama, e verificar sua associação com parâmetros clínicos determinantes de pior prognostico da doença.

2.2 Específicos

- Caracterizar os aspectos clinicopatológicos das pacientes atendidas no Hospital do Câncer – CEONC de Francisco Beltrão – PR no período do estudo.

- Quantificar os níveis de p-ANCA em amostras de plasma e tecido mamário coletadas

- Correlacionar os níveis de p-ANCA com parâmetros clinicopatológicos como idade, status menopausal, índice de massa corpórea, subtipos moleculares, status linfonodal, grau histológico e estratificação de risco das pacientes.

3. METODOLOGIA

3.1 Desenho do Estudo

Neste trabalho foram voluntariadas 150 mulheres atendidas no Hospital do Câncer – Ceonc, Francisco Beltrão-PR portadoras de imagens suspeitas para neoplasia mamária, atendidas entre maio de 2015 e março de 2018. 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) (disponível no Anexo 01). Dados clinicopatológicos e sócio-demográficos foram obtidos através de consulta aos prontuários médicos e conversa com as pacientes. Foram incluídas pacientes atendidas no Ceonc no período do estudo, com lesões suspeitas para câncer de mama. Foram excluídas pacientes atendidas pelo Ceonc neste período portando qualquer outra neoplasia.

Amostras contendo 10 mL de sangue total em EDTA ou heparizinado foram obtidas por punção venosa periférica e transportadas para processamento e armazenamento. A obtenção de plasma foi realizada através de centrifugação à

4000 rpm por 5 minutos, com posterior armazenamento à -20°C até o momento das análises. Foi coletado da amostra tumoral destinada à biópsia um pequeno retalho para análises realizadas em tecido. Essa amostra tumoral foi armazenada em microtubo e congelada à -20°C até o momento das análises.

3.2 Quantificação de p-ANCA circulante e tecidual

Para análise dos níveis de p-ANCA, as amostras das pacientes foram agrupadas em pools (amostras compostas), sendo cada pool plasmático formado por 20 amostras de plasma e cada pool tecidual formado por 10 amostras de tecido. Estes pools foram categorizados segundo critérios clinicopatológicos de interesse: diagnóstico da biópsia normal, diagnóstico da biópsia lesão benigna, diagnóstico da biópsia carcinoma ductal *in situ* (CDIs), diagnóstico da biópsia carcinoma ductal invasivo (CDI), idade ao diagnóstico <45 anos ou >45 anos, status menopausal ao diagnóstico, índice de massa corpórea (eutrófico, sobrepeso ou obeso), classificação do subtipo molecular do tumor (Luminal A, Luminal B, HER2, Luminal-HER ou Triplo Negativo), status linfonodal (positivo ou negativo), grau de diferenciação histológica da biópsia (baixo/intermediário ou alto) e estratificação de risco da paciente (baixo, intermediário ou alto). Cada *pool* plasmático foi criado através da junção de 20µl de vinte (20) pacientes diferentes. Já para a criação dos *pools* teciduais, foi utilizado um retalho tecidual de até dez (10) pacientes diferentes para cada grupo, e a obtenção do sobrenadante tecidual foi feita, respectivamente, através da homogeneização, maceração e centrifugação dos tecidos por 10min à 4.000rpm. Foi procurado repetir as mesmas pacientes em cada critério clinicopatológico nas análises de plasma e tecido.

A quantificação dos níveis plasmáticos e do centrifugado tecidual de p-ANCA foi realizada através de enzima imunoensaio (Kit Myeloperoxidase-Ab ELISA, IBL International). Esta abordagem permite a determinação quantitativa de anticorpos IgG contra a MPO presente no soro humano. A placa fornecida pelo kit encontra-se previamente sensibilizada com MPO humana (antígeno), onde se ligam os p-ANCA presentes nas amostras de soro e centrifugado analisadas, quantificadas após a adição de anticorpo secundário conjugado a enzima, que, com adição do

substrato gera uma coloração proporcional à quantidade de p-ANCA na amostra analisada. As leituras das absorbâncias foram realizadas em leitora de microplacas (Polaris®, Celer Biotecnologia) no comprimento de onda de 412 nm. O cálculo da concentração de p-ANCA na amostra é feito com base em padrões contendo concentrações conhecidas de p-ANCA fornecidos pelo kit.

3.3 Análise dos resultados

Os dados obtidos foram expressos como média±erro padrão da média e apresentados na forma de gráficos e tabelas. Após análise da normalidade, os grupos foram comparados por teste t de Student, teste de Mann-Whitney ou one-way ANOVA com teste de Bonferroni como pós-hoc. As análises estatísticas foram conduzidas no software GraphPad Prism 6.0 e considerou-se um valor de $p \leq 0,05$ como significante.

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5. ARTIGO CIENTÍFICO

ANTI-NEUTROPHIL ANTIBODIES ARE ASSOCIATED WITH POOR PROGNOSIS IN HUMAN BREAST CANCER

p-ANCA presence in BC poor prognosis

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TABLE OF CONTENTS

ABSTRACT

Background: Anti-neutrophil cytoplasmic antibodies (ANCA) are capable of activating neutrophils by binding to their antigens, such as myeloperoxidase (p-ANCA), thus inducing neutrophil degranulation and respiratory bursts in sterile environments. This leads to cell and tissue damage, which may be involved in breast cancer (BC) development and progression. Methods: The presence of p-ANCA in serum and tissue samples of 150 (BC = 92, benign disease = 58) women attended in Francisco Beltrão Cancer Hospital, Paraná, Brazil, from May 2015 to December 2017. IgG p-ANCA quantification in both serum and tissue pools was carried out by the Myeloperoxidase-Ab enzyme-linked immunoassay, according to clinicopathological characteristics. Results: Higher p-ANCA levels were detected in groups presenting negative BC characteristics, such as high histological grade tumors (serum $p=0.0465$, tissue $p<0.0001$) positive lymph node invasion (serum $p<0.001$, tissue $p=0.034$) and risk factors such as BMI (tissue $p<0.001$), menopausal status (tissue $p<0.001$) and <45

years old (serum p=0.0006, tissue p<0.0001) at diagnosis. Conclusion: The presence of p-ANCA may be associated to poor prognosis BC characteristics.

