AEDRA CARLA BUFALO KAWASSAKI

EXPOSIÇÃO AOS AGROTÓXICOS E NÍVEIS PLASMÁTICOS DE CORTISOL, CITOCINAS E PERFIL REDOX EM PACIENTES COM CÂNCER DE MAMA

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

Orientador: Prof. Dr. Wander R. Pavanelli
Co-orientadora: Profa. Dra. Carolina Panis

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"O que importa não é a jornada, nem o final, mas no que nos transformamos no percurso".

Autor desconhecido

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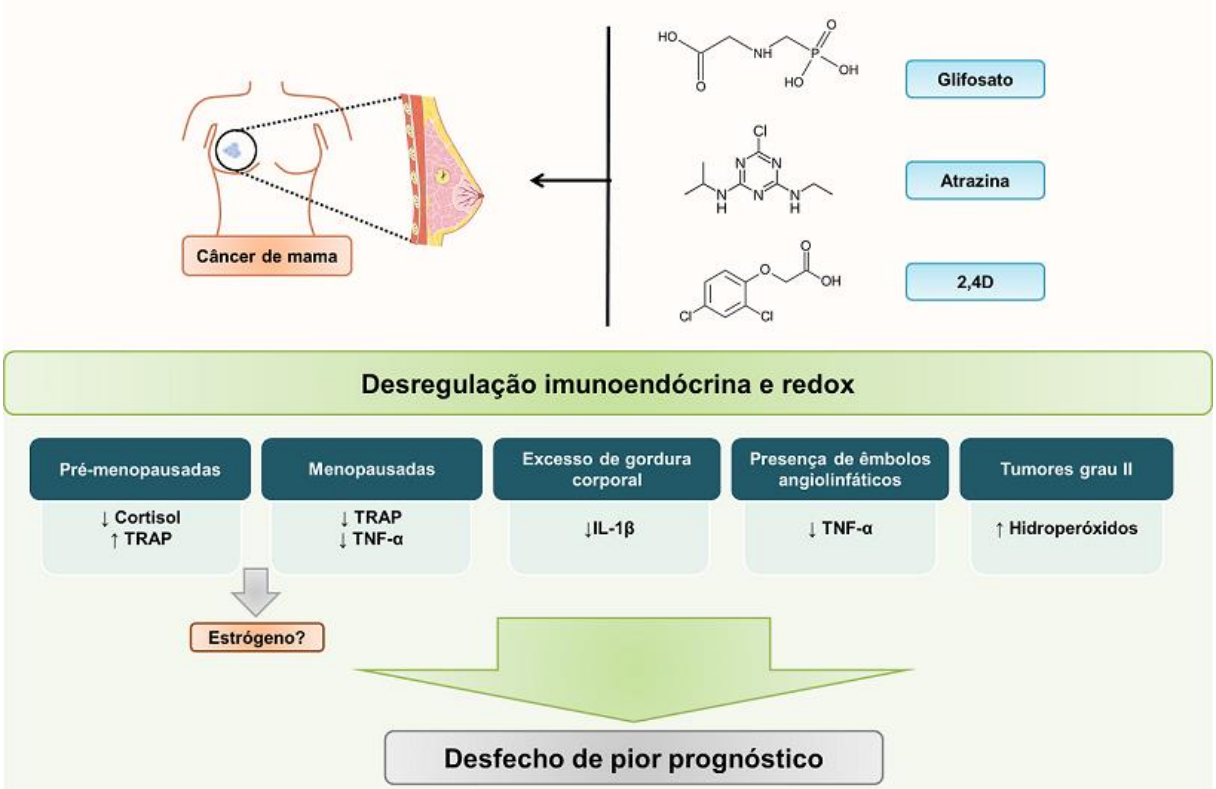
RESUMO

O câncer de mama é conhecido pela sua alta incidência entre as mulheres no mundo e muito já se conhece sobre os diversos fatores envolvidos no seu surgimento e desenvolvimento. Entre os fatores ambientais, os agrotóxicos vêm ganhando destaque por se mostrarem capazes de atuar na progressão tumoral e gerar quadros de pior prognóstico. Vários mecanismos de lesão ao DNA podem estar envolvidos, mas, pouco se sabe sobre os efeitos dos pesticidas nos níveis plasmáticos de citocinas pró-inflamatórias, hormônios e substâncias envolvidas com o perfil redox, as quais são importantes no contexto da carcinogênese, e, a relação destas substâncias com características prognósticas importantes no câncer de mama. Além disso, fatores relacionados ao estilo de vida das pacientes, como a obesidade, também parecem contribuir para um desfecho de pior prognóstico, principalmente pela existência de um ambiente pró-inflamatório de baixo grau crônico instalado, o qual promove alterações hormonais importantes, como no eixo do cortisol. Assim este trabalho teve como objetivos: avaliar se mulheres portadoras de câncer de mama expostas cronicamente aos agrotóxicos apresentam relação entre desregulação imunoendócrina e desbalanço redox com desfechos de pior prognóstico e, analisar a correlação dos níveis de cortisol sistêmico e parâmetros de pior prognóstico em pacientes com câncer de mama, levando em consideração a categorização das pacientes de acordo com IMC (Índice de Massa Corpórea). A população de estudo foram mulheres com indicativo de câncer de mama atendidas no Hospital do Câncer de Francisco Beltrão, Paraná, Brasil, entre os períodos de 2015 e 2017. Durante a cirurgia para retirada de material para biópsia, no período das 14 às 17 horas, amostras de sangue (10 mL) foram coletadas assim como, das pacientes com diagnóstico positivo para câncer de mama também foram coletadas características clinicopatológicas dos prontuários médicos. Todos os dados foram analisados através do Graph Pad Prism 7.0 e SPSS Statistic 20.0, considerando significância estatística $p \leq 0,05$. Para demonstrar se há relação dos agrotóxicos com marcadores endócrinos, imunológicos e de estresse oxidativo, iniciamente as pacientes (n=73), que responderam a um questionário sobre exposição direta aos agrotóxicos, foram categorizadas como expostas ou não-expostas. Das amostras de sangue destas pacientes foram dosados: cortisol, citocinas (TNF-α e IL-1β) e o perfil redox (hidroperóxidos, NO e TRAP), para posterior correlação com parâmetros clinicopatológicos. Observamos que os níveis de TNF-α, cortisol e TRAP variaram em relação ao status menopausal das pacientes. Os níveis de TNF-α e TRAP foram aumentados nas pacientes menopausadas não expostas aos pesticidas quando comparadas às expostas (p=0,0443 e p=0,0488, respectivamente). Já nas pacientes na pré-menopausa, estas apresentaram um aumento significativo nos níveis de cortisol no grupo não exposto em relação aos expostos (p=0,0262). Ao contrário, o TRAP nas mulheres na pré-menopausa expostas apresentaram níveis aumentados em comparação com as não expostas (p=0,0006). Além disso, as pacientes não expostas portadoras de tumores de grau 2 apresentaram níveis reduzidos de hidroperóxidos quando comparadas as pacientes expostas do mesmo grau (p=0,0488). Outra diferença observada foi à redução dos níveis de TNF-α nas pacientes expostas quando comparadas as não expostas, em relação a presença de êmbolos angiolinfáticos (p = 0,0128). Em relação a IL-1β, pacientes obesas não expostas apresentaram níveis aumentados em relação às expostas aos pesticidas (p=0,0247). Desta forma, podemos concluir que a exposição crônica aos pesticidas em mulheres com câncer de

mama pode contribuir com desfechos de pior prognóstico, em decorrência de desregulação imunoendócrina e redox, influenciada principalmente pelo status menopausal das pacientes. Em seguida, foi analisado se o nível de cortisol plasmático está associado a parâmetros de pior prognóstico quando as pacientes são categorizadas de acordo com o IMC (n=115), sem levar em consideração a exposição aos agrotóxicos. Observou-se que o cortisol sistêmico foi significativamente aumentado nas pacientes com câncer de mama obesas em relação às eutróficas (p=0,051). Pacientes obesas apresentaram cortisol elevado em associação com tumor Luminal B em relação às eutróficas (p=0,0211) e as sobrepesos (p=0,0191), enquanto as sobrepesos apresentaram níveis elevados de cortisol em associação com tumores Triplo Negativo em relação às eutróficas (p=0,0310) e as obesas (p=0,0296). Além disso, a presença de metástase linfonodal, de êmbolos angiolinfáticos e de tumores de alto grau foi relacionada com níveis aumentados de cortisol sérico em pacientes com excesso de peso corporal. Assim, IMC elevado parece ter um efeito negativo sobre o câncer de mama, levando em consideração os níveis de cortisol sérico e parâmetros de pior prognóstico.

Palavras-chave: Câncer de mama. Pesticidas. Desregulação imunoendócrina. Balanço redox. Antioxidante. Estresse oxidativo. Prognóstico. Excesso de peso.

RESUMO GRÁFICO



KAWASSAKI, Aedra C.B. **Exposition to pesticides and plasma levels of cortisol, cytokines and redox profile in patients with breast cancer**. 2019. 105 f. Teshis. Universidade Estadual de Londrina, Londrina. 2019.

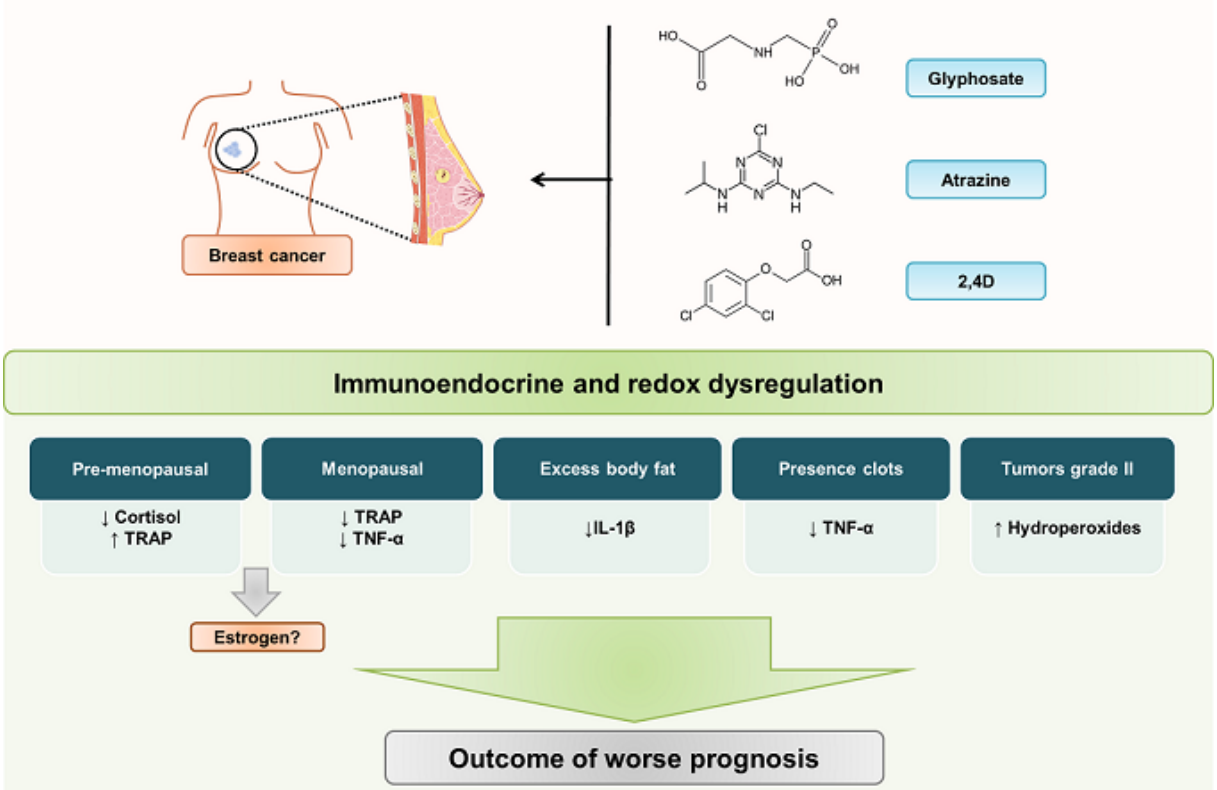
ABSTRACT

Breast cancer is known for its high incidence among women in the world and much is already known about the various factors involved in its emergence and development. Among the environmental factors, the pesticides have been gaining prominence because they are able to act in the tumor progression and generate pictures of worse prognosis. Several mechanisms of DNA damage may be involved, but little is known about the effects of pesticides on the plasma levels of proinflammatory cytokines, hormones and substances involved with the redox profile, which are important in the context of carcinogenesis, and the of these substances with important prognostic features in breast cancer. In addition, factors related to patients' lifestyle, such as obesity, also seem to contribute to a worse prognosis outcome, mainly due to the existence of a chronic low-level pro-inflammatory environment, which promotes important hormonal changes, such as cortisol axis. The objective of this study was to evaluate whether women with breast cancer who are chronically exposed to pesticides present a relationship between immunoendocrine dysregulation and redox imbalance with worse prognosis, and to analyze the correlation between systemic cortisol levels and worse prognostic parameters in patients with breast cancer, taking into consideration the categorization of patients according to BMI (Body Mass Index).The study population were women with indicative of breast cancer attended at the Francisco Beltrão Cancer Hospital, Paraná, Brazil, between the periods of 2015 and 2017. During the surgery to remove material for biopsy, from 2 pm to 5 pm, blood samples (10 mL) were collected, as well as the clinicopathological characteristics of the patients' medical records with positive diagnosis for breast cancer. All data were analyzed using Graph Pad Prism 7.0 and SPSS Statistic 20.0, considering statistical significance $p \leq 0.05$. In order to demonstrate the relationship between pesticides with endocrine, immunological and oxidative stress markers, patients (n = 73), who responded to a questionnaire on direct exposure to pesticides, were initially categorized as exposed or non-exposed. Of the blood samples from these patients, cortisol, cytokines (TNF-α and IL-1β) and the redox profile (hydroperoxides, NO and TRAP) were measured for posterior correlation with important clinicopathological parameters in breast cancer. We observed that levels of TNF-α, cortisol and TRAP varied in relation to the menopausal status of the patients. TNF-α and TRAP levels were increased in menopausal patients non-exposed to pesticides when compared to exposed ones ($p = 0.0443$ and $p = 0.0488$, respectively). In pre-menopausal patients, these presented a significant increase in cortisol levels in the non-exposed group compared to those exposed ($p = 0.0262$). In contrast, TRAP in exposed pre-menopausal women had increased levels compared to non-exposed ($p = 0.0006$). In addition, non-exposed patients with grade 2 tumors had reduced levels of hydroperoxides when compared to exposed patients of the same grade ($p = 0.0488$). Another difference observed was the reduction of TNF-α levels in exposed patients when compared to non-exposed patients, in relation to the presence of angiolymphatic emboli ($p = 0.0128$). Regarding IL-1β, obese non-exposed patients presented increased levels in relation to those exposed to pesticides ($p = 0.0247$). In this way, we can conclude that there is a relation between chronic exposure to pesticides in women with breast cancer and outcomes of worse prognosis, due to immunoendocrine and redox dysregulation, mainly influenced by the menopausal status of the patients. Next, it was analyzed whether the level of plasma cortisol is associated with worse

prognostic parameters when the patients are categorized according to the BMI (n = 115), without taking into account exposure to pesticides. It was observed that systemic cortisol was significantly increased in patients with obese breast cancer in relation to eutrophic (p = 0.051). Obese patients had elevated cortisol in association with Luminal B tumor in relation to eutrophic (p = 0.0211) and overweight (p = 0.0191), whereas overweight had high levels of cortisol in association with Negative Triple tumors in relation to eutrophic (p = 0.0310) and obese (p = 0.0296). In addition, the presence of lymph node metastasis, clots and high-grade tumors was related to increased levels of serum cortisol in patients with excess body weight. Thus, high BMI has a negative effect on breast cancer, taking into account serum cortisol levels and worse prognostic parameters

Keywords: Breast cancer. Pesticides. Immuneendocrine dysregulation. Redox balance. Antioxidant. Oxidative stress. Prognosis. Excess weight.