Keywords: Antineutrophil antibodies, Myeloperoxidase, Breast Neoplasms, Prognosis

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INTRODUCTION

Neutrophils play key roles in host defence against extracellular invaders, such as bacteria and fungi[1], and are activated by molecules derived and associated with cell damage, recognized as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) present in several cells[2,3].

DAMPs can originate from a variety of sources, with physiological functions inside the cell and invisible to immune cells until exposed to the extracellular environment[4,5]. DAMPs are expressed not only by necrotic cells but also by cells suffering high stress under "danger"[6], with the ability to trigger sterile inflammation[7]. DAMPs activate neutrophils under recruitment to tissue injury[8,9], leading to the release of pro-inflammatory molecules, including myeloperoxidase (MPO), an enzyme present in neutrophil azurophilic granules, responsible for the production of toxic reactive species (RS), such as hypoclorous acid ($HOCl^{\bullet}$)[10–12]. DAMP overproduction contributes to autoimmune and inflammatory diseases, from Alzheimer's disease to cancer[6,13–15].

MPO release into the inflammatory milieu by neutrophil degranulation results in protein and DNA damage, favouring the inflammatory environment[16,17]. In addition, MPO is a key molecule associated to the formation of neutrophil extracellular traps (NETs)[18,19] during a process called NETosis, generating a web-like structure composed of chromatin and histones derived from the nucleus, along with granule proteins[20].

Increased NET formation is associated to the pathogenesis of chronic diseases, due to their ability to cause tissue damage, thrombosis, regulate inflammatory cytokines and favour the production of autoantibodies[20,21], such as anti-neutrophil cytoplasmic antibodies (ANCAs)[22,23]. ANCAs are categorized as either cytoplasmic, when specific for proteinase 3 (c-ANCA), or perinuclear, when produced against MPO (p-ANCA or MPO-ANCA)[24]. p-ANCAs are capable of activating neutrophils and promoting their degranulation and NET formation, even in sterile environments[25,26], and are associated with vasculitis and correlated

diseases[27–32]. Furthermore, p-ANCAs are capable of producing a feedback loop through neutrophil activation, promoting antigen access for auto-antibodies[30,33,34].

NETs are associated with poorer oncological tumor outcomes35, due to the capability of cancer cells to promote NET formation[36], enhancing distant metastasis[37–39], thrombosis[40], and recent recurrence, by awakening dormant tumour cells through inflammation[41]. In this context, no information regarding the role of p-ANCAs in the prognosis of breast cancer or other solid tumours has been reported in the literature. Therefore, determining the presence of circulating and tissue p-ANCA in breast cancer patients and investigating their relationship with disease prognosis may aid in the understanding their role in cancer cases.

MATERIALS AND METHODS

One hundred and fifty women were enrolled in this prospective study, approved by the Unioeste Ethics Committee under number CAAE 5524814.4.0000.0107, in accordance to the Declaration of Helsinki. All patients included in the study signed written informed consent forms. This study was carried out on patients attending the Francisco Beltrão Cancer Hospital (Ceonc), Parana, Brazil, from May 2015 to December 2017. Patients did not receive any treatment prior to sample collection.

Tissue samples were surgically collected, while blood was collected by peripheral venous punction. Breast tissue and blood samples were obtained from patients diagnosed with either breast cancer (n = 92) or benign lesions (n = 58). Clinicopathological data were obtained through medical records and are detailed in Table 1 (breast cancer patients) and Table 2 (benign lesions). Blood samples were centrifuged at 4,000 rpm for five minutes, and subsequently stored at -20°C until analysis. Biopsies were directly frozen at -20°C until analysis.

IgG p-ANCA quantification in both serum and tissue pools (pooled serum n=20 and tissue n=10 each) were performed by enzyme-linked immunoassays (Kit Myeloperoxidase-Ab ELISA, IBL International, RE75601), according to the following clinicopathological characteristics: biopsy diagnosis (ductal breast carcinoma or benign breast disease), age at diagnosis (under or over 45 years old), menopausal status at diagnosis, body mass index (BMI) (eutrophic, overweight and obese), tumour molecular subtype (accordingly to the Saint Gallen Consensus[42]), lymph node invasion, tumour histological grade and risk stratification. After a data normality assessment, the results were compared by t Student's test, Mann-Whitney test or a one-way ANOVA with Bonferroni's post-hoc test. Serum and tissue results were not compared between each other. The statistical analyses were carried out using the GraphPad Prism 6.0 and $p \leq 0.05$ was considered significant.

RESULTS

Figure 1A displays the p-ANCA levels regarding biopsy diagnosis. Patients presenting in situ carcinoma exhibited higher circulating p-ANCA levels (5.92±0.29 UI/mL) compared to controls (5,06±0,29 UI/mL, p=0.05), benign disease (2,62±0,23 UI/mL, p=0.0029) and invasive breast tumours groups (0,52±0,29 UI/ mL, p<0.001). p-ANCA tissue levels were increased in benign disease samples (21,28±0,63 UI/mL) compared to invasive breast tumours (17,22±0,61 UI/mL, p=0.0103), in situ breast carcinomas (13,24±0,62 UI/mL, p=0.0008) and control (8,10±0,58 UI/mL, p=0.0001).

Figure 1B presents comparisons according to breast tumour molecular subtypes in serum samples. Luminal A group (12,22±0,61 UI/mL) and triple negative (11,01±0,57 UI/mL) group presented significantly higher p-ANCA levels when compared to the luminal B (6,06±0,58 UI/mL, p=0.0019 for LumA x LumB and p=0.0038 for LumB x TN), luminal-HER (5,21±0,58 UI/mL, p=0.0011 for LumA x Lum-HER and p = 0.0020 for Lum-HER x TN) and HER2 (4,84±0,44 UI/mL, p= 0.0006 for LumA x HER2 and p= 0.0011 for TN x HER2) groups. Concerning tissue analysis (Figure 1C), significantly higher p-ANCA levels were found in the luminal-HER group (17,80±0,11 UI/mL) when compared to the triple negative (9,01±0,11 UI/mL, p<0.001), HER2 (6,78±0,11 UI/mL, p<0.001), luminal A (5,81±0,11 UI/mL, p<0.001) and luminal B (4,32±0,11 UI/mL, p<0.0001) groups.

Higher p-ANCA levels detected in the serum of patients presenting high-grade tumors when compared to those with low/intermediate grade cancers (Figure 2, 17,03±1,15 UI/mL for high-grade and 13,27±0,63 UI/mL for low/intermediate, p=0.0465). Similar results were observed when analysing tissue samples (9,51±0,28 UI/mL detected in high-grade tumours and 2,52±0,28 UI/mL in low/intermediate tumors, p<0.0001).

Regarding lymph node invasion (Figure 3), higher p-ANCA levels were detected in the serum of patients with positive lymph node invasions when compared to negative lymph node invasion (1,62±0,06 UI/mL and 0,25±0,02 UI/mL, respectively at, p<0.001). The same pattern was observed for tissue, (6,08±0,58 UI/mL for the positive group and 1,97±0,31 UI/mL for the negative group, at p=0.034).

According to the BMI classification (Figure 4A), p-ANCA levels were higher in the serum of eutrophic patients in relation to overweight and obese individuals (7,05±0,58 UI/mL for the eutrophic group, 3,21±0,61 UI/mL for the overweight group and 2,00±0,57 UI/mL for the obese group, p=0.0105 for eutrophic x overweight and p= 0.035 for eutrophic x obese), while the overweight and obese groups did not differ between each other. Regarding tissue, however, the overweight group presented significantly different p-ANCA levels

when compared to both the eutrophic and obese groups (22,71±0,11 UI/mL in the overweight group , 4,82±0,11 UI/mL in the eutrophic group and 6,62±0,11 UI/mL in the obese group, $p<0.001$ for overweight x eutrophic and $p<0.001$ for overweight x obese).

Figures 4B and 4C displays the results according to the age at diagnosis and menopausal status of the assessed breast cancer patients respectively. Higher p-ANCA levels were detected in the serum of the younger individuals (<45 years old) when compared to the older group (>45 years) (4,30±0,11 UI/mL and 3,12±0,03 UI/mL, $p=0.0006$, respectively). The tissue analysis also indicated higher p-ANCA levels in the younger group (24,26±0,63 UI/mL in younger group and 2,63±0,68 UI/mL in the older group, $p<0.0001$). Regarding menopausal status, higher p-ANCA levels in the serum of the menopausal group were detected compared to the non-menopausal group (12,00±0,57 UI/mL and 5,76±0,40 UI/mL, $p<0.0009$, respectively). The opposite was observed for tissue, where menopausal patients presented reduced p-ANCA levels (8,92±0,11 UI/mL) compared to non-menopausal patients (23,02±0,57 UI/ml, $p<0.001$).

Figure 5 displays the results according to patients risk stratification. High p-ANCA levels were detected in serum samples from the high-risk group (11,95±0,57 UI/mL) compared to the intermediate-risk (9,07±0,29 UI/mL, $p=0.0116$) and low-risk (6,94±0,26 UI/mL, $p=0.0014$) groups. Regarding tissue, the intermediate-risk group had displayed higher p-ANCA levels (19,23±0,62 UI/mL) compared to both the high-risk (11,06±0,58 UI/mL, $p=0.0007$) and low-risk groups (4,76±0,14 UI/mL, $p<0.001$).

DISCUSSION

The role of ANCAs in cancer has been recently described for a wide of topographies, as well as their clinicopathological significance out of the autoimmunity context. To the best of our knowledge, this is the first study to demonstrate the presence of anti-neutrophil antibodies in patients with breast cancer, as well as in both blood and normal/tumor breast tissue samples, and point out its clinicopathological significance. The data reported herein indicate that p-ANCAs are differentially expressed under poor prognosis conditions, such as in the case of triple negative tumor subtypes, histological high-grade tumor, lymph node invasion and patients presenting a high-risk of recurrence. Additionally, significant variations regarding age at diagnosis, BMI and menopausal status, crucial risk factors for both breast cancer development and progression, were also noted.

The mechanism of action of p-ANCAs during the immune response include the activation of cytokine-primed neutrophils and monocytes, leading to cellular damage and inducing the activation of the complement pathway, creating a positive feedback loop[25,43]. This process results in oxidative burst, degranulation and

NETosis, which leads to inflammation amplification and significant damage to endothelial cells[21,44]. In addition, tumor infiltrated neutrophils (TAN) can play a dual role in breast cancer development and progression, as discussed ahead.

The data reported herein indicate that patients with breast cancer present both circulating and tissue ANCA levels, at distinct levels according to clinicopathological categorization, suggesting that these molecules are produced in this pathology and may have some prognostic meaning.

The first analysis aimed to compare p-ANCA levels between normal breast tissue, benign breast tumors, in situ breast carcinoma and invasive breast cancer cases (Fig 1A). The lowest p-ANCA levels were detected in the the plasma of patients presenting invasive cancers when compared to the other assessed conditions.

p-ANCA levels in tissue were higher than in the blood, suggesting a local production of this antibody. The highest levels were observed in benign tumors, while in situ carcinomas presented reduced levels, and invasive carcinomas increased levels, suggesting that, while in situ, cancer may escape immunosurveillance. In situ breast carcinomas are reported as high-leukocyte density tissues[45], suggesting that these cells may play a role in breast cancer progression. When they begin their invasion progression, antigens that might activate immune response are then exposed. However, the disease progress, in spite of this antigen unmasking, suggests the existence of mediators that supress local immune responses[46]. These findings reinforce the fact that leukocytes favour tumor progression in breast tissue, and that the presence of p-ANCAs reported herein suggests that neutrophils can play a role in this network. Furthermore, the reduced p-ANCA levels observed in the serum of patients presenting invasive breast cancer may be a reflection of the reduced recognition of cancer antigens resulting from systemic immunosuppressive stimuli derived from the local inflammatory milieu.

Augmented MPO levels have been reported in benign colorectal carcinoma cases when compared to both malignant adenomas and normal mucosa, increasing significantly throughout carcinogenesis[47]. MPO can act on leukocytes and stimulate the release of pro-inflammatory cytokines, such as IL-8 and TNF-α[48], facilitating metastasis[49]. Concerning breast cancer, neutrophilic infiltrates have been reported in both in situ and invasive carcinomas compared to normal breast tissue, suggesting that the in situ transition to invasive tumor is the most critical progression step, requiring immunoediting mechanisms displaying significant differences between the in situ and invasive disease[46].