GRAPHICAL ABSTRACT



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Pesticide chronic exposition is associated with immunoendocrine deregulation and redox imbalance in women with breast cancer.

Systemic cortisol and poor prognosis in overweight/obese women with breast cancer.

LISTA DE TABELAS

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Systemic cortisol and poor prognosis in overweight/obese women with breast cancer.

LISTA DE ABREVIATURAS E SIGLAS

ADAPAR	Agência de Defesa Agropecuária do Paraná
HER2	Receptor do Fator de Crescimento Epidérmico Humano 2
HPA	Hipotálamo-Pituitária-Adrenal
IL-1β	Interleucina -1beta
IMC	Índice de Massa Corpórea
INCA	Instituto Nacional do Câncer
NF-κB	Fator Nuclear Kappa B
NO	Óxido Nítrico
ROS	Espécie Reativa de Oxigênio
TEM	Transição Epitélio-Mesenquimal
TNF-α	Fator de Necrose Tumoral - alfa
TNM	T - é relativo ao tamanho ou profundidade do tumor / N - diz respeito à presença ou ausência de metástases nos linfonodos / M - diz respeito à presença ou ausência de metástase à distância
TRAP	Capacidade Antioxidante Total do Plasma

SUMÁRIO

1 INTRODUÇÃO

1.1 CÂNCER DE MAMA: ASPECTOS CLINICOPATOLÓGICOS E FISIOPATOLOGIA

O câncer de mama é a neoplasia maligna de maior incidência entre as mulheres e, do ponto de vista clínico, de diagnóstico e tratamento complexo devido a sua origem multifatorial e heterogeneidade de subtipos. No Brasil, o Instituto Nacional do Câncer (INCA) publicou que para o biênio 2018-2019, a estimativa de incidência é de 600 mil novos casos de câncer para cada ano e, excluindo o câncer de pele não melanoma que corresponde a 170 mil novos casos, os de próstata, pulmão, mama feminina, cólon e reto são os mais incidentes, sendo as regiões Sul e Sudeste as principais acometidas (70% das ocorrências). Os dados mostram que o câncer de mama no Brasil corresponde a 29,5% do número total de casos de câncer (59.700 novos casos), com risco estimado de 56,33 para cada 100.000 habitantes. Entretanto, um comparativo entre as regiões do Brasil mostra que a região Sul concentra uma grande parte destes casos (28,7% ou 11.030 novos casos) com risco estimado bem acima da média nacional de 73,07 casos para cada 100.000 habitantes (INCA, 2018) e, ainda, uma das maiores taxas de mortalidade (SOARES *et al*., 2015). Quando se observa a região Sul, o Paraná é responsável por 3.730 novos casos com risco estimado de 64,70 para cada 100.000 habitantes (INCA, 2018).

A classificação clínica desta doença refere-se principalmente a localização do tumor e sua capacidade de invasão a outros tecidos. No câncer de mama não invasivo, o tumor permanece dentro dos ductos ou lóbulos mamários. Já o tipo invasivo ou infiltrante ocorre quando as células tumorais saem dos ductos e lóbulos e invadem tecidos estromais da mama. Este ainda pode ser sub-classificado em: carcinoma ductal invasivo e carcinoma lobular invasivo, que acometem 90% a 95% dos casos e, os menos freqüentes como carcinoma medular, carcinoma mucinoso, carcinoma tubular, carcinoma inflamatório e Doença de Paget (AKRAM *et al*., 2017).

Para fins de tratamento e prognóstico da doença, o Sistema de Estadiamento TNM (tamanho do tumor primário, comprometimento linfonodal e metástase à distância) criado pelo Comitê Conjunto Americano sobre Câncer e a União Internacional de Controle do Câncer, é considerado o mais importante. Nesta classificação, os estágios I e II referem-se a tumores pequenos (até 5cm) com presença ou não de micrometástases linfonodal enquanto que, no estágio III as pacientes têm tumores maiores (maior de 5cm) e metástase em linfonodo. O estagio IV é o mais agressivo no qual ocorre a presença de metástase em órgão distante. Entretanto, outros fatores intrínsecos ao tumor são importantes para a conduta médica em relação ao tratamento e risco de recorrência. Essas características "prognósticas" incluem o subtipo molecular e grau histológico (CHAVEZ-MACGREGOR *et al*., 2017; GIULIANO *et al*., 2017; CSERNI *et al*., 2018).

Em relação aos subtipos moleculares o câncer de mama é uma doença heterogênea e esta classificação tem como base o grau de expressão de receptores de membrana e de genes de proliferação. O subtipo Luminal A apresenta alta expressão de genes relacionados ao receptor de estrógeno, baixa expressão de genes de proliferação (Ki-67 ≤14%), sendo responsável por aproximadamente 40% dos casos determina bom prognóstico. O subtipo Luminal B assemelha-se ao padrão Luminal A em relação à expressão de receptores estrogênicos, mas, difere pela alta expressão de genes de proliferação (Ki-67 > 14%), apresentando características de pior prognóstico. Dentre os mais agressivos encontramos o subtipo HER2 com superexpressão do gene HER2 e de genes de proliferação e o subtipo Triplo Negativo, o qual apresenta ausência de expressão de receptores estrogênicos e ausência da superexpressão do gene HER2, caracterizando um padrão de extrema agressividade e de pior prognóstico quando comparado aos outros subtipos. Essa classificação molecular é de extrema importância na conduta médica em relação ao tratamento e prognóstico de cada paciente (RAKHA; GREEN, 2017; FENG *et al*., 2018; HORTON *et al*., 2018). Trabalhos

demonstram que o subtipo molecular do tumor está associado à taxa de sobrevida da paciente, ao grau de incidência e localização de metástase, tipo de resposta imune no microambiente tumoral, além de que cada subtipo apresenta um comportamento metabólico variado e está relacionado a diferentes fatores de risco (TURKOZ *et al*., 2013; PRAT *et al*., 2015; SAVCI-HEIJINK *et al*., 2015; MONACO, 2017; HOLM *et al*., 2017; SOLINAS *et al*., 2017; LI *et al*., 2017).

O grau histológico representa a característica morfológica do tumor e baseia-se no grau de diferenciação das células tumorais sendo classificado em: grau I de células bem diferenciadas; grau II de células de moderada diferenciação; e grau III de células altamente indiferenciadas (RAKHA *et al*., 2010). Esta graduação tem íntima relação com os fatores de risco sendo os subtipos moleculares de melhor prognóstico, como Luminal A, os que apresentam na maioria das vezes tumores de baixo grau enquanto que, os de pior prognóstico como o Triplo Negativo apresentam tumores de células indiferenciadas (ABUBAKAR *et al*., 2018).

A indiferenciação das células tumorais permite uma maior capacidade destas células realizarem invasão angiolinfática e assim, metástase à distância (CHUI, 2013; FIDALGO *et al*., 2015). Entretanto, esta migração das células tumorais depende de vários fatores físicos e químicos. O microambiente tumoral, principalmente pela privação de oxigeno (hipóxia), secreta fatores de transcrição conhecidos como fatores induzidos por hipóxia (DALES *et al*, 2005) que, dentre outras funções, levam a neoangiogênese propiciando um aumento da densidade microvascular no tumor, o que está relacionado a uma maior incidência de metástase (ZHONG *et al*., 1999), já que estes novos vasos possuem uma anatomia anormal (HASHIZUME *et al*, 2000). Além disso, o processo de metástase pode favorecer a formação de tromboembolismo venoso, uma condição que inclui trombose venosa profunda e embolia pulmonar. Estudos demonstraram que células tumorais circulantes podem ativar a cascata de

coagulação e, indiretamente, serem responsável pela formação de tromboembolismo venoso (MEGO *et al*., 2015), o que em mulheres com câncer de mama está relacionado a menor taxa de sobrevida (CHEW *et al*., 2007). Vários trabalhos relatam a grande incidência desta situação clínica em pacientes com câncer e sua relação com a agressividade e malignidade do tumor.

O processo de metástase depende de diversos eventos que ocorrem ordenadamente sendo a Transição Epitélio-Mesenquimal (TEM) das células tumorais o responsável por promover a estas células um fenótipo migratório. Vários fatores que regulam a proliferação celular, a apoptose e, também, a expressão de proteínas de adesão celular como a E-caderina controlam a TEM. Qualquer desregulação destes mecanismos pode levar a essa transição e assim, a metástase (SHAO *et al*., 2015; YIN *et al*., 2017; QUI *et al*., 2018). Um dos fatores que é conhecido por induzir a TEM é a citocina pró-inflamatória TNF-α. Esta quando produzida pelo microambiente tumoral parece induzir esta transição via NF-κB (Fator Nuclear kappa B) e interage com outras substâncias que também produzem este efeito, como o fator de crescimento endotelial, o estrogênio e a IL-1β, potencializando este efeito (SORIA *et al*., 2011; LI *et al*., 2012; WEITZENFELD *et al*., 2013; LEIBOVICH-RIVKIN *et al*., 2013). Além disso, tanto a IL-1β quanto o TNF-α no ambiente microtumoral estão fortemente associados à indução da migração de células tumorais (FILIPPI; CARRARO; NALDINI, 2015; WOLCZYK *et al*., 2016).

Paradoxalmente, não podemos esquecer que as citocinas pró-inflamatórias, como TNF-α e IL-1β, são importantes no contexto da patogênese do câncer, pois, também atua no combate a proliferação das células tumorais, principalmente nos estágios iniciais da doença. O TNF- α é secretado principalmente por macrófagos e células dendríticas e, a IL-1β por neutrófilos, células endoteliais e células epiteliais. Ambas participam tanto da resposta inata quanto da resposta adaptativa do sistema imune (FERNANDES *et al*, 2015). Além disso, é de

grande importância dentro do contexto da resposta imune que haja um infiltrado de células T CD8+ no tumor para que estas células possam destruir as células tumorais, fato que está relacionado diretamente com uma diminuição do risco relativo de morte (ALI *et al*., 2014). Estas células imunes são estimuladas de diversas formas como pela secreção de TNF-α por células T CD4+ auxiliares, que participam ativamente no microambinte tumoral para regressão do tumor (RAKHRA *et al*., 2010). Entretanto, em estágios mais avançados da doença os níveis plasmáticos de TNF-α parecerem estar aumentados (MA *et al*. 2017), o que parece contribuir para a progressão co câncer.

Assim, a presença destas e de outras citocinas pró-inflamatórias de forma crônica estão envolvidas com a progressão e metástase tumoral. De acordo com Hanna *et al.* (2017), altos níveis de substâncias pró-inflamatórias no tecido mamário aumentam o risco de desenvolver câncer de mama. Além disso, aumento dos níveis séricos de marcadores inflamatórios, como proteína C-reativa, também está associado a menor taxa de sobrevida após o diagnóstico (WULANINGSIH *et al*., 2015). Um dos mecanismos pelo qual a inflamação contribui na fisiopatologia do câncer de mama é pela indução de estresse oxidativo (ROQUE *et al*., 2015).

O desequilíbrio oxidativo sistêmico é um marcador importante de progressão tumoral e, altos níveis circulantes de espécies reativas de oxigeno (ROS) no câncer de mama estão relacionados a estágios mais avançados (PANIS *et al*., 2012). O quadro de inflamação crônica fornece meios para a ocorrência de estresse oxidativo e, conseqüentemente, dano ao DNA criando uma instabilidade genômica que contribui para o desenvolvimento tumoral (NIU *et al*., 2012; OHNISHI *et al*., 2013).

Desta forma, a partir das pesquisas realizadas nas últimas décadas, o câncer de mama se tornou uma grande preocupação dentro da área de saúde pública e muito vem sendo

estudado sobre fatores de risco envolvidos com o surgimento e desenvolvimento deste câncer. Os principais fatores envolvidos são: idade, histórico familial, fatores reprodutivos, fatores hormonais e estilo de vida (SUM *et al*., 2017; FENG *et al*., 2018). Unlu *et al*. (2017), demonstraram que mulheres com faixa etária de 35 anos em status pré-menopausal são diagnosticadas com tumores de mama mais agressivos do que mulheres com idade de 60 anos em status pós-menopausal.

Dentre os diversos fatores de risco para o surgimento e progressão do câncer de mama, a o excesso de gordura corporal e o status hormonal da paciente vêm se destacando pela complexidade na interpretação dos achados clínicos.

1.2 Excesso de gordura corporal e status menopausal como fatores prognósticos no câncer de mama

O excesso de gordura corporal está relacionado à origem e a comorbidade de várias doenças (ALLOT; HURSTING, 2015), dentre elas o câncer (ACKERMAN *et al*., 2017). A presença de obesidade está relacionada a um maior risco de desenvolvimento de câncer de mama (KABAT *et al*., 2017) e mudanças de hábitos que modificam o perfil metabólico, como alimentação e atividade física, diminuem o risco desta neoplasia (HAN *et al*., 2018).

A obesidade é definida como uma doença crônica que ocorre pelo excesso de gordura corporal. O tecido adiposo em grandes quantidades secreta fatores, como adipocinas, que induzem uma maior secreção de citocinas pró-inflamatórias que resulta na formação de um ambiente sistêmico inflamatório crônico de baixo grau (PARK *et al.*, 2014). Assim, como visto anteriormente, a inflamação crônica promove um ambiente indutor da geração de estresse oxidativo persistente que contribui para a progressão do câncer de mama nas pacientes com excesso de gordura corporal (MADEDDU *et al*., 2014).

Além disso, este quadro de inflamação crônica sistêmica é capaz de promover uma desregulação de eixos regulatórios como o do cortisol, tornando-o hiperativado (RODRIGUEZ *et al*., 2015). Este hormônio, dentre várias funções, tem ação antiinflamatória, mas quando presente em níveis sistêmicos elevados pode contribuir com o crescimento e progressão tumoral (SCHREPF *et al*., 2013; SHIN *et al*., 2016). Pacientes com câncer de mama metastático que apresentam níveis de cortisol sistêmico elevados de forma contínua tendem a apresentar mortalidade precoce e parece estar relacionado à imunossupressão gerada pelo cortisol sobre células Natural Killer (SEPHTON *et al*., 2000). Pacientes com câncer de mama Triplo Negativo que apresentam alto nível de expressão de receptores glicocorticóide em células tumorais possuem um padrão TEM diferencial sugestivo de tumores de maior agressividade (PAN; KOCHERGINSKY; CONZEN, 2011).

O status menopausal da paciente com câncer de mama também é de extrema importância para o prognóstico. A variação hormonal estrogênica que ocorre entre os períodos de pré e pós-menopausa alteram o perfil metabólico, a resposta imune e outros eixos hormonais das mulheres (ITO *et al*., 2001; O'CONNOR *et al.*, 2009; MURPHY *et al*., 2009; LAMBERT *et al*., 2004).

Pacientes com câncer de mama em menopausa ao diagnóstico tendem a apresentar tumores de menor agressividade como Luminal A. Estudos demonstram que este tipo de tumor pode estar relacionado à utilização de terapia de reposição hormonal por estas pacientes (DALLAL *et al*., 2014; CHLEBOWSKI *et al*., 2015) ou a presença de obesidade (MONTAZERI *et al*., 2008). No trabalho de Mutlu *et al*. (2013) eles demonstraram que a relação do status menopausal foi associado aos subtipos moleculares dos tumores de mama. Pacientes pré-menopausadas apresentaram subtipos mais agressivos como Triplo Negativo e HER2 enquanto que as pós-menopausadas apresentaram subtipos hormônios dependentes.

A partir dessas informações, observa-se que o câncer de mama pode ser considerado um câncer de difícil prognóstico e tratamento, pois há muitas particularidades em cada tumor que devem ser observadas para tomada da conduta médica. Além disso, vários estudos vêm abordando a participação de fatores extrínsecos ao tumor como fatores ambientais, que também devem ser considerados quanto ao prognóstico e risco de recorrência (GRAY *et al*., 2017).

1.3 EXPOSIÇÃO AOS AGROTÓXICOS, CÂNCER DE MAMA E DESREGULAÇÃO IMUNOENDÓCRINA

Os agrotóxicos, pesticidas ou agroquímicos são substâncias utilizadas, principalmente na agricultura, com objetivo de exterminar ervas daninhas e pragas (insetos, fungos e bactérias). Existem vários tipos de agrotóxicos que podem ser classificados de acordo com o organismo alvo (herbicidas, inseticidas, fungicidas, bactericidas e rodenticidas) ou de acordo com a estrutura química (organofosfatados, organoclorados, carbamatos, piretróides, fenilamidas, alcolatosfenoxi, trazinas, ácido benzóico e ftalamidas) (LIMA *et al.*, 2016).

As regiões brasileiras que consomem cerca de 70% dos agrotóxicos vendidos no país são o Sudoeste e o Sul, sendo também as que apresentam maior número de casos de intoxicação aguda por contato com estes agentes e maiores incidências de desfechos negativos à saúde (CREMONOSE, 2014). No estado do Paraná, a mesorregião do Sudoeste (cerca de 480 mil habitantes) corresponde ao segundo maior valor agropecuário do Estado, baseado na agricultura familiar. Os 27 municípios que compõe esta região, em 2015, foram responsáveis pelo consumo de mais de 5000 toneladas de agrotóxicos (GABOARDI; CANDIOTTO, RAMOS, 2019). Segundo a Agência de Defesa Agropecuária do Paraná (ADAPAR), nesta região é extensivo o uso de herbicidas e inseticidas de significante toxicidade, como piretróides e organofosforados, com destaque ao glifosato, 2,4D e atrazina (ADAPAR, 2014; IBGE, 2014).

A grande utilização destes compostos acarreta uma maior exposição humana a essas substâncias tanto de forma direta quanto indireta. Os efeitos gerados variam de agudos, que promovem casos de intoxicação que podem ser fatais, a efeitos crônicos que podem ocasionar distúrbios em vários sistemas do organismo humano (GUIMARÃES *et al*., 2014).

Dentro do contexto da patogênese do câncer de mama, existem vários trabalhos que demonstram a associação da exposição aos agrotóxicos e o maior risco de desenvolver câncer de mama (ARREBOLA *et al*., 2015; LERRO *et al*., 2015; HE *et al*., 2017). Entretanto, os agrotóxicos devem ser considerados também em relação a sua participação no desenvolvimento de tumores mais agressivos e de pior prognóstico (PARADA *et al*., 2016; ELLSWORTH *et al*., 2018). Yang *et al*. (2015) observaram que os maiores níveis de agrotóxicos encontrados no plasma e tecido mamário de pacientes com câncer de mama foram associados com carcinoma ductal invasivo. Os mecanismos pelos quais estes compostos participam da fisiopatologia do câncer envolvem principalmente dano ao DNA (ALLEVA *et al*., 2017) e modificação da expressão de genes relacionados a proliferação celular (CALAF; ECHIBURU-CHAU; ROY, 2009). No entanto, os agrotóxicos são reconhecidos por sua ação como desregulador imunoendócrino e gerador de ROS, o que pode contribuir para o desenvolvimento tumoral.

Trabalhos demonstram que os agrotóxicos são capazes de promover alterações no eixo do hormônio cortisol. Koakoski *et al.* (2014) demonstraram que a exposição de peixes Fish de forma aguda e crônica a agrotóxicos promove o enfraquecimento do eixo hipotálamo-pituitária-adrenal (HPA), com uma maior dificuldade de adaptação a situações de estresse. Sabe-se que pacientes com câncer de mama que apresentam um padrão de secreção de cortisol anormal com níveis aumentados apresentam tumores de progressão mais avançada (ZEITZER *et al*., 2016). Além disso, estas substâncias agrotóxicas apresentam potenciais efeitos desreguladores sobre o sistema imunológico (FRIEDRICH, 2013). Corsini *et al.*

(2007) demonstraram que exposição *in vitro* e *in vivo* a herbicidas é capaz de promover imunomodelação sobre a secreção de citocinas como IL-1β, IL-6 and TNF-α. Assim, os efeitos cumulativos de desreguladores ambientais como os agrotóxicos podem alterar a secreção de cortisol e promover alterações do sistema imune (THOMPSON *et al.*, 2015).

Ademais, Lerro et al. (2017) demonstraram que exposição humana aos agrotóxicos atrazina e 2,4D são responsáveis pela geração de estresse oxidativo sistêmico. Este desequilíbrio redox poder ocasionar dano ao DNA (GUNDOGAN *et al*., 2018), efeito este relacionado diretamente com o câncer de mama. Entretanto, esta relação dos agrotóxicos como desreguladores imunoendócrinos/redox e câncer de mama ainda não está totalmente elucidada.

Desta forma, considerando que a região Sudoeste do Paraná caracteriza-se pela elevada utilização ocupacional e exposição aos agrotóxicos e que de acordo com dados da 8ª Regional de Saúde do Paraná, esta região apresenta elevada incidência de neoplasias quando comparada à média nacional, especialmente o câncer de mama (estimado em 80 casos/100.000 mulheres), o papel dos agrotóxicos na evolução clinicopatológicado do câncer de mama ainda é pouco compreendido, tornando-se altamente relevante investigar de que forma este histórico de exposição crônica e continuado pela atividade agrícola ocupacional das pacientes com câncer de mama pode afetar o desfecho clínico da doença a partir de modificações no eixo imunoendócrino/redox.

2 OBJETIVO GERAL

Avaliar se mulheres portadoras de câncer de mama expostas cronicamente aos agrotóxicos apresentam desregulação imunoendócrina e desbalanço redox em relação aos desfechos de pior prognóstico e, analisar a correlação dos níveis de cortisol sistêmico e parâmetros de pior prognóstico em pacientes com câncer de mama, levando em consideração

a categorização das pacientes de acordo com IMC, independente da exposição aos agrotóxicos.

2.1 OBJETIVOS ESPECÍFICOS

- Categorizar o perfil das mulheres com suspeita de neoplasia mamária atendidas pelo Hospital do Câncer de Francisco Beltrão, Paraná, no período de 2015 a 2017, quanto a presença de câncer na biópsia e suas características clinicopatológicas.

- Categorizar as pacientes com câncer de mama de acordo com a exposição aos agrotóxicos em expostas ou não expostas, conforme contato ocupacional a essas substâncias.

- Determinar os níveis circulantes de citocinas (TNF-α e Il-1β) e perfil redox (capacidade antioxidante total plasmática, hidroperóxidos e NO) nestas pacientes.

- Medir os níveis plasmáticos de cortisol nasmulheres portadoras ou não de câncer de mama.

- Estabelecer a potencial correlação dos parâmetros mensurados com dados clinicopatológicos determinantes de pior prognóstico (idade, status menopausal, subtipo molecular, grau histológico do tumor, índice de massa corpórea, índice de proliferação celular, tamanho tumoral, presença de metástase linfonodal e presença de êmbolos angiolinfáticos).

A partir dos objetivos apresentados e dos resultados encontrados, esta tese expõe a seguir dois artigos científicos intitulados “Pesticides chronic exposition is associated with immunoendocrine deregulation and redox imbalance in women with breast câncer” e “Systemic cortisol and poor prognosis in overweight/obese women with breast câncer”.

PESTICIDE CHRONIC EXPOSITION IS ASSOCIATED WITH IMMUNOENDOCRINE DEREGULATION AND REDOX IMBALANCE IN WOMEN WITH BREAST CANCER

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ABSTRACT

Pesticides are compounds which activity in the human body have been widely discussed as carcinogenic and promoter of systemic deregulation. In the persent study, we analyzed the relationship between pesticides chronic exposition and the occurrence of worse prognosis in rural women with breast cancer, based on changes in immunological, hormonal and redox parameters. Peripheral blood samples were collected from 73 patients and evaluated to quantify cortisol, cytokines (TNF-α and IL-1β) and redox balance (total antioxidante capacity – TRAP, nitric oxide metabolites - NO and hydroperoxides). Results were correlated to clinicopathological data and analyzed in GraphPad Prism 7.0. In relation to TNF-α levels, it was observed an increase in circulating levels from menopausal patients non-exposed to pesticides when compared to the exposed ones. In the pre-menopausal group, women showed a significant increase in cortisol levels in the non-exposed group in relation to the exposed ones. Exposed patients in menopause had a significant reduction of TRAP when compared to those non-exposed. IL-1β levels were significant difference when comparing the pattern of exposure in obese patients. The levels of plasma hydroperoxides in non-exposed patients with grade 2 tumors were reduced when compared to the group of exposed patients of the same grade. Finally, when in the presence of clots, patients not exposed to pesticides presented higher levels of circulating TNF-α compared to exposed patients. Our data show for the first time that pesticide exposure is related to poor prognosis in breast cancer, and are potential immunoendocrine and redox balance deregulators in such patients.

KEYWORDS: Pesticides, breast cancer, immunoendocrine dysregulation, redox imbalance.

1 INTRODUCTION

Brazil is the major consumer of pesticides worldwide ([1]). Pesticides exposition in several degrees have been widely discussed as a risk factor for the development of cancer, including breast tumors ([2]). Chronic exposure to pesticides contribute to carcinogenesis by inducing damage to DNA ([3]), hormones deregulation, andrising of metabolites that activate oncogenes ([4]).

Pesticides have deregulatory effects on the immune system, which may lead to immunosuppression. This effect is extremely relevant because it consists of reducing the fight against tumor cells, which can lead to a higher incidence of cancer ([5]). Exposure to chemical compounds such as hexachlorobenzene generates immunotoxicity observed by reducing the amount of cells in the immune system and altering levels of circulating cytokines ([6]). In addition, agrochemicals such as atrazine may promote genetic damage in lymphocytes after exposure ([7]).

Pesticides also promote effects on the redox balance. Occupational exposure to pesticides, such as organophosphates, induces oxidative stress and DNA damage ([8]), which may lead to changes in the expression of genes related to metabolizing enzymes ([9]). Moreover, chronic exposure of wistar rats to malathion promoted formation of reactive oxygen species inducing oxidative stress ([10]). Considering that oxidative imbalance is closely related to carcinogens, chronic exposure to pesticides may contribute to cancer progression.

Pesticides can also act as endocrine disruptors ([11]), and can act on cortisol axis ([12]). In breast cancer, patients with abnormal secretion of cortisol present acelerated disease progression ([13]). In spite of this, few studies have investigated the immunoendocrine and redox homeostasis alterations induced by pesticides regarding its clinicopathological meaning

in breast cancer. In this context, the present work aimed to evaluate whether women with breast cancer exposed to pesticides have immunoendocrine dysregulation and redox imbalance in relation to the outcomes of worse prognosis.

2 MATERIALS AND METHODS

2.1 Study design, clinicopathological data and sample collection

The present study is a cross-sectional observational study conducted at the Cancer Hospital of Francisco Beltrão (CEONC), with information collected from the period 2015 to 2017. Women breast cancer were recruited from the following inclusion criteria: volunteer patients who arrived at the CEONC with altered imaging tests (BIRADS 4 and 5) and who underwent biopsy surgery; only female patients; those who presented the clinicopathological data collected from the patients' medical records; patients with staging II; and operable tumors. This study was approved by the National Commission for Ethics in Research (CONEP), protocol number 35524814.4.0000.0107 and all participants signed Informed Consent Term (TCLE).

Aiming to characterize patients' exposition to pesticides, information regarding their direct contact with the agrochemicals and individual protective equipments of contamination, time and duration of exposition lifelong, as well as data concerning indirect expostion (contamination by neighbourhood) were collected. Therefore, women were interviewed and categorized into 2 groups, as exposed or non-exposed. The schematic design of the study is shown in Figure 1.

Clinicopathological data were collected from patients' medical records. Each parameter was categorized in the following groups: age (≤ 50 years or> 50 years); histological grade of the tumor (low, intermediate or high grade); molecular subtypes of tumors (Luminal A for positive estrogen and/or progesterone with ki 67 index under 14%, Luminal B for

positive estrogen and/or progesterone with ki 67 index above 14%, negative estrogen; and the triple negative, without estrogen, progesterone and HER-2 receptors); lymph nodal metastasis (presence or absence); clots (presence or absence); menopause at diagnosis (presence or absence); tumor size (≤ 2cm,> 2cm and <5cm, ≥ 5cm); cell proliferation index (Ki-67) (<14% or ≥14%); and body mass index (BMI) with defined values for adults by the World Health Organization - a eutrophic (BMI ≤ 24.9 kg/m^2), overweight (BMI ≥ 25.0 kg/m^2 or ≤ 29.9 kg/m^2); or obese (BMI ≥ 30.0 kg/m^2). The Information regarding weight and height (for calculating BMI), menopausal status and age at diagnosis were collected through inter views at the moment they were approached to participation in the project.

For all participants, prior to the surgical procedure for biopsy, a peripheral blood sample (10 mL) was collected through venous puncture in tubes with anticoagulant (heparin or EDTA) during the period 2 pm to 5 pm. The samples were then subjected to a five-minute centrifugation at 4000 rpm and the plasma frozen until analysis.

2.2 Cortisol measurements

Blood samples (10 mL) collected from participating patients prior to biopsy through venous puncture in tubes with anticoagulant (heparin or EDTA) were subjected to a five-minute centrifugation at 4000 rpm to obtain plasma. Cortisol levels in samples were measured at 450 nm by using a commercial chlorimetric Enzyme-linked kit (AccuBind ELISA kit, USA). Blood was collected between 2 pm to 5 pm and was used for cortisol dosage out of the morning peak. The plasma levels of cortisol were expressed as µg/dL.

2.3 Cytokines TNF-α and IL-1β measurements

The TNF-α and IL-1β cytokines were measured from the plasma of the patients by using a Elisa Kit (GSR ELISA specific for commercial antibodies eBiosciences, San Diego,

USA). The absorbance of the plates was read at wave length of 450 nm and the plasma levels of TNF-α and IL-1β were expressed as ρg/mL.