Therefore, since p-ANCAs in both plasma and breast tissue were detected, the following step comprised an assessment if different breast cancer subtypes could exhibit distinct p-ANCA expression patterns.

In this context, the data indicate that patients presenting the luminal A and triple negative subtypes exhibited the highest levels of circulating p-ANCAs (Fig. 1B). Tissue analysis (Fig. 1C) demonstrated that p-ANCA levels were differentially expressed among subtypes, highlighting Luminal-HER2 and triple negative as those displaying the highest levels.

Particularly concerning the triple negative subtype, the prevalence of neutrophils in blood and their infiltration in breast cancer tissue seems to be strongly linked to poorer clinical outcomes. Almost 90% of these cancers are infiltrated by TAN[50], and present NETs inside[38]. It is possible that, in this case, p-ANCAs will be formed against NETs. Another observation regarding triple negatives is that high neutrophil to lymphocyte ratios have been reported in their bloodstream, which have been pointed as a poor prognosis with regard to predictor parameters, such as lower 5-year disease free survival[51], overall survival[52] and disease recurrence rates[53]. Furthermore, triple negative breast cancers are usually composed of highly undifferentiated cells. Tumor histological grade (Fig. 2) are a pivotal parameter that reflects cancer aggressiveness, since undifferentiated cells can easily metastasize. The patients assessed herein presenting high-grade tumors displayed increased levels of p-ANCA in blood when compared to those with low-grade breast cancer. In the same way, the tissue analysis revealed that high-grade breast cancer cases exhibited higher p-ANCA levels in comparison to low-grade tumors. These findings indicate that p-ANCAs may play a role in the development of triple negative breast cancers.

Lymph node status is another determinant condition for the prognosis of breast cancer cases. The data indicate (Fig. 3) that patients with lymph node invasion presented higher p-ANCA levels in both plasma and breast cancer tissue. Neutrophils can enter lymphoid organs and modulate immune responses, while MPO is able to inhibit dendritic cells (DCs) activation and proliferation by inhibiting CD86, MHC-II and CCR7 expression, decreasing CD4+ T cell activity and antigen responses[10,54]. This inhibition occurs mainly through the formation of $HOCl^{\bullet}$ and hypobromous acid ($HOBr^{\bullet}$), both powerful oxidants[55]. Furthermore, p-ANCAs may also be able to stimulate MPO release from infiltrated neutrophils inside lymph nodes by the same mechanism, suppressing cytokines production, including IL-12 and IL-23, responsible for T cell activation and differentiation into Th1 and Th17, decreasing the release of IL-17A and IFNγ[55,56]. Furthermore, IL-12 and IFNγ are also responsible for NK cell activation, so this response is, thus, impaired by DC inhibition[57]. TNF-α, IL-8 and IL-6 have been reported as increased in patients presenting lymph node metastasis[49], known as potent neutrophil chemotactic factors and activators, especially IL-8, frequently produced by tumor cells[58].

The p-ANCA analysis regarding breast cancer-related risk factors also demonstrated interesting results (Fig. 4). Obese women (Fig. 4A) presented the lowest p-ANCA levels in blood, while overweight patients displayed the highest levels in breast cancer tissue. Adipose tissue induces proinflammatory reactions by immune cells, associated with the development of dyslipidaemia and insulin resistance[59]. As in classical immune responses, neutrophils infiltrate early inflammation stages due to cytokines produced by adipose tissue, such as IL-6, IL-8 and TNF-α[17,60]. This process induces neutrophil activation and MPO release through NET formation and degranulation[61], creating a self-amplifying cascade and, thus, sustaining the sterile inflammatory process, increasing NF-κB signalling and TNF-α production by macrophages[17,62].

Younger women with breast cancer presented higher p-ANCA levels in both blood and tissue (Fig 4B). Elderly individuals, on the other hand, present impaired innate immune system activity, such as a lower ability to present antigens and diminished NET release by neutrophils, in turn lowering circulating inflammation[63]. These results could be related to higher neutrophil invasion and NET formation in young/middle aged patients, which are associated to a migratory phenotype and characterized by local angiogenesis and tumour survival and progression, as reviewed by some authors[21,64].

Regarding patient menopausal status (Fig. 4C), an inverse relationship concerning p-ANCA levels was observed, where non-menopausal women presented reduced ANCAs in plasma and increased levels in breast cancer tissue. In this context, a recent study[66] has demonstrated that estrogen exacerbates mammary inflammation during breast involution by recruiting and reprograming neutrophils, transforming them into pro-tumoral cells. Thus, high ANCA levels in breast cancer tissue could represent this neutrophil deregulation, which may then secrete more NETs and induce the local production of anti-neutrophil antibodies. It is important to highlight that the mammary tissue can produce small amounts of estrogen by the aromatase pathway[65], which is exacerbated by excessive body fat[67], and that the patients assessed herein were mostly overweight and obese.

When considering the main prognostic factors, risk stratification can be considered a trustworthy tool to evaluate breast cancer patient prognosis. High-risk patients exhibited the highest p-ANCA levels in blood, while intermediate risk patients displayed the highest levels in breast cancer tissue (Fig. 5). These findings reinforce that poor prognosis parameters are associated with high p-ANCA levels in breast cancer patients.

The hypothesis of this study is that high MPO exposure occurs due to inflammation, attracting p-ANCA and maintaining a favourable environment for tumour malignancy. Although NET formation or neutrophil infiltration within the tumor were not assessed, it is postulated that this mechanism could explain

the findings reported herein, according to the reported results, and knowing that p-ANCA are capable of inducing neutrophil activation and its recruitment[25].

In spite of its limitations, the current study is the first to point out an initial interpretation on the clinical significance of p-ANCAs in breast cancer patients. A better understanding of the mechanistic interplay that leads the innate immune system to produce p-ANCA in breast cancer remains a challenge. A tolerance break due to a shift from non-pathogenic to pathogenic antibody production induced by changes in epitope specificity in inflamed tissue may be one of the answers to process[68].