2.4 Redox balance investigation

To evaluate the potential of pesticides in deregulating the systemic redox balance, the total antioxidant capacity of the plasma (TRAP), nitric oxide (NO) and hydroperoxides levels were measured.

To evaluate the antioxidant profile of the patients the total antioxidant capacity of the plasma (TRAP) was measured as described by [14]. In this technique the plasma antioxidants inhibit the degradation of ABAP, a free radical compound that emits photons in their decompositionmeasured in a Glomax luminometer (Turner Designs TD 20/20) over 30 min at 5 readings/s. Plasma levels of total antioxidants were expressed in nM trolox.

In addition, the oxidative power of plasma was evaluated by measuring levels and nitric oxide and hydroperoxides, The levels of circulating hydroperoxides were measured by the chemiluminescence method ([15,16]). During the formation of these compounds by the reaction of tert-butyl with lipids, the emission of photons occurs that are captured in a Glomax luminometer (Turner Designers TD 20/20) over 40 minutes at 1 readings/s. Results were expressed as relative units of light (RUL).

The determination of NO concentration in the samples was performed by the technique described by [17]. The method is based on the reduction of nitrate to nitrite mediated by oxo-reduction reactions occurring between the nitrate of the sample and the Cadmo-copper system of the reactants, with subsequent diazotization and colorimetric detection of the azocomposite formed by the addition of the Griees. Absorbance measured at 550 nm on a spectrophotometer microplate reader and plasma levels of nitric oxide were expressed in micromolars (μM) of nitrite.

2.5 Statistical Analysis

All results were analyzed using the Grubbs Test for checking outliers. Student's t-test was applied for parametric data, Mann-Whitney test for non-parametric data or Analysis of Variance (ANOVA) with Bonferroni post-test for comparison of three or more groups. The GraphPad Prism 7.0 Program was used to analyze the quantitative variables. Statistical significance was considered $p \leq 0.05$.

3 RESULTS

A total of 73 patients were included in the presente study, and categorized according to pesticide exposure as exposed (n=42) and non-exposed (n=31) to pesticides.

Table 1 presents the clinicopathological characteristics of all breast cancer patients included in the study. In both groups there were predominance of patients over 50 years of age, overweight, menopausal and who had no metastasis in lymph node and angiolymphatic emboli. However, in the exposed group, we found worse prognostic features such as larger tumors, high histological grade, high proliferation index and triple negative subtype, whereas in non-exposed patients these characteristics presented less aggressive patterns.

Figure 2 shows the circulating levels of TNF-α, cortisol and TRAP according to the menopausal status of patients of each expostion group. In relation to TNF-α levels (Figure 2A), it was observed an increase in its circulating levels in menopausal patients non-exposed to pesticides (127.2 ± 13.99 pg/mL) when compared to the menopausal exposed ones (94.6 ± 8.52 pg/mL, $p = 0.0443$). No significant diferences were observed between pre-menopausal patients (105.4 ± 10.43 pg/mL to exposed and 92.4 ± 16.49 pg/mL to non-exposed, $p = 0.4931$).

As shown in Figure 2B pre-menopausal patients showed a significant increase in cortisol levels in the non-exposed group in relation to the exposed ones (18.34 ± 0.74 μg/dL and 10.38 ± 1.73 μg/dL, p = 0.0262). Further, when compared to non-exposed women in relation to menopausal status, pre-menopausal women (18.34 ± 0.74 μg/dL) had a significant increase in cortisol levels compared to menopause (10.36 ± 1.08 μg/dL, p = 0.0006). No diferences were detected when comparing patients at menopause in relation to pesticide exposition (11.95 ± 1.36 μg/dL to exposed and 10.36 ± 1.08 μg/dL to non-exposed, p = 0.3762).

In relation to antioxidants (Figure 2C) pre-menopausal women that were exposed to pesticide had significantly increased levels of TRAP compared to the non-exposed (445.7 ± 49.84 nM trolox to the exposed group and 221.7 ± 23.19 nM trolox to the non-exposed, p = 0.0006). On contrary, exposed patients in menopause had a significant reduction of TRAP when compared to the non-exposed (368.1 ± 33.06 nM trolox, 480.0 ± 45.91 nM trolox, respectively, p = 0.0488). When comparing only the non-exposed groups, pre-menopausal women (221.7 ± 23.19 nM trolox) presented reduced TRAP if compared to those in menopause (480.0 ± 45.91 nM trolox, p = 0.0043).

As shown in Figure 3, a significant difference was observed in IL-1β levels when comparing the patter of exposition in obese patients (42.78 ± 4.64 ρg/mL to the exposed and 85.10 ± 14.52 ρg/mL to the non-exposed, p = 0.0247). Moreover, when analyzing IL-1β levels at different BMI in exposed patients, we observed a significant reduction of IL-1β levels was observed in obese women (42.78 ± 4.64 pg/mL) in relation to the eutrophic group (70.58 ± 8.73 pg/mL, p = 0.0225).

Concerning the pro-oxidant profile, Figure 4 shows the levels of plasma hydroperoxides distributed according to the histological grade of the tumors. It was observed

that non-exposed patients with grade 2 tumors had reduced levels of hydroperoxides ($6.13x10^5 \pm 1.06x10^5$ RUL) when compared to the group of exposed patients of the same grade ($13.4x10^5 \pm 2.15x10^5$ RUL, $p = 0.0448$). In the intragroup comparison, the exposed patients did not presented significant differences, however, among the non-exposed ones, patients bearing tumors with histological grade 1 ($14.6x10^5 \pm 1.77x10^5$ RUL) presented increased levels of hydroperoxides compared to those with histological grade 2 ($6.13x10^5 \pm 1.06x10^5$ RUL, $p = 0.0054$).

In Figure 5, when in the presence of clots, patients non-exposed to pesticides presented higher levels of circulating TNF-α compared to exposed patients (151.0 ± 20.99 pg/mL for non-exposed patients, 89.82 ± 11.31 pg/mL for exposed patients, $p = 0.0128$). In the absence of clots, there was no significant difference between exposure or not to pesticides (104.1 ± 8.13 pg/mL for exposed patients, 112.8 ± 16.34 pg/mL for non-exposed patients, $p = 0.9211$), as well as intragroup comparisons ($p = 0.3281$ to exposed and $p = 0.2141$ to non-exposed).

Supplementary tables containing all data of TNF-α, IL-1β, cortisol, TRAP, hydroperoxides and NO in relation to the statitically non-significant clinical data available (Tables 2, 3, 4, 5, 6 and 7).

4 DISCUSSION

This is the first study that describes a prognostic relationship between pesticide exposure and breast cancer parameters. Our main findings demonstrate that pesticides are immunoendocrine disruptors that also cause redox imbalance, in association with relevant clinicopathological features.

One relevant finding of our study was the significant variations found regarding patients' menopausal status. Here we demontrated that TNF-α, cortisol and antioxidants can

vary significantly in the blood of menopausal patients. Menopause is a period of significant estrogenic changes ([18]), which impact both metabolic pathways and immune function. It is known that estrogens modulate T lymphocytes Th1/Th17 responses ([19]), and control the secretion of TNF-α by macrophages ([20]).

We observed that patients exposed to agrochemicals exhibit reduced levels of TNF-α in plasma when compared to non-exposed patients, suggesting that pesticide exposition life long can alter the secretion pattern of this cytokine in breast cancer. It is expected that in menopause occurs an increase in TNF-α production as a response to estrogen de privation, as well as a compensatory mechanism against the decreasing of imune cells in menopause ([21,22]), on contrary of the data that we found. It is important to highlight that TNF-α is extremely important in the inhibition of tumor growth in physiological tumor responses ([23]). Environmental factors such as pesticides may reduce or inhibit its secretion ([24]), which can be considered as a promoter of worse prognosis in cancer. Therefore, disruption in TNF-α mechanisms by pesticides may be associated with poor prognosis disease in breast cancer.

Since there is a interplay between TNF-α and cortisol responses ([25]), another point of interest investigated in our study was the cortisol profile and its relationship with agrochemicals exposure. Once again, pre-menopausal women non-exposed to pesticides presented significant variations, displaying increased cortisol when compared to the exposed ones. Estrogen is a positive regulator of the immune response ([26]), able to contribute to the formation of a pro-inflammatory environment in the presence of tumor, which stimulates the cortisol axis to counterbalance the excessive inflammatory response ([27]). In addition, estrogenic hormones also control the secretion of cortisol by regulating the hypothalamic-pituitary-adrenal axis ([28]). As the pesticides known to deregulate the cortisol axis ([29]), the anti-inflammatory response of cortisol has been reduced in exposed patients, which can

generate an environment that contributes to the promotion of cancer. Interestingly we noted that breast cancer patients exposed to pesticides presented the same levels of cortisol independently of their menopausal status. These findings suggest that the exposure to pesticides abrogates the potential regulatory effects of estrogen physiologically expected on cortisol ([30]). We further detected that agrochemicals exposure affected the antioxidante status of menopausal breast cancer patients. According to the literature it is expected that TRAP levels be higher in post-menopausal women ([31]). However, when considering pesticides exposition, our data shown the contrary, suggesting that the mechanisms underliyng the antioxidante system control be deregulated in exposed women with breast cancer. We suggest that exposure to pesticides during pre-menopause promotes estrogenic dysregulation, would affect the general antioxidant activity ([32]) mobilizing other forms of antioxidant compounds production. However, in the menopausal the pesticides are responsible for generating a redox imbalance, which is the result of the increased consumption of these antioxidant molecules, which are also consumed by the presence of the tumor ([33]) .

Considering that the inflammatory axis seems to be unbalanced in exposed patients, we further investigated the profile of IL-1β and hydroperoxides levels. Plasmatic IL1-β was found reduced in obese patients that were exposed to pesticides when compared to exposed euthrophic patients and also in relation to non-exposed obese patients. IL1-β plays a controversial role in the development and progression of cancer ([34]), and immune cells secreting IL-β may be affected by exposure to pesticides. *In vitro* exposure of macrophages to these substances reduces their lysosomal activity and also negatively regulates the secretion of IL-1β and TNF-α. Some pesticides still appear to affect IL-1β levels in the spleen and thymus of exposed mice, enhancing their immunotoxic potential ([35]). It is also suggested that chronic exposure to pesticides promotes the spontaneous secretion of IL-1β by human

monocytes present in the peripheral blood ([36]), which in the long run could result in the exhaustion or deregulation of this system. These findings suggest that pesticides may exhibit immunosuppressive activity on such cells ([37]).

Thus, in the possibility of chronic dysregulation of IL-1β production by the pesticides before or after the development of the cancer, the reduction of its production would favor the progression of cancer by compromising the immune response. In addition, considering that obesity constitutes a major risk factor for the worst prognosis of breast cancer ([38]), the reduction of IL1-β in these patients due to exposure to pesticides could constitute an additional factor that would contribute to the increase of the aggressiveness of the cancer. Considering the inflammatory millieu of breast cancer, and its modification by pesticide exposure, other factors may be affected in this context.

Once rural workers exposed to pesticides have increased levels of oxidative stress ([8,39]), we further investigated if the exposed patients from our study presented some variation in pro-oxidants, and if this analysis had clinicopathological significance. We found that non-exposed patients bearing grade 2 tumors exhibited reduced levels of hydroperoxides when compared to exposed women with the same tumor grade. Moreover, non-exposed patients with grade 2 tumors differed from non-exposed patients with grade 1 tumors. Breast tumor grade have been also analyzed by microarrays by a method name as index of histological graduation based on gene expression. ([40]), This tool suggests that grades 1 and 3 comprise tumors of different prognoses, while most histological grade 2 correspond to a transient phase that expresses genes associated with high risk of recurrence, similar to histological grade tumors 3 ([41]).

In this context, the progression of grade 2 to grade 3 tumors depends on the accumulation of genomic instability, promoted by mediators of the inflammatory response,

mainly from oxidative stress, that have been associated with the development of undifferentiated tumors ([42]). Thus, our findings suggest that patients not exposed to pesticides present reduced hydroperoxide levels in relation to those with grade 1 and 3 tumors, suggesting that there is a physiological adaptation of grade 2 tumors to oxidative stress levels, before progressing to grade 3. It was observed that this reduction of stress levels does not occur in grade 2 when patients are exposed to pesticides, indicating that such tumors are adapted to high levels of stress, necessary to ensure the genomic instability and progress to grade 3. This relationship between the occurrence of oxidative stress, genomic instability and exposure to agrochemicals ([43]) has already been demonstrated in the literature. Within this hypothesis, such patients may present a more aggressive disease throughout the clinical follow-up period (5-10 years).

Finally, exposure to pesticides affected the clot formation in patients with breast cancer. It was observed that non-exposed patients with intratumoral clots had significantly higher TNF-α levels than those who were exposed and also presented intratumoral clots, suggesting that this cytokine is a factor that modifies the vascular endothelium and contributes to the formation of emboli ([44]). However, women exposed to pesticides presented both reduced levels of circulating TNF- α intratumoral clots. In spite we can not point out the clear relevance of such phenomenon, it is important to highlight that clot formation allows metastasis ([45]). These findings indicate that other componentes that alter vascular reactivity may be enrolled in breast cancer when patients are under pesticide exposure.

Limitations of this include limited sample siz and lack of repeated measures of cortisol, cytokines and oxidative stress in tumor.

Altogether, our data indicate that pesticides are potential immunoendocrine and redox balance deregulators in patients with breast cancer. We observed that these deregulations were

mainly associated with the menopausal status of the patients, with a potential impairment of the mechanisms of tumor growth control by the reduction of circulating cytokine such as TNF-α and by consumption extra antioxidant compounds. Further investigation needs to be conducted to understand the meaning of immuno-endocrine-redox changes in breast cancer induced by pesticide chronic exposition, especially regarding the long-term follow up of such patients.

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FIGURES

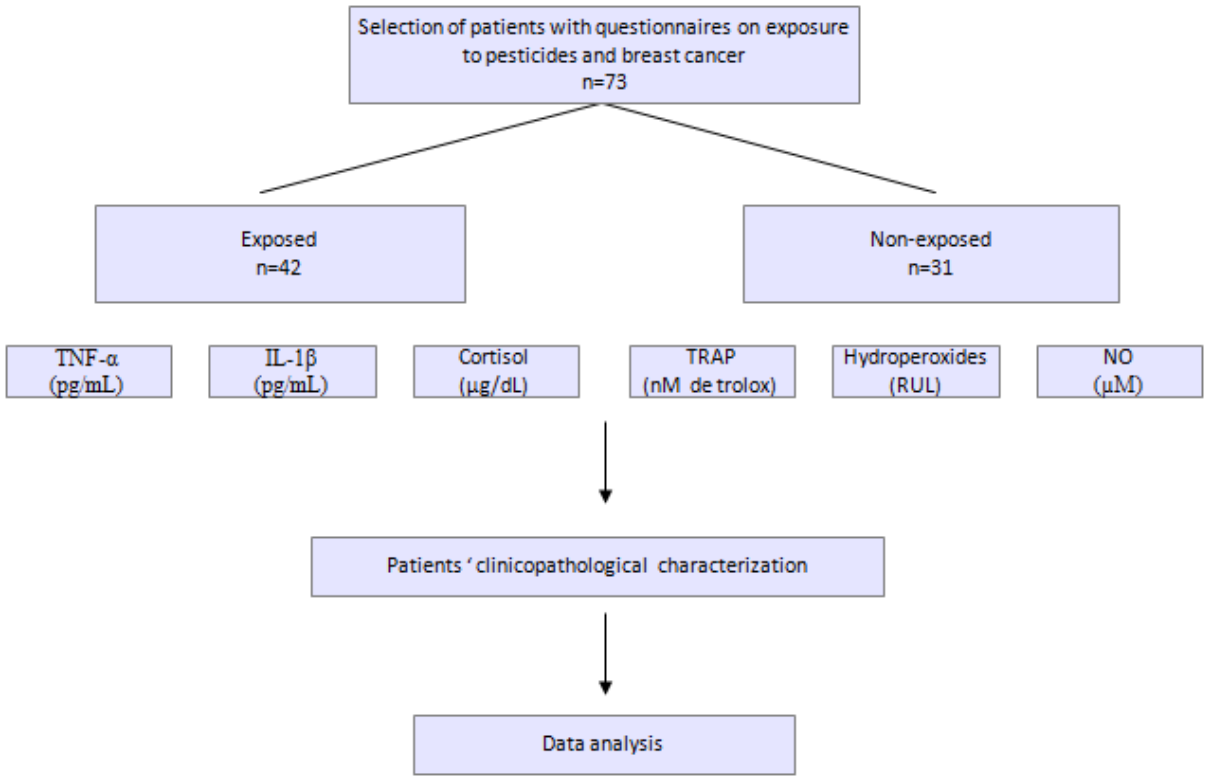

Figure 1 – Design of the study.

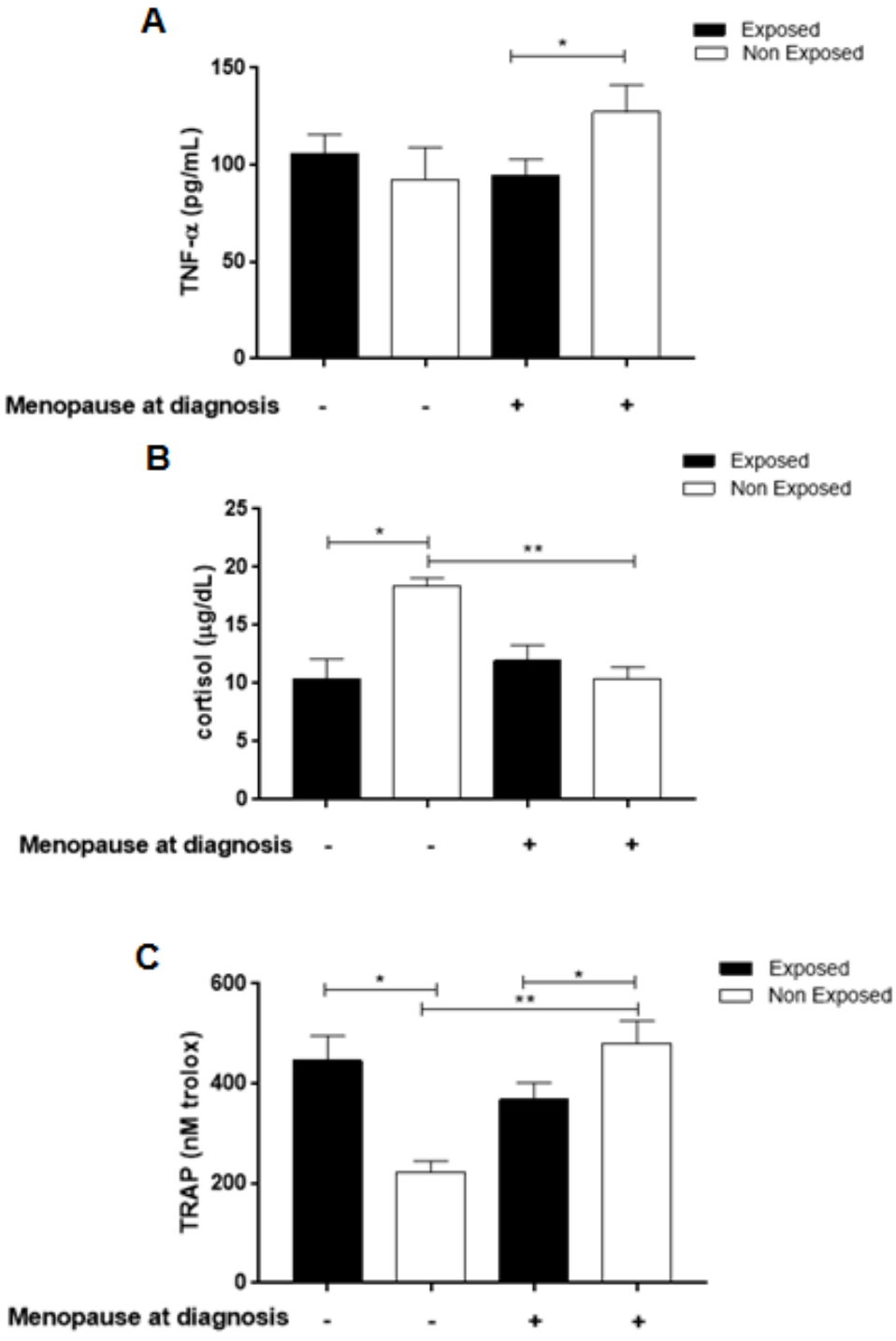

Figure 2 – Circulating levels of tumor necrosis factor-alpha (TNF-α) (A), cortisol (B) and total plasma antioxidant capacity (TRAP) (C) in patients with breast cancer, according to exposure to pesticides and menopausal status at diagnosis. The data represent the mean ± standard error of the mean. (*) and (**) indicates a statistically significant difference ($p \leq 0.05$).

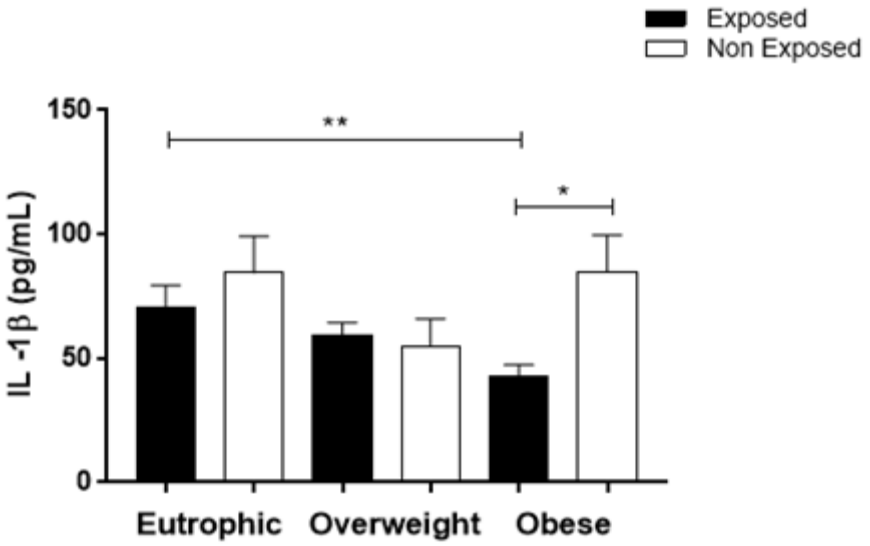

Figure 3– Circulating levels of IL-1β in breast cancer patients according to exposure to pesticides and body mass index. The data represent the mean ± standard error of the mean. (*) and (**) indicates significant statistical difference (p≤0.05). Interleukin -1beta (IL-1β)

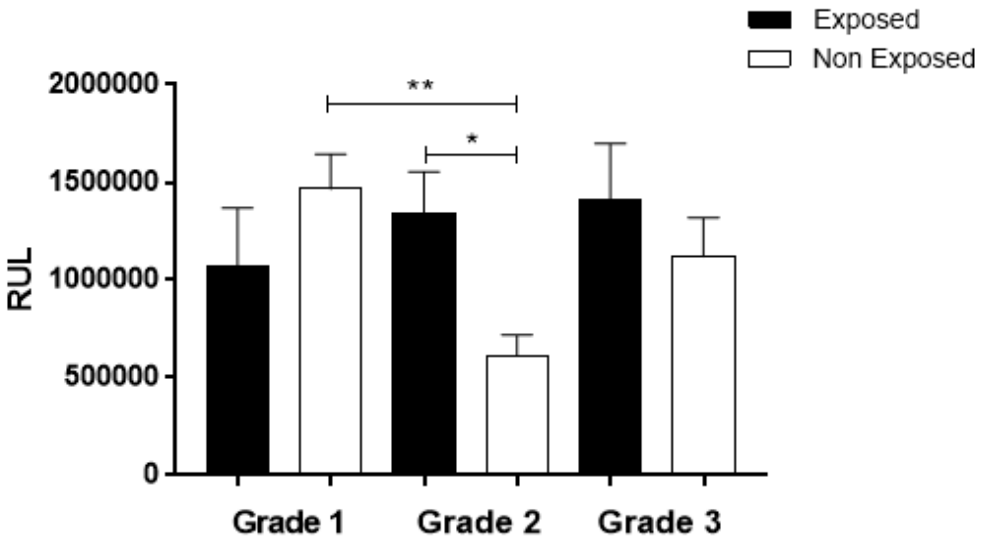

Figure 4– Levels of hydroperoxides measured by chemiluminescence of high sensitivity in patients with breast cancer, according to exposure to pesticides and histological grade of the tumor. The data represent the mean ± standard error of the mean. (*) and (**) indicates significant statistical difference (p≤0.05). Relative Unit of Light (RUL)

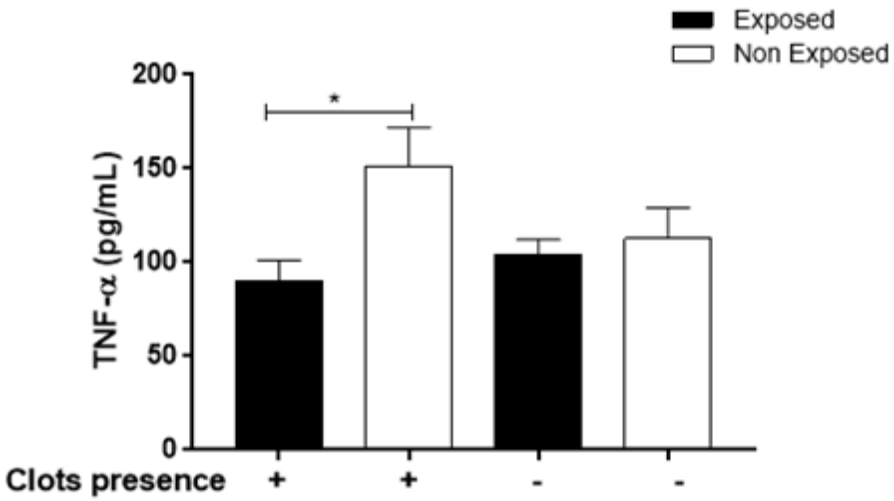

Figure 5– Levels of tumor necrosis factor-alpha (TNF-α) in breast cancer patients, according to exposure to pesticides and presence of clots. Data represent the mean ± standard error of the mean. (*) indicates statistically significant difference ($p \leq 0.05$).

Table 1 – Patients' clinicopathological data with breast cancer exposed or non-exposed to pesticides.

	Exposed	Non- Exposed
Number of individuals	**N=42 (57.5%)**	**N=31 (42.5%)**
Age at diagnosis		
≤ 50 anos	**19 (45.2%)**	**12 (41.1%)**
> 50 anos	**23 (54.8%)**	**19 (58.9%)**
Histological grade		
Grade I	**11 (27.5%)**	**14 (49.1%)**
Grade II	**19 (47.5%)**	**10 (30.9%)**
Grade III	**10 (25.0%)**	**07 (20.0%)**
Molecular subtype		
Luminal A	**09 (24.3%)**	**07 (25.0%)**
Luminal B	**10 (27.0%)**	**14 (50.0%)**
Triple negative	**14 (37.8%)**	**07 (25.0%)**
Lymph nodes metastasis		
Absence	**25 (69.4%)**	**15 (68.2%)**
Presence	**11 (30.6%)**	**07 (31.8%)**
Clots		
Absence	**26 (68.4%)**	**18 (75.0%)**
Presence	**12 (31.6%)**	**06 (25.0%)**
Menopause at diagnosis		
Absence	**15 (36.6%)**	**08 (26.7%)**
Presence	**26 (63.4%)**	**22 (73.3%)**
Tumor size		
≤ 2cm	**13 (40.6%)**	**10 (38.5%)**
> 2cm e < 5cm	**08 (25.0%)**	**14 (53.8%)**
≥ 5cm	**11 (34.4%)**	**02 (7.7%)**
Ki-67		
< 14%	**12 (30.0%)**	**11 (35.5%)**
≥ 14%	**24 (60.0%)**	**20 (64.5%)**
Body mass index (kg/m^2)		
Eutrophic	**13 (31.0%)**	**10 (33.3%)**
Overweight	**20 (47.6%)**	**12 (40.0%)**
Obese	**09 (21.4%)**	**08 (26.7%)**

Table 2 – Circulating levels of nitric oxide (NO) in breast cancer patients exposed or non-exposed to pesticides

	NO (µM)		
	Exposed (n=16)	Non-Exposed (n=18)	p valor
Circulating levels	59.57±3.30	64.57±7.09	0.9389
Age at diagnosis			
≤ 50 anos	63.55±6.07	54.80±2.99	0.2051
> 50 anos	56.11 (49.15-63.07)	58.50 (51.11-80.02)	0.5936
Histological grade			
Grade I	57.20 (46.33-63.93)	77.63 (51.76-101.10)	0.1807
Grade II	53.86±3.23	57.10±4.60	0.5888
Grade III	68.17±4.02	45.46±8.49	0.1551
Molecular subtype			
Luminal A	54.30±3.38	66.98±11.87	0.3622
Luminal B	62.85 (46.98-82.52)	52.96 (48.83-59.64)	0.2673
Triple negative	57.63±3.80	53.79±2.36	0.5002
Lymph nodes metastasis			
Absence	58.79±5.29	67.39±5.31	0.2690
Presence	56.00±6.84	52.09±0.32	0.6257
Clots			
Absence	60.38±5.39	63.02±5.70	0.7426
Presence	59.20±2.90	59.37±7.19	0.9812
Menopause at diagnosis			
Absence	61.47±8.06	55.89±4.13	0.5714
Presence	56.11(49.15-63.07)	58.50 (51.43-79.42)	0.5155
Tumor size			
≤ 2cm	50.40±5.17	47.85±4.56	0.7722
> 2cm e < 5cm	61.28±4.34	60.35±5.93	0.9000
≥ 5cm	57.41±5.65	80.15±15.40	0.3127
Ki-67			
< 14%	53.07 (50.35-62.20)	77.63 (57.52-90.57)	0.4206
≥ 14%	60.52±5.02	56.88±2.79	0.5238
Body mass index (kg/m^2)			
Eutrophic	62.78±7.31	41.27±7.24	0.0815
Overweight	59.00±4.26	70.02±6.19	0.1783
Obese	54.51±4.22	56.71±2.90	0.6754

Table 3- Circulating levels of tumor necrosis factor alpha (TNF-α) in breast cancer patients exposed or non-exposed to pesticides