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TABLE 1 – Clinicopathological characteristics of patients presenting breast cancer

Patients presenting Breast Cancer	N = 92
Median age at diagnosis	±55,63 years
(range)	(33 – 82)
Sample classification	
Invasive ductal carcinoma	91,30%
Ductal carcinoma *in situ*	9,69%
Histological grade	
I/II	71,73%
III	22,82%
Molecular Subtype	
Luminal A	22,08%
Luminal B	35,86%
Luminal-HER2	9,78%
HER2†	10,86%
Triple Negative	19,56%
Risk stratification	
Low	9,78%
Intermediate	55,43%
High	30,43%
Not stratified	4,34%
Lymph node invasion	
Positive	34,78%
Negative	53,26%
Unknown	11,95%
BMI‡	
Underweight	1,08%
Eutrophic	23,91%
Overweight	41,30%
Obese	27,17%
Unknown	5,43%
Menopausal at diagnosis	
Yes	64,13%
No	33,69%
Unknown	2,17%

Abbreviations: †HER2, Human Epidermal Growth Factor 2; ‡BMI, Body Mass Index

TABLE 2 - Clinicopathological characteristics of patients presenting benign disease

Patients presenting Benign Disease	N = 58
Median age at diagnosis	±44,01 years
(range)	(15 – 74)
Sample classification	
Fibroadenoma	51,72%
Cysts (controls)	48,28%
BMI†	
Eutrophic	32,75%
Overweight	22,41%
Obese	18,96%
Unknown	25,86%
Menopausal at diagnosis	
Yes	51,72%
No	43,10%
Unknown	5,17%

Abbreviations: †BMI, Body Mass Index.

Figure Legends

Figure 1 – p-ANCA levels according to the diagnosis results and molecular breast cancer subtype. In Fig. 1A, serum and tissue p-ANCA levels in differential diagnoses related to breast disease, from cystic disease to invasive carcinoma. * = significant difference from normal breast. ** = significant difference from benign lesions. *** significant difference from ductal carcinoma in situ. **** = significant difference from invasive ductal carcinoma. Circulating (Fig. 1B) and tissue (Fig. 1C) p-ANCA levels in patients with breast cancer categorized by molecular subtype. * = significant difference from Luminal A. ** = significant difference from Luminal B. *** significant difference from Luminal-HER. **** = significant difference from HER2. ***** = significant difference from Triple Negative. Results are expressed by units of p-ANCA per mL (U/mL) in both serum and tissue. The bars are representative of the mean p-ANCA levels in each pool, expressed as standard deviation. The symbol represents statistical difference ($p \leq 0.05$)

Figure 2 – p-ANCA serum and tissue levels according to breast cancer histological grade at diagnosis. Results are expressed by p-ANCA units per mL (U/mL) in both serum and tissue. The bars are representative of the mean p-ANCA levels in each pool, expressed by standard deviation. The symbol represents statistical difference ($p \leq 0.05$).

Figure 3 – p-ANCA serum and tissue levels according to lymph node status at diagnosis of breast cancer. Results are expressed by p-ANCA units per mL (U/mL) in both serum and tissue. The bars are representative of the average of p-ANCA levels in each pool, expressed as standard deviation. The symbol represents statistical difference ($p \leq 0.05$).

Figure 4 – p-ANCA serum and tissue levels determined in breast cancer patients grouped according to risk factors characteristics for breast cancer development. Fig. 4A displays circulating and tissue p-ANCA levels in groups categorized by BMI index. * = statistical difference from eutrophic. ** = statistical difference from overweight. *** = statistical difference from obese. Fig. 4B presents the analysis of serum and tissue in groups classified by age at diagnosis, and Fig. 4C indicates p-ANCA levels in groups categorized according to menopausal status. Results are expressed by p-ANCA units per mL (U/mL) in both serum and tissue. The bars are representative of the mean p-ANCA levels in each pool, expressed as standard deviation. The symbol represents statistical difference ($p \leq 0.05$).

Figure 5 – p-ANCA serum and tissue levels according to the clinical risk stratification of breast cancer patients. Results are expressed by p-ANCA units per mL (U/mL) in both serum and tissue. The bars are representative of the mean p-ANCA levels in each pool, expressed as standard deviation. The symbol represents statistical difference ($p \leq 0.05$).