	TNF-α (pg/mL)		
	Exposed (n=39)	**Non-Exposed (n=28)**	**p valor**
Circulating levels	**102.0±7.96**	**118.0±11.06**	**0.2077**
Age at diagnosis			
≤ 50 anos	**98.14±9.23**	**94.04 ±13.50**	**0.7972**
> 50 anos	**96.50 ±9.25**	**123.90 ±12.61**	**0.0808**
Histological grade			
Grade I	**114.40 ±13.00**	**118.80 ±15.67**	**0.8347**
Grade II	**112.90 ±17.88**	**157.90 ±36.32**	**0.2526**
Grade III	**100.70 ±10.95**	**110.90 ±21.06**	**0.6530**
Molecular subtype			
Luminal A	**97.90 (47.15-138.60)**	**146.40(77.90-245.00)**	**0.1416**
Luminal B	**110.70 (75.03-121.80)**	**109.30(93.95-133.60)**	**0.8357**
Triple negative	**91.14±8.51**	**90.25±20.03**	**0.9615**
Lymph nodes metastasis			
Absence	**104.30 (79.33-130.70)**	**121.50 (86.05-177.20)**	**0.1778**
Presence	**93.76 ±2.24**	**97.22±16.12**	**0.8646**
Tumor size			
≤ 2cm	**101.80 ±9.03**	**104.20 ±14.09**	**0.8810**
> 2cm e < 5cm	**87.29±13.45**	**111.90 ±15.42**	**0.2663**
≥ 5cm	**111.00±16.24**	**121.50 ±2.15**	**0.7359**
Ki-67			
< 14%	**86.73±10.46**	**116.60±20.59**	**0.1649**
≥ 14%	**125.90±13.28**	**111.00 ±10.63**	**0.4061**
Body mass index (kg/m^2)			
Eutrophic	**93.60 (67.88-119.30)**	**136.40 (92.90-224.30)**	**0.0848**
Overweight	**108.60 ±10.25**	**121.20±18.50**	**0.5247**
Obese	**78.78 ±10.49**	**98.31±16.26**	**0.3190**

Table 4 - Circulating levels of cortisol in breast cancer patients exposed or non-exposed to pesticides

	Cortisol (µg/dL)		
	Exposed (n=42)	**Non-Exposed (n=31)**	**p valor**
Circulating levels	**11.9±1.02**	**11.26±1.04**	**0.6773**
Age at diagnosis			
≤ 50 anos	**12.98±1.8**	**9.98±1.5**	**0.2141**
> 50 anos	**11.2±1.26**	**12.31±1.42**	**0.5690**
Histological grade			
Grade I	**11.8±1.51**	**13.5±1.85**	**0.4792**
Grade II	**13.28±1.78**	**10.94±1.65**	**0.3794**
Grade III	**10.17±2.32**	**9.39±2.15**	**0.8124**
Molecular subtype			
Luminal A	**9.34±1.70**	**12.58±2.50**	**0.3312**
Luminal B	**12.64±1.43**	**11.42±2.11**	**0.6252**
Triple negative	**12.99±2.73**	**9.25±1.92**	**0.2748**
Lymphonodes metastasis			
Absence	**13.84±1.32**	**12.4±1.33**	**0.4769**
Presence	**11.54±2.20**	**9.59±2.11**	**0.5471**
Clots			
Absence	**12.46±1.39**	**11.62±1.35**	**0.6743**
Presence	**11.05±1.99**	**10.23±2.10**	**0.8046**
Tumor size			
≤ 2cm	**12.54±1.9**	**8.91±1.80**	**0.1843**
> 2cm e < 5cm	**11.95±1.52**	**11.0±2.12**	**0.7103**
≥ 5cm	**6.49±0.19**	**10.53±2.66**	**0.4874**
Ki-67			
< 14%	**11.78±1.802**	**11.48±1.81**	**0.9095**
≥ 14%	**11.94±1.27**	**11.08±1.37**	**0.6522**
Body mass index (kg/m^2)			
Eutróphic	**11.28±1.82**	**9.41±1.85**	**0.4879**
Overweight	**10.83±1.50**	**12.61±1.52**	**0.4406**
Obese	**15.03±2.05**	**10.93±2.29**	**0.2062**

Table 5 - Total plasma antioxidant capacity (TRAP) in patients with breast cancer exposed or non-exposed to pesticides

	TRAP (nM de trolox)		
	Exposed (n=40)	**Non- Exposed (n=27)**	**p valor**
Circulating levels	**412.3±31.81**	**415.1±40.38**	**0.9557**
Age at diagnosis			
≤ 50 anos	**491.6±55.08**	**389.0 ±68.23**	**0.2739**
> 50 anos	**347.3±31.02**	**428.2 ±51.14**	**0.1680**
Histological grade			
Grade I	**357.7 ±49.38**	**402.8 ±56.30**	**0.5605**
Grade II	**421.9±44.19**	**467.5 ±95.95**	**0.6256**
Grade III	**342.1 (296.60-380.80)**	**373.2 (211.00-569.30)**	**0.9551**
Molecular subtype			
Luminal A	**422.9±79.69**	**406.7±91.58**	**0.8966**
Luminal B	**368.7±41.01**	**425.7±70.36**	**0.5175**
Triple negative	**438.8±54.09**	**379.2±72.27**	**0.5254**
Lymph nodes metastasis			
Absence	**350.2±32.20**	**417.4±63.94**	**0.3022**
Presence	**506.2±68.29**	**484.5±69.85**	**0.8336**
Tumor size			
≤ 2cm	**416.9 ±39.48**	**442.8 ±90.55**	**0.7349**
> 2cm e < 5cm	**489.3±66.58**	**376.6 ±50.56**	**0.1867**
≥ 5cm	**480.6±126.20**	**691.0 ±6.99**	**0.4241**
Ki-67			
< 14%	**499.3±58.27**	**375.7±69.84**	**0.1894**
≥ 14%	**339.5 (273.5-403.4)**	**373.2 (275.8-663.5)**	**0.2772**
Body mass index (kg/m^2)			
Eutrophic	**429.2±72.67**	**418.8±96.57**	**0.9331**
Overweight	**362.0 (271.3-422.5)**	**440.1 (229.9-661.3)**	**0.3410**
Obese	**466.8 ±61.61**	**317.2±32.18**	**0.0686**

Table 6 - Circulating levels of interleukin-1beta (IL-1β) in breast cancer patients exposed or non-exposed to pesticides.

	IL-1β (pg/mL)		
	Exposed (n=42)	**Non-Exposed (n=29)**	**p valor**
Circulating levels	**58.85 (40.75-72.88)**	**64.20 (38.45-108.80)**	**0.3067**
Age at diagnosis			
≤ 50 anos	**57.30 (41.90-66.50)**	**63.50 (38.10-108.80)**	**0.4257**
> 50 anos	**62.75 ±6.55**	**72.94 ±11.00**	**0.4007**
Histological grade			
Grade I	**64.80 ±6.71**	**77.43 ±10.57**	**0.3445**
Grade II	**60.87 ±6.63**	**62.84 ±15.53**	**0.8913**
Grade III	**54.78 ±7.47**	**69.96 ±16.35**	**0.3642**
Molecular subtype			
Luminal A	**51.76±9.84**	**78.09±18.79**	**0.2067**
Luminal B	**60.40(48.05-74.65)**	**63.50(38.45-110.00)**	**1.000**
Triple negative	**56.82±6.37**	**53.83±10.36**	**0.8036**
Lymph nodes metastasis			
Absence	**52.55±7.76**	**80.67±15.63**	**0.8293**
Presence	**61.35±5.70**	**58.88±11.38**	**0.0895**
Clots			
Absence	**57.30(36.73-69.05)**	**64.20(36.55-108.80)**	**0.6013**
Presence	**59.18±6.75**	**72.45±15.31**	**0.3695**
Menopause at diagnosis			
Absence	**55.01±5.03**	**64.34±12.27**	**0.4079**
Presence	**61.55(37.10-79.05)**	**64.20(36.55-100.00)**	**0.4559**
Tumor size			
≤ 2cm	**47.37 ±5.822**	**49.29 ±5.37**	**0.8280**
> 2cm e < 5cm	**57.58±6.41**	**72.20±10.46**	**0.2773**
≥ 5cm	**61.75±9.85**	**29.60 ±24.60**	**0.1960**
Ki-67			
< 14%	**55.00(35.98-65.93)**	**59.60(36.93-103.30)**	**0.3162**
≥ 14%	**67.61±5.47**	**71.39 ±9.48**	**0.7181**

Table 7- Plasma hydroperoxide levels in patients with breast cancer patients exposed or non-exposed to pesticides

	Hidropéróxidos (RUL)		
	Exposed (n=41)	**Non-Exposed (n=29)**	**p valor**
Circulating levels	**12.7 x 10^5 ±1.4 x 10^5**	**11.8x 10^5 ±1.2x 10^5**	**0.6510**
Age at diagnosis			
≤ 50 anos	**12.6x 10^5 ±1.7x 10^5**	**11.5x 10^5 ±2.0x 10^5**	**0.6821**
> 50 anos	**12.9x 10^5 ±2.2x 10^5**	**12.1x 10^5 ±1.6x 10^5**	**0.7811**
Molecular subtype			
Luminal A	**15.5x 10^5 ±4.3x 10^5**	**17.2x 10^5 ±3.3x 10^5**	**0.7621**
Luminal B	**11.5x 10^5 ±2.2x 10^5**	**7.2x 10^5 ±0.8x 10^5**	**0.3100**
Triple negative	**11.2x 10^5 ±2.3x 10^5**	**17.1x 10^5 ±4.0x 10^5**	**0.2016**
Lymph nodes metastasis			
Absence	**11.6x 10^5 ±1.9x 10^5**	**12.9x 10^5 ±2.0x 10^5**	**0.6496**
Presence	**10.8x 10^5 ±2.6x 10^5**	**11.4x 10^5 ±2.5x 10^5**	**0.8762**
Clots			
Absence	**12.4x 10^5 ±1.6x 10^5**	**12.3x 10^5 ±1.8x 10^5**	**0.9848**
Presence	**10.9x 10^5 ±2.8x 10^5**	**10.9x 10^5 ±2.3x 10^5**	**0.9913**
Menopause at diagnosis			
Absence	**11.6x 10^5 ±1.9x 10^5**	**12.9x 10^5 ±2.0x 10^5**	**0.6496**
Presence	**12.3x 10^5 ±1.6x 10^5**	**13.0x 10^5 ±2.0x 10^5**	**0.8762**
Tumor size			
≤ 2cm	**15.6x 10^5 ±3.56x 10^5**	**17.4x 10^5 ±4.4x 10^5**	**0.7625**
> 2cm e < 5cm	**12.11x 10^5 ±2.0x 10^5**	**15.4x 10^5 ±2.3x 10^5**	**0.2953**
≥ 5cm	**10.1x 10^5 ±2.6x 10^5**	**12.0x 10^5 ±3.1x 10^5**	**0.5333**
Ki-67			
< 14%	**15.7x 10^5 ±2.2x 10^5**	**13.9x 10^5 ±2.7x 10^5**	**0.6328**
≥ 14%	**13.9x 10^5 ±2.37x 10^5**	**9.5x 10^5 ±1.2x 10^5**	**0.3315**
Body mass index (kg/m^2)			
Eutrophic	**10.1x 10^5 ±2.2x 10^5**	**9.93x 10^5 ±2.0x 10^5**	**0.9476**
Overweight	**15.3x 10^5 ±2.8x 10^5**	**13.8x 10^5 ±2.2x 10^5**	**0.6459**
Obese	**12.1x 10^5 ±2.5x 10^5**	**14.7x 10^5 ±3.9x 10^5**	**0.6093**

SYSTEMIC CORTISOL AND POOR PROGNOSIS IN OVERWEIGHT/OBESE WOMEN WITH BREAST CANCER

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ABSTRACT

Excessive body fat promotes several negative effects on the pathogenesis of breast cancer. Studies have shown that both the factors of excessive fat and cancer modify the physiological effects of the hormone cortisol, which could possibly affect cancer progression and disease prognosis. Our study analyzed the correlation between systemic levels of cortisol and prognostic parameters in patients with breast cancer categorized according to the body mass index (BMI). We collected peripheral blood samples from 224 women out of the peak of morning cortisol, between 2 p.m. and 5 p.m., aiming at investigating whether cortisol levels were deregulated during the day. Cortisol levels were measured using an enzyme immunoassay kit and correlated to clinicopathological data. Results were analyzed through GraphPad Prism 7.0 and SPSS Statistic 20, $p \leq 0.05$. Circulating cortisol levels were significantly higher in obese women patients in relation to the eutrofic ones. Overweight patients exhibited high cortisol in association with Luminal A and triple negative tumors in relation to euthrophic women with breast cancer. Higher cortisol levels were found in obese patients with Luminal B tumors linked to both overweight and euthrophic women. Overweight patients also exhibited augmented cortisol when diagnosed with high grade tumors. In addition, the presence of lymph nodes metastasis correlated to high cortisol and overweight/obesity, as well as for patients with the presence clots. These data indicate that excessive body fat has a negative effect on the disease prognosis as well as that cortisol levels can be associated with poor prognosis in breast cancer.

Key Words: breast cancer, cortisol, prognosis, overweight, obesity.

Abbreviations: BMI, body mass index; HPA, Hypothalamic-Pituitary-Adrenal; (IL)-6, interkeukin 6; (TNF)-α, tumor necrosis factor alfa; 11B-HSD, 11B-hydroxysteroid dehydrogenase; CEONC, Cancer Hospital of Francisco Beltrão; CONEP, National Commission of Ethics in Research; HER-2, receptor of the human epidermal growth factor 2;

SEM, standard error; ANOVA, analysis of variance; TNM, size, number of lymph nodes, metastasis

1 INTRODUCTION

Obesity is the basis of several diseases that cause important comorbidities [1], including cancer [2,3]. Excessive body fat is related to enhanced mortality rates in cancer patients[4] by promoting the onset and development of tumors through many different known mechanisms[5,6].These mechanisms include the following: increased levels of growth factors, such as insulin-like growth factor[7]; higher plasma concentrations of sex steroid hormones, mainly estrogen, altering body metabolism[8]; release of factors from adipose tissue which affect the activity of macrophages and thus the immune system functioning[9]; raised pro-inflammatory substances, such as tumor necrosis factor (TNF)-α[10], in addition to an imbalanced production of reactive oxygen species caused by excess fat [11]. Therefore, overweight and obesity provide an inflammatory milieu that bolster cancer success[5]. Endocrine changes derived from excessive adipose tissue forming a chronic pro-inflammatory systemic environment [4], which modifies cellular communication among adipocytes, immune components, and epithelial cells [12] leading to the initiation, promotion, and progression stages of carcinogenesis[13].

Even though adipose tissue is able to alter sexual hormonal axes, some studies have reported that other axes, such as cortisol, may also be affected. A number of studies have indicated that pro-inflammatory mediators secreted by fatty tissues influence the activity of the Hypothalamic-Pituitary-Adrenal (HPA) axis by promoting hyperstimulation[14,15]. In addition to such an effect, adipocytes also synthesize higher levels of cortisol–from cortisone–through enhanced activation of the 11B-hydroxysteroid dehydrogenase (11B-HSD) enzyme in obese patients, which interferes with the hormone plasma concentration [16,17].