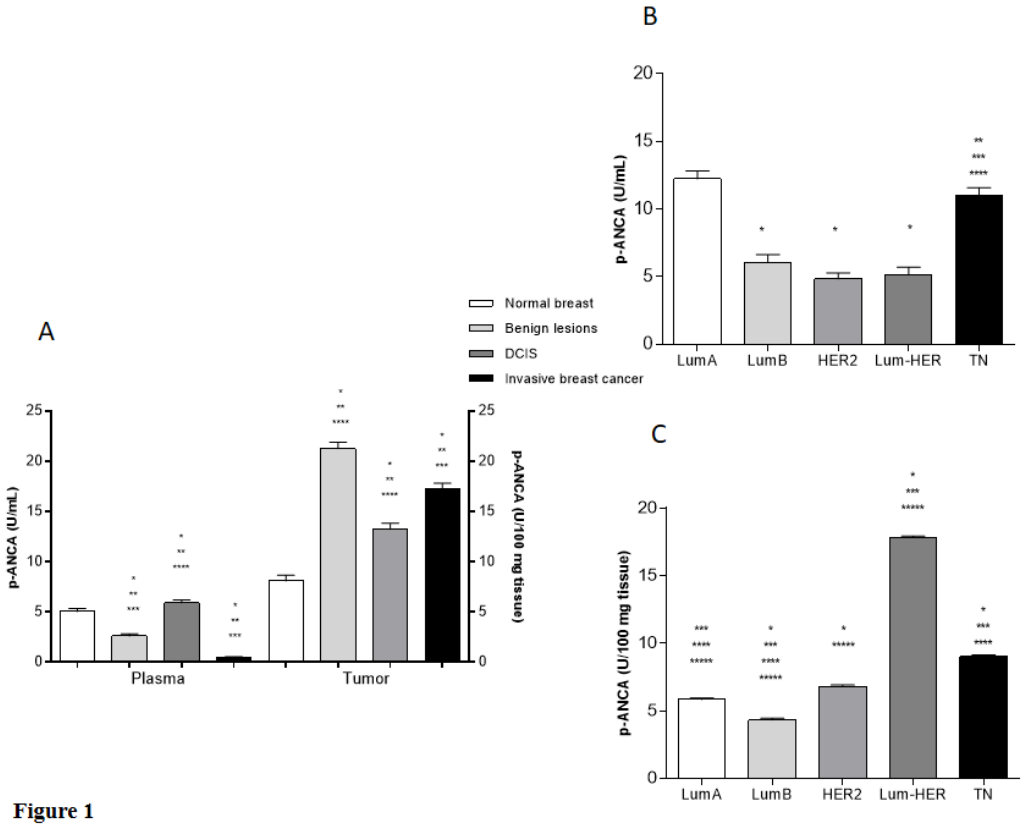

Figure 1

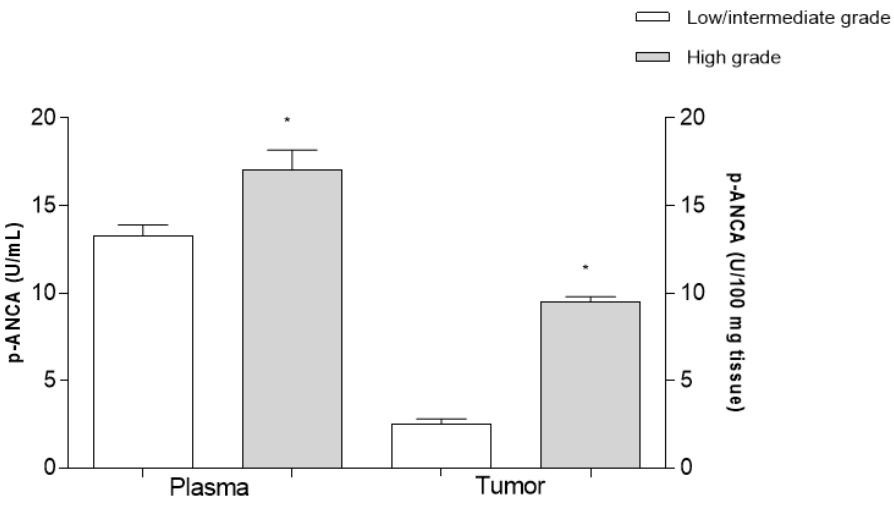

Figure 2

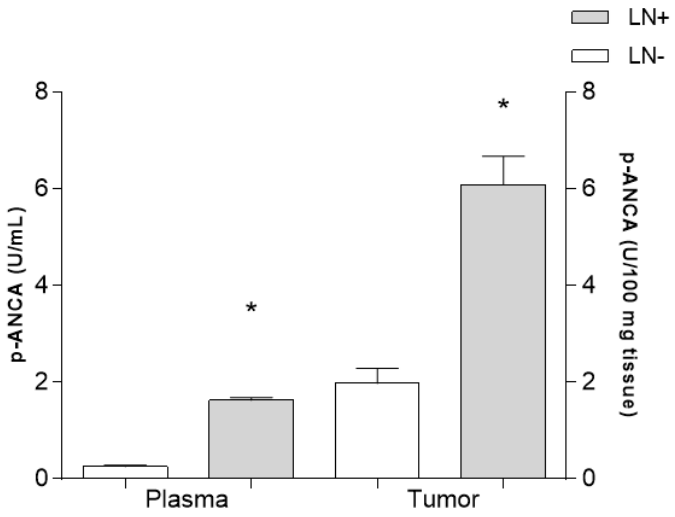

Figure 3

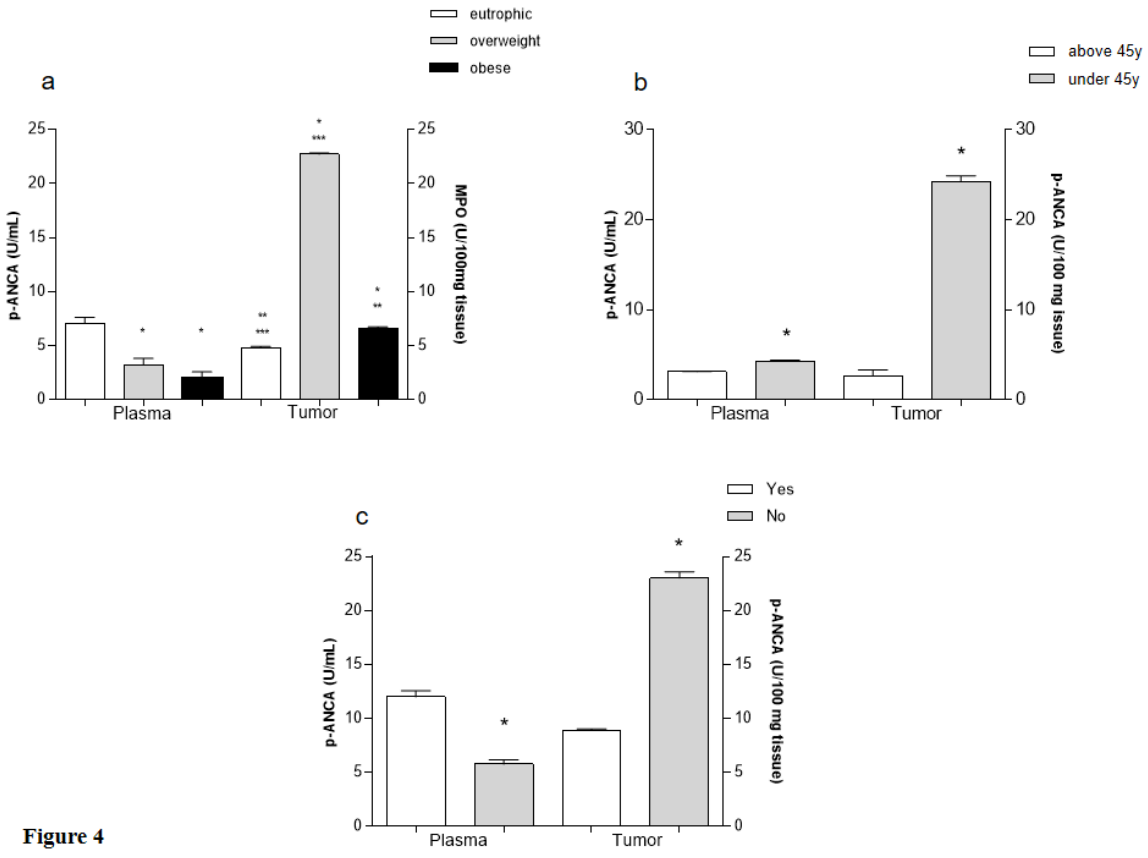

Figure 4

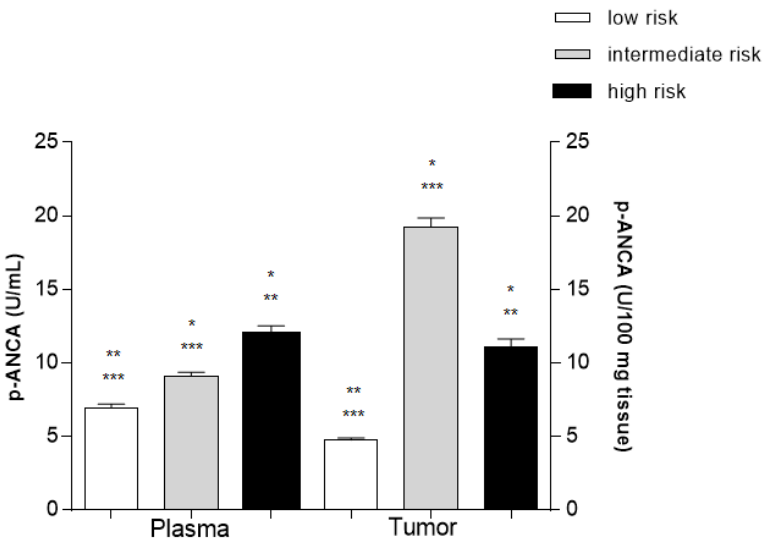
low risk
intermediate risk
high risk
p-ANCA (U/mL)
p-ANCA (U/100 mg tissue)
0
5
10
15
20
25
Plasma
Tumor

Figure 5

ANEXOS

ANEXO I – Normas da Revista *Cancer*, Ed. Wiley

Author Guidelines

Sections

1. Submission
2. Aims and Scope
3. Manuscript Categories and Requirements
4. Preparing the Submission
5. Editorial Policies and Ethical Considerations
6. Author Licensing
7. Publication Process After Acceptance
8. Post Publication
9. Editorial Office Contact Details

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vii. A conflict of interest statement that includes details of potential conflicts for all authors (or indicates that there are none);

viii. Author contributions statement (required for Original Articles only);

ix. Acknowledgments;

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xi. Abstract and keywords;

xii. Total number of each: 1) text pages (including title page(s), abstract, main text, references, and figure legends); 2) tables; 3) figures; 4) and supporting files for publication;

xiii. Main text;

xiv. References;

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Quality improvement studies: SQUIRE

Economic evaluations: CHEERS

Animal pre-clinical studies: ARRIVE

Study protocols: SPIRIT

Clinical practice guidelines: AGREE

We also encourage authors to refer to and follow guidelines from:

- Future of Research Communications and e-Scholarship (FORCE11)
- National Research Council's Institute for Laboratory Animal Research guidelines
- The Gold Standard Publication Checklist from Hooijmans and colleagues
- Minimum Information Guidelines from Diverse Bioscience Communities (MIBBI) website
- FAIRsharing website

Species Names

Upon its first use in the title, abstract, and text, the common name of a species should be followed by the scientific name (genus, species, and authority) in parentheses. For well-known species, however, scientific names may be omitted

from article titles. If no common name exists in English, only the scientific name should be used.

Genetic Nomenclature

Sequence variants should be described in the text and tables using both DNA and protein designations whenever appropriate. Sequence variant nomenclature must follow the current HGVS guidelines; seevarnomen.hgvs.org, where examples of acceptable nomenclature are provided.

Sequence Data

Nucleotide sequence data can be submitted in electronic form to any of the three major collaborative databases: DDBJ, EMBL, or GenBank. It is only necessary to submit to one database as data are exchanged between DDBJ, EMBL, and GenBank on a daily basis. The suggested wording for referring to accession-number information is: 'These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number U12345'. Addresses are as follows:

DNA Data Bank of Japan (DDBJ): www.ddbj.nig.ac.jp

EMBL Nucleotide Archive: ebi.ac.uk/ena

GenBank: www.ncbi.nlm.nih.gov/genbank

Proteins sequence data should be submitted to either of the following repositories:

Protein Information Resource (PIR): pir.georgetown.edu

SWISS-PROT: expasy.ch/sprot/sprot-top

Cell Line Authentication

To ensure the highest standards of quality and accuracy, Cancer strongly encourages the authentication of cell lines used in the research submitted to the journal. Please include the RRID (see Resource Identification Initiative above) for each cell line used. In addition, manuscripts based on research using cell lines must include a statement addressing the following points in the Methods section of the manuscript:

1. Where the cells were obtained from
2. Whether the cell lines have been tested and authenticated
3. The method by which the cells were tested

If cells were obtained directly from a cell bank that performs cell line characterizations and passaged in the user's laboratory for fewer than 6 months

after receipt or resuscitation, re-authorization is not required. In these cases, please include the method of characterization used by the cell bank. If the cell lines were obtained from an alternate source, authors must provide authentication of the origin and identity of the cells. This is best achieved by DNA (STR) profiling. The DNA profile should be cross-checked with the DNA profile of the donor tissue (in case of a new cell line) or with the DNA profile of other continuous cell lines.

Biomarker Papers

For papers that report new biomarkers or algorithms, authors should identify the unmet clinical need that the diagnostic test could fill. Data obtained with a discovery ("learning") set of specimens should be confirmed with a separate validation set. Statistical analysis should define limits on sensitivity, specificity, positive predictive value, and negative predictive value. Sufficient detail should be provided regarding the clinical characteristics of the donors of the assayed specimens to be certain that they are representative of the population in which an unmet need is to be targeted. Similarly, methods for assay and analysis should be presented in sufficient detail to permit replication by other investigators.

Statistical Analysis

The following guidelines should be followed:

1. All statistical methods should be described in the Methods section of the manuscript.
2. Assignment of individuals/patients to groups should be clearly described (e.g. randomized assignment, and any stratification factors; self-selection).
3. Report the sample size (n) for each study and each group/subgroup for each analysis presented.
4. Provide a sample size justification, such as a power calculation, if appropriate.
5. Describe all statistical methods, including approaches for verifying or testing assumptions for statistical methods.
6. Report descriptive statistics for center (e.g., mean, median) and dispersion (e.g. range, IQR, standard deviation) for all continuous variables.
7. Graphical displays should include individual data points for studies with small sample sizes per group (<10 per group).
8. Report n and the sample proportion for binary variables.