Cancer itself is also capable to modify cortisol secretion pattern, which, according to the literature, can occur in the context of varied scenarios. Most cancer patients need psychosocial support since the stress of having discovered the disease, in addition to the treatment process, leads to dysregulation of the HPA axis, which can cause disturbances in the circadian rhythm and an impact on sleep pattern as well as induce depressive symptoms [18]

Regardless of its origin, this altered axis and increased cortisol secretion are responsible for inducing the increased proliferation and invasion of cancer cells[19]. Patients with lung cancer who have lower diurnal cortisol variation demonstrated circadian rhythm rupture and accelerated tumor progression[20]. Likewise, high levels of systemic cortisol in patients with oral cancer are related to more advanced stages of the disease [21]. Moreover, women with ovarian cancer who did not present recurrence within one year after the end of treatment had cortisol levels closer to normal [22] and negative correlation between salivary cortisol levels and survival rate [23].

For breast cancer– the most common malignant neoplasia in women worldwide– evidence has proved obesity to bea worsening factor by demonstrating an association between weight loss and increased survival [24]. The mechanisms involved in such a scenario are the same described for other types of cancers–such as the presence of high levels of adipokines and pro-inflammatory factors[25] - but with the addition of altered lipid metabolism and formation of compounds, such as oxysterol 27-hydroxycholesterol,which promotes tumor growth and metastasis [26].Furthermore, it has been well-established that breast cancer alters HPA axis [27], which, in turn, has been associated with reduced survival rates [28].

Despite the studies in the literature associating obesity with breast cancer and its clinical outcome, little is known regarding its impact on cortisol levels. In this context, our

goal was to analyze the profiling of systemic cortisol levels and parameters related to poor prognosis in patients with breast cancer considering their categorization according to BMI.

2. MATERIALS AND METHODS

2.1 Study Population

This is a cross-sectional observational study whose participant women aged 15–86 years with indicative of cancer breast were recruited from Cancer Hospital of Francisco Beltrão (CEONC), Paraná, Brazil, over the period from 2015 to 2017. We applied the following criteria for women to be included in our study: 1) volunteer patients who arrived at the CEONC with altered imaging tests indicating breast cancer (BIRADS 4 to 5) and who underwent biopsy surgery; 2) only female patients; and 3) those who presented the clinicopathological data collected from the patients' medical records. Participants meeting the following criteria were excluded: 1) those who were unable to perform the BMI calculation. Our analysis was approved by the National Commission of Ethics in Research (CONEP), protocol number 35524814.4.0000.0107 and all participants signed informed consent terms.

2.2 Study design, clinicopathological and social data

After the results of the breast biopsy, the patients were categorized as patients with benign breast disease or breast cancer patients. Patients with benign disease were compared to the group of breast cancer patients regarding plasma cortisol concentration. Patients with mammary neoplasia were selected to be categorized according to their BMI – values defined for adults by the World Health Organization– as eutrophic (BMI $\leq$ 24.9Kg/m^2), overweight (BMI $\geq$ 25.0 Kg/m^2 or $\leq$ 29.9 Kg/m^2); or obese (BMI $\geq$ 30.0 Kg/m^2) (Figure 1). Clinicopathological and social data were collected from all of the breast cancer patients' medical records to be categorized in the following groups: molecular subtypes of tumors (Luminal A for positive estrogen and/or progesterone with ki 67 index under 14%, Luminal B

for positive estrogen and/or progesterone with ki 67 index above 14%, negative estrogen and progesterone and amplified to the receptor of the human epidermal growth factor 2 (HER-2); and the triple negative, without estrogen, progesterone and HER-2 receptors); histological grade of the tumor (low, intermediate or high grade); lymph nodal metastasis (presence or absence), in addition to presence or absence of clots. Other parameters such as age (≤ 50 years or >50 years) and presence or absence of menopause at diagnosis were also considered since these may be confounding factors when studying possible prognoses in breast cancer patients. The information regarding weight and height (for calculating BMI), menopausal status and age at diagnosis were collected through an interview at the moment they were approached to participatein the project. All patients included in the study were staged as TNM II a or b.

2.3 Sample collection and cortisol measurements

At the time the participants (with or without breast cancer) were submitted to biopsy surgery, peripheral blood (10 mL) was collected through venous puncture in tubes with anticoagulant (heparin or EDTA) during the period between 2 pm to 5 pmin order to assess the systemic cortisol levels out of the morning basal cortisol range. Therefore, the protocol of sample collection was specifically designed to avoid the awakening cortisol response. Samples were then subjected to a five-minute centrifugation at 4000 rpm and the plasma frozen until analysis. Cortisol levels in samples were measured at 450 nm by using acommercial chlorimetric Enzyme-linked kit (AccuBind ELISA kit, USA).

2.4 Statistical Analysis

For the study analysis, plasma cortisol levels (μg/dL) were compared between groups of patients with benign condition and those with breast cancer. Subsequently, the cortisol levels of breast cancer patients were compared within each variable and the results are

presented as mean ± standard error (SEM). Table 1 presents the data as number and percentage of breast cancer patients according to the subgroup of each clinicopathological variable. We used the GraphPad Prism 7.0 software to analyze the quantitative variables. Firstly, we determined data distribution and groups normality. Data was assessed through Grubbs test to verify outliers. We applied the Student t test (parametric data), Mann-Whitney test (non-parametric data) or analysis of variance (ANOVA), and regression test using SPSS Statistic 20 to performe data distribution and compared the parameters according to their normality. Significant results were considered when $p \leq 0.05$.

3. RESULTS

Our study recruited 224 patients out of which 89 had a diagnosis of benign breast condition and 135 had breast cancer. Figure 1 illustrates the study design. Table 1 presents the patients' clinicopathological and social characteristics with breast cancer. Most of the patients were aged over 50 years in all groups and had tumors predominantly at intermediate histological grade (grade II). The distribution regarding the molecular subtypes of breast cancer was similar in all groups and most of the patients had been undergoing menopause at diagnosis.

Initially, we determined the circulating cortisol levels in plasma samples from all patients by comparing both benign (11.09±0.57 μg/dL) and malign conditions (11.28±0.49 μg/dL, Figure 2A). Since no differences were found in cortisol levels regarding either the presence or absence of breast cancer ($p=0.8023$), we chose to investigate only women carrying breast tumors. Figure 2B shows the distribution of cortisol levels in all patients with breast cancer enrolled in the study (11.28±0.49 μg/dL, ranging from 0.92 to 23.68 μg/dL). Regarding the analysis of cortisol levels, according to relevant clinicopathological data, when breast cancer patients were categorized according to their BMI, cortisol levels varied significantly when comparing eutrophic and obese groups (10.02±0.88 μg/dL to eutrophic,

11.98±0.83μg/dL for overweight, and 12.46±0.9 μg/dL for obese, p=0.051, Figure 2C). Based on this finding, we have decided to categorize our data according tothe BMI and establish a comparison to verify whether other clinicopathological parameters could change there after.

We found differences among the molecular subtypes of breast cancer regarding (Figure 3) their cortisol levels by comparing eutrophic and overweight patients who carried Luminal A tumors (5.9±0.82 μg/dL for eutrophic and 14.05±1.63 μg/dL for overweight, p = 0.0108, Figure 3A). For Luminal B subtype, obese women presented higher levels than either eutrophic (p=0.0211) or overweight (p=0.0191) women (16.78±1.13 μg/dL for obese, 11.2±1.5μg/dL for overweight, and 11.58±1.49 μg/dL for eutrophic, Figure 3B), while for HER-2 tumors, cortisol did not change according to the BMI group (11.07±1.82 μg/dL for eutrophic, 13.05±2.36 μg/dL for overweight, and 11.23±1.64 μg/dL for obese, Figure 3C). Overweight patients bearing triple negative tumors had augmented cortisol levels in relation to both eutrophic (p=0.0310) and obese (0.0296) groups (9.89±2.25 μg/dL for eutrophic, 17.13±1.35 μg/dL for overweight, and 10.62±1.69 μg/dL to obese, Figure 3D).

In contrast, the groups did not present different classical risk factors (Figure 4) associated with disease prognosis, like age (10.24±1.37 μg/dL in eutrophic, 13.76±1.39 for overweight and 12.63±1.52 for obese under 50 years and 10.33±1.23 μg/dL in eutrophic, 13.07±1.16 μg/dL in overweight and an 11.79±1.18 μg/dL for obese over 50 years, Figure 4A) and menopausal status (10.12±1.08 μg/dL for eutrophic, 13.06±1.16 μg/dL in overweight and 11.66±1.17 μg/dL in obese women at menopause and 11.64±2.13 μg/dL for eutrophic, 13.96±1.48 μg/dL for overweight and 13.6±1.58 μg/dL for obese women without menopause, Figure 4B).

Overweight patients bearing tumors with high histological grade (Figure 5) displayed higher cortisol levels (16.9±1.48 μg/dL) in relation to both eutrophic and obese women (9.17±2.52 μg/dL, p=0.0401 for eutrophic, 10.21±1.19μg/dL, p=0.0024 for obese, Figure 5C).

Eutrophic patients with lymph nodal invasion (Figure 6A) presented reduced cortisol (7.6±1.74 μg/dL) in relation to those who were either overweight (12.54±1.45 μg/dL, p=0.0449) or obese (13.82±1.15 μg/dL, p=0.0052). Regarding the presence of intratumoral clots (Figure 6B), cortisol was found significantly increased in obese women with clots inside tumor vessels in relation to the eutrophic ones (8.14±1.52 μg/dL for eutrophic p=0.0060, 11.86±1.20for overweight, and 14.05±1.22μg/dL for obese). In contrast, overweight (13.48±1.43 μg/dL, p<0.0001) and obese patients (10.25±1.40 μg/dL, p<0.0001) presented significantly reduced cortisol levels compared to eutrophic patients (21.71±0.40 μg/dL) in the absence of clots (data not shown).

Significant correlations between cortisol and lymph nodal invasion (R=0.406 and p=0.035) (Table 2) were found in eutrophic patients. For obese women, significant correlations were found regarding cortisol and histological grades (R=0.411 and p=0.031), as well as between cortisol and clots (R=0.418 ad p=0.037). Linear data regression (Table 3) revealed the existence of important associations of cortisol levels in eutrophic patients regarding lymph nodal invasion (R=-0.429, p=0.032) and obese patients for histological grade (R=0.366, p=0.028), in addition to the presence of clots (R=0.368, p=0.035).

4. DISCUSSION

Our study indicated that systemic cortisol levels are dysregulated in women with breast cancer out of the morning basal cortisol peak and correlate to poor prognosis

parameters in those with excessive body fat. From the best of our knowledge, this represents the first report showing such an association in the literature.

In breast cancer cases, patients may experience some changes in serum cortisol levels during the course of the disease[29], as previously demonstrated [30]. Our study found no difference between patients with or without breast cancer regarding circulating cortisol concentrations. The women included in our investigation were diagnosed during the early stages of the disease, with operable tumors, which may affect cortisol levels in some manner. Although the literature presents few data regarding cortisol status in breast cancer, it is known that changes in cortisol levels are more significant in patients with advanced disease [29,31], which is related to both early mortality [32] and poor prognosis[33].

However, cortisol categorization results through BMI revealed the existence of distinct levels among euthophic, overweight, and obese patients. It is well-established the association between hypercortisolism and obesity[34,35]; however, it is not clear in the case of breast cancer. The available data in the literature is quite old and have conflicting information. For example, although it was demonstrated that obese women with breast cancer can have high levels of free cortisol [36], no association was reported for cortisol and obesity in obese women with metastatic breast cancer[37]. Thus, this study complements the literature regarding the understanding on cortisol patterns in the plasma of women with breast cancer carrying tumors during the early disease stages.

Breast cancer is not the only disease but at least four distinct molecular subtypes determine distinct prognosis (good for hormone positive tumors with low proliferative rate and poor prognosis for hormone negative tumors or positive with high proliferation). In this context, we investigated the differential expression of circulating cortisol and the molecular subtypes according to patients' BMI and found that eutrophic patients bearing tumors with Luminal A subtype presented significantly lower cortisol levels than those with overweight.

This suggests that a better clinical prognosis could be associated with low levels of circulating cortisol.

Ingestion of exogenous estrogen increases circulating cortisol [38], so it is expected that patients with Luminal A tumors, who have an increased expression of estrogenic receptors[39–42], have high levels of circulating cortisol. The eutrophic patients had reduced levels of circulating cortisol when compared to overweight patients, suggesting that the presence of a dependent estrogen tumor is not able to produce significant increases in circulating cortisol in patients with normal BMI. However, we observed that overweight patients had twice as much circulating cortisol as eutrophic ones, which is in accordance withdata from the literature regarding excess adipose tissue generatinga low-grade inflammatory process to deregulate the cortisol axis. In this context, we seek to understand why obese patients did not present such a response pattern. According to the literature, overweight and obese people have different metabolic profile san expressions of several proteins, characterizing overweight as an altered pattern of signaling pathways and cell-cell interaction, while obesity has a deregulation of biochemical pathways[43].

We also found that overweight breast cancer patients had higher levels of cortisol than either obese or eutrophic patients who carriedtriple negative tumors. Furthermore, the obese group had increased levels of circulating cortisol in patients with Luminal B tumors– significantly different from eutrophic and overweight patients. Other studies corroborated with our findings and proved an association of obesity with triple negative and Luminal B tumors[44–46]. Studies suggest that overweight is a risk factor for tumor development of poor prognosis since excessive fat would affect the response to treatment, tumor growth, proliferation, and metastatization[47–49], thus favoring luminal B and triple negative tumors [44–46]. Possible mechanisms may include proinflammatory mediators capable to sustainchronic inflammation and potentiating Th1 cytokines, which are well-established as

cancer promoters[50,51]. In addition,it has been demonstrated that BMI is related to molecular subtypes of breast cancer when considering that each subtype has a different metabolic pattern. Patients with excess fat appear to be more likely to develop triple-negative tumors resulting from the chronicity of low-grade inflammation, which would lead to changes in molecular patterns[52], such as metabolic reprogramming[53]. In contrast, the presence of luminal subtype in these same patients is associated with positive expression of estrogen receptors and insulin resistance[52].

Our data also showed an association among high grade tumors, high circulating cortisol, and excessive body fat in breast cancer patients. The cortisol axis has an effect on cancer progression by affecting the tissue morphological characteristics ofthe tumor nuclear grade. Although it is not established for breast cancer, a study carried out with patients with polyps and colorectal carcinomas shows that the expression of 11-HSD1 and 11-HSD2 enzymes, responsible for the local production of cortisol in tumors, is increased in patients with carcinoma, but reduced in patients atthe pre-malignant stages[54], which could result in high cortisol production in patients with high histological grade tumors.A study involving patients with renal cell carcinoma revealed a correlation between circulating cortisol levels and tumor grade, in which those with poorly differentiated tumors had higher cortisol levels[55]. Therefore, our data suggest that the combination among breast cancer, excessive body fat, and high circulating cortisol may favor less differentiated tumor in such patients. The more undifferentiated the more aggressive cancer cells; in addition, invasion and metastasis may also emerge.

In our study, overweight and obese patients presenting lymph nodal metastasis had higher cortisol levels in relation to the eutrophic ones. Metastasis is the clinical parameter related to cancer evolution and patient survival. In fact, changes in cortisol levels have been related to such an event[33,56]. Women with metastatic breast cancer and higher mean

concentrations of day-time cortisol have important suppression of immune functions[57], which could foment cancer spreading to lymph nodes. Although our data indicated a distinct profiling among eutrophic, overweight and obese women, we found no differences in cortisol when comparing lymph nodal status within groups, except for eutrophics (unmarked data in graph with *). These findings indicate that in overweight/obese women, once the signaling triggered by excessive fat is installed, cortisol levels remain unaltered regardless of lymphnodal invasion.