9. For hypothesis tests, provide alpha (the probability of a Type I error); specify whether tests are one- or two-sided.
10. Adequately explain more sophisticated statistical procedures such a multivariable logistic regression, Cox proportional hazard regression model, multivariate modeling, and verify the assumptions of each such procedure.
11. Confidence intervals should be reported for parameter estimates. P values (reported to two significant digits) may also be reported. Explain what is meant by statistical significance. For studies that use Bayesian statistics, report analogous results (e.g. credible intervals; tail probabilities) using the same guidance.
12. When reporting P values, include the estimated clinical effect. That is, include the point estimate of the parameter that is being tested recognizing that clinical and statistical significance are both important.
13. Discuss and describe adjustments (or rational for lack of adjustments) for multiple testing.

Conflict of Interest

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2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

In addition to being accountable for the parts of the work he or she has done, an author should be able to identify which co-authors are responsible for specific other parts of the work. In addition, authors should have confidence in the integrity of the contributions of their co-authors.

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2. When specified authors assume responsibility for an entire group (eg, Jane E. Doe, John L. Smith, Mark F. Jones for the Pediatric Oncology Group), only the specified authors must meet the criteria for authorship previously outlined. All members of the group may be listed in a footnote but are not acknowledged as authors. In this case, the corresponding author must state in the cover letter that she/he has written permission from each group member to list her/his name as a member of the group.

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The list of contributions should use the following format:

AUTHOR CONTRIBUTIONS

Author Full Name: List of contributions.

Sample Author Contribution Section

Author Contributions: John Smith: Conceptualization, formal analysis, funding acquisition, software, and writing – review and editing. Sarah Jones: Data

curation, methodology, project administration, writing – original draft, and writing – review and editing.

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The journal encourages authors to share the data and other artefacts supporting the results in the paper by archiving it in an appropriate public repository. Authors should include a data accessibility statement, including a link to the repository they have used, in order that this statement can be published alongside their paper.

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Authors who discuss their work with the media in the week prior to publication must ensure that the media representatives know the embargo policy and the embargo date.

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• Please do not distribute copies of the manuscript, tables, or figures.

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250 Williams Street NW
Atlanta, Georgia 30303 USA
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ANEXO II – Comprovante de Submissão

CNCR-19-0354 Submitted to Cancer

Cancer <onbehalfof@manuscriptcentral.com>
11:07

Para: thalitascandolara@hotmail.com; lytomita@gmail.com; carlasilvajanaina.nutri@gmail.com; elaineminattidias@hotmail.com; jessica.malanow...

05-Feb-2019

Dear Dr. PANIS,

Thank you for your interest in Cancer. Your manuscript entitled "ANTI-NEUTROPHIL ANTIBODIES ARE ASSOCIATED WITH POOR PROGNOSIS IN HUMAN BREAST CANCER" has been successfully submitted online and is presently being given full consideration for publication.

Your manuscript number is CNCR-19-0354. Please mention this number in all future correspondence regarding this submission.

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ANEXO III – 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 IV – Comprovante do Comitê de Ética

UNIOESTE - CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE DA UNIVERSIDADE

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

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2. Readequação do título para: Mapeamento do câncer de mama e estudo de associação de risco com a exposição ocupacional aos agrotóxicos no Paraná: mecanismos moleculares e implicações clínicas - ACATADO

3. No item metodologia, incluir que será realizada a coleta de 20 mL de amostra de urina, tanto das pacientes voluntárias como de membros de sua família (caso a mesma seja exposta aos agrotóxicos). Tal coleta será realizada pelo próprio indivíduo, através de micção espontânea em frasco plástico, após assinatura do TCLE modificado em anexo - ACATADO

4. Inclusão do instrumento de coleta para investigação da exposição aos agrotóxicos, composto das questões em anexo (Instrumento de coleta de dados – Agrotóxicos) - ACATADO

5. Etapa de validação do estudo: Inclusão da análise de material de biópsias de mama e linfonodos oriundos de pacientes portadoras de câncer de mama atendidas em Londrina-PR. Este material é composto de blocos de parafina e encontra-se arquivado sob guarda do laboratório Logos, que irá colaborar no projeto (declaração em anexo) - ACATADO

Considerações sobre os Termos de apresentação obrigatória:
Todos os termos foram anexados, incluindo a modificação do TCLE e o termos de aprovação do responsável pelo campo de estudo - Laboratório Logos.

Recomendações:
Não há

Conclusões ou Pendências e Lista de Inadequações:
Todas as modificações solicitadas pela pesquisadora estão fundamentadas e foram acrescidas aos projeto integral, assim como anexados documentos referentes às respectivas modificações quanto ao instrumento de coleta, TCLE e termo assinado pelo responsável pelo laboratório Logos.

Assim, não há pendências e a pesquisadora poderá dar seguimento, estando de acordo com as normas regulamentadoras de pesquisas envolvendo seres humanos.

Considerações Finais a critério do CEP:

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Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_978825_E2.pdf	18/08/2017 16:55:42		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_modificado.pdf	18/08/2017 16:54:28	CAROLINA PANIS	Aceito
Projeto Detalhado / Brochura Investigador	brochura_pesquisador_modificada.pdf	18/08/2017 16:54:05	CAROLINA PANIS	Aceito
Recurso Anexado pelo Pesquisador	Solicita_emendas.pdf	18/08/2017 16:37:40	CAROLINA PANIS	Aceito
Declaração do Patrocinador	resultado_ppsus_aprovacao.pdf	18/08/2017 16:37:26	CAROLINA PANIS	Aceito
Declaração de Pesquisadores	declaracao_Logos.pdf	18/08/2017 16:35:52	CAROLINA PANIS	Aceito
Outros	Instrumento_de_coleta_de_dados.pdf	18/08/2017 16:34:52	CAROLINA PANIS	Aceito
Folha de Rosto	folha_de_rosto_assinada.pdf	18/08/2017 16:32:07	CAROLINA PANIS	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CASCAVEL, 02 de Outubro de 2017

Assinado por:
Fausto José da Fonseca Zamboni
(Coordenador)

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