The literature reports that the presence of emboli in cancer patients is a risk factor of increased mortality [58]. By comparing the groups categorized according to the BMI, we found that obese patients presented increased levels of cortisol in relation to eutrophic patients. In the presence of emboli, excessive fat and cortisolare triggered by mechanisms that lead to endothelial dysfunction, which, in turn, alters vascular functioning[59,60]. Thus, our results suggest that cortisol levels found in obese patients favor the formation of emboli, which would lead these patients to a worse prognosis. We also found high cortisol levels in eutrophic patients without clots in relation to the remaining groups (unmarked data in graph with*). Even though inflammation intensifies endothelium reactivity, our data suggest these levels of cortisol disfavor clot formation in eutrophic women with breast cancer.

The main limitations of our study include limited sample size, lack of repeated measures of cortisol in blood, and the need of cortisol evaluation in other samples, such as saliva or tumors. Furthermore, we found that the literature has very few records on circulating cortisol and breast cancer prognosis.

5. CONCLUSIONS

This results indicates that excessive body fat have a negative effect on the disease prognosis as well as that cortisol levels can be associated with poor prognosis in breast

cancer. Thus, for further investigations and evidence, the calculation of BMI and plasma cortisol dosage in these patients may be used as parameters to evaluate the prognosis of breast cancer and consequently influence medical conducts.

Declaration of interest

The authors declare the absence of conflict of interest that could impair the impartiality of the research reported.

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Author contributions

WRP and CP designed the experiments and data analysis. ACBK carried out cortisol measurements and collected the data. EDM cortisol measurements. DR, oncologist, sample obtention. All authors reviewed and edited the manuscript as well ashad final approval of the submitted version.

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FIGURES

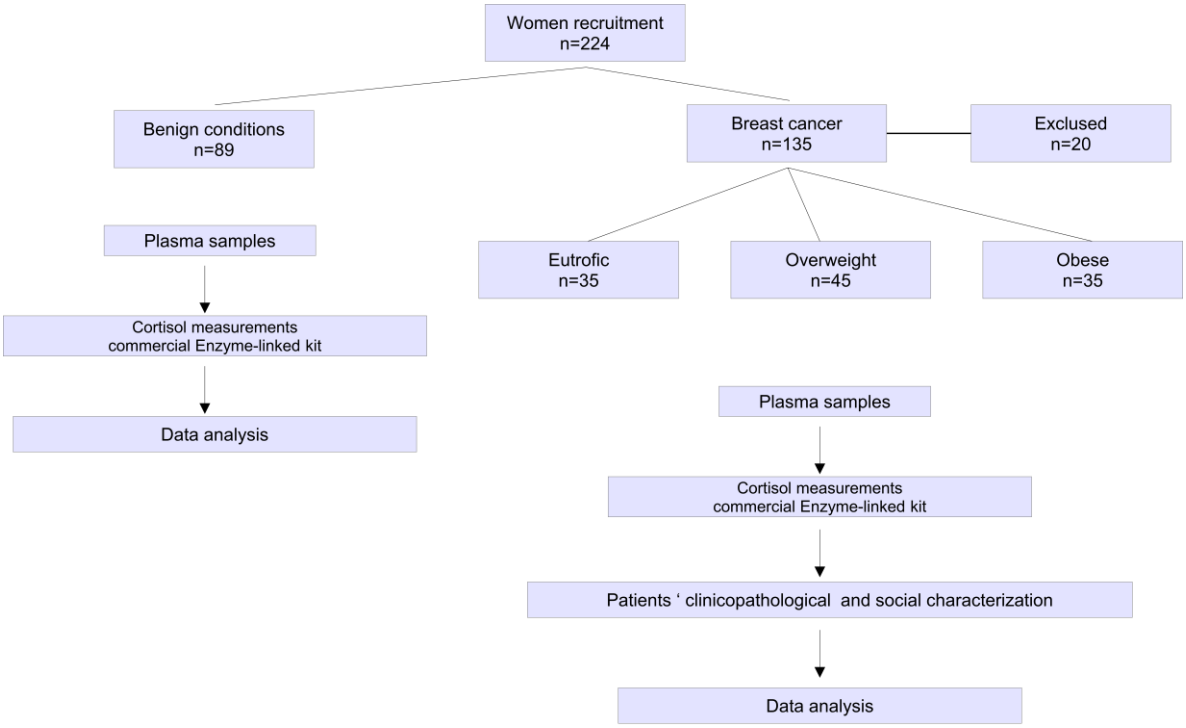

Figure 1 – Design of the study.

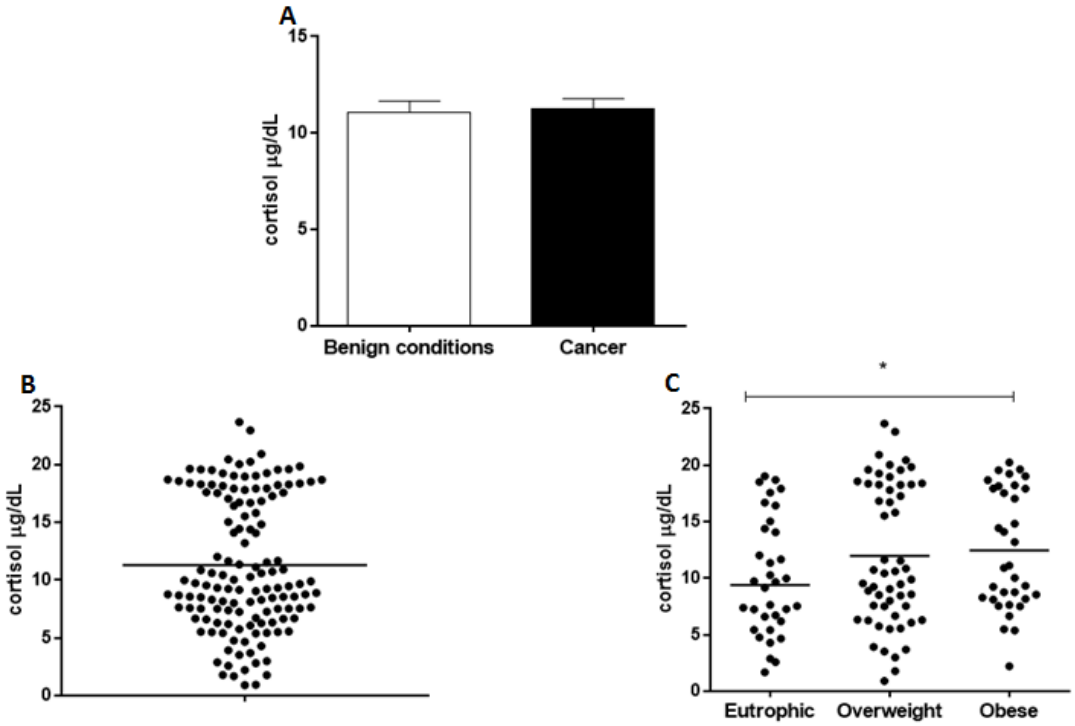

Figure 2 – Circulating cortisol levels. In A, a comparative analysis of cortisol levels in plasma from women with bening breast conditions and women with breast cancer. In B, is shown the dispersion of cortisol levels in all enrolled breast cancer patients. In C, cortisol levels were significantly different when breast cancer patients were categorized according to body mass index (BMI). * $p \leq 0.05$ vs. eutrophic

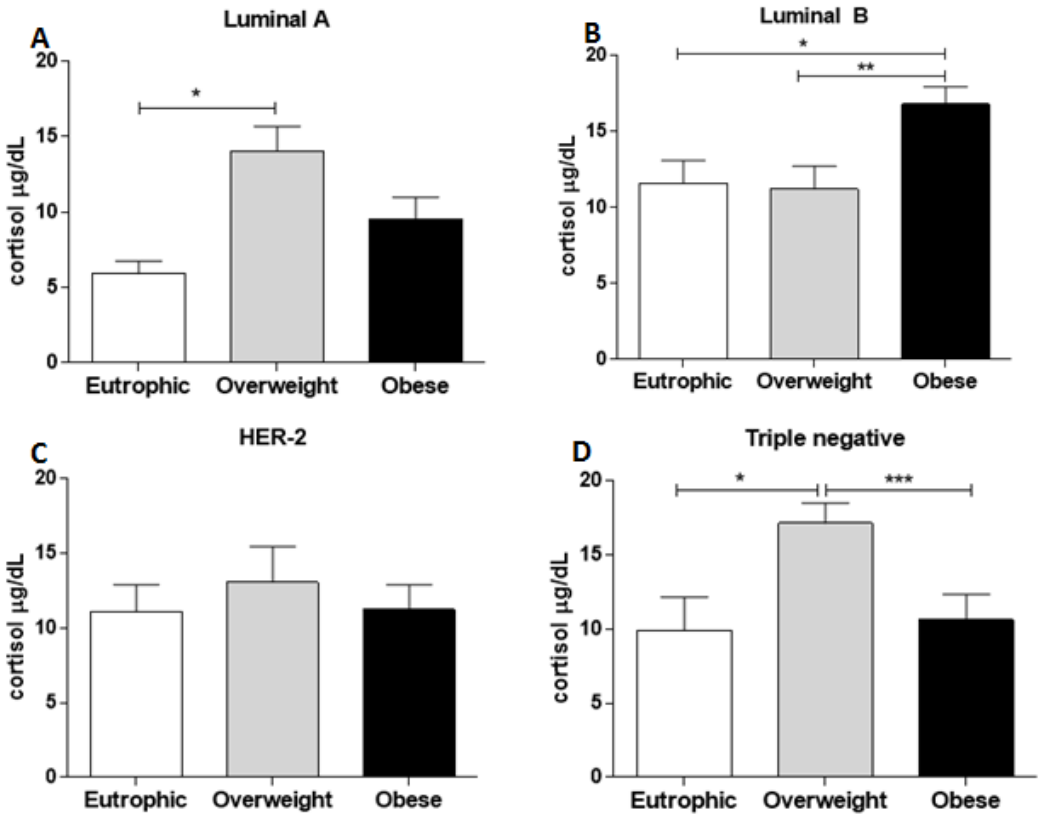

Figure 3 – Comparative analysis of circulating cortisol levels in patients with breast cancer, according to its molecular subtypes and body mass index (BMI) categories. Cortisol levels in plasma from breast cancer patients bearing tumors subtyped as Luminal A (A), Luminal B (B), HER-2 amplified (C) and Triple negative (D). * p≤ 0.05 vs. eutrophic; ** p≤ 0.05 vs. overweight, *** p≤ 0.05 vs. obese.

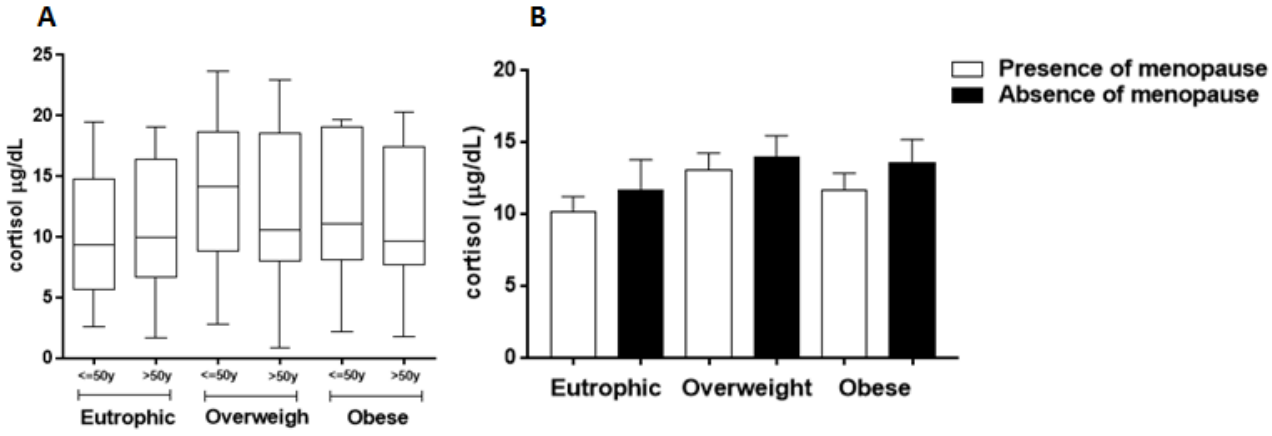

Figure 4 – Circulating cortisol levels in patients with breast cancer, according to age at diagnosis (A) and menopausal status (B), considering the body mass index (BMI) categories. Patients were categorized according to age at diagnosis in ≤ 50 years (<=50y) or > 50 years (>50y) and according to presence or absence of menopause at diagnosis.

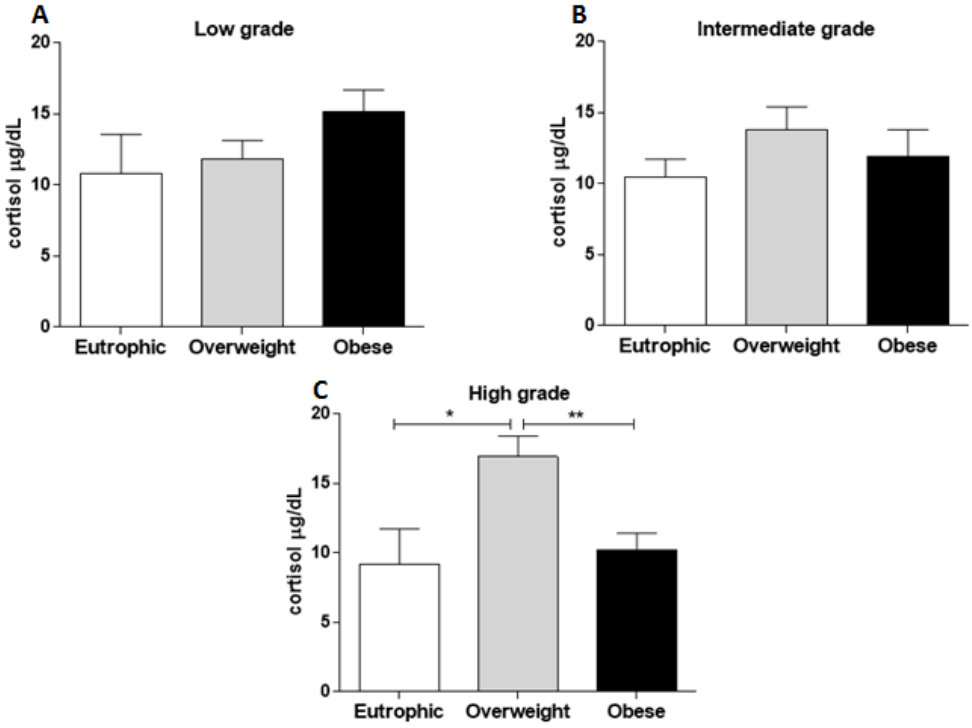

Figure 5 – Cortisol levels and histological grade in tumors from patients with breast cancer. Cortisol levels from different body mass index (BMI) categories are shown according tumor histological grade as low (A), intermediate (B) and high (C) grade. * p≤ 0.05 vs. eutrophic; ** p≤ 0.05 vs. obese.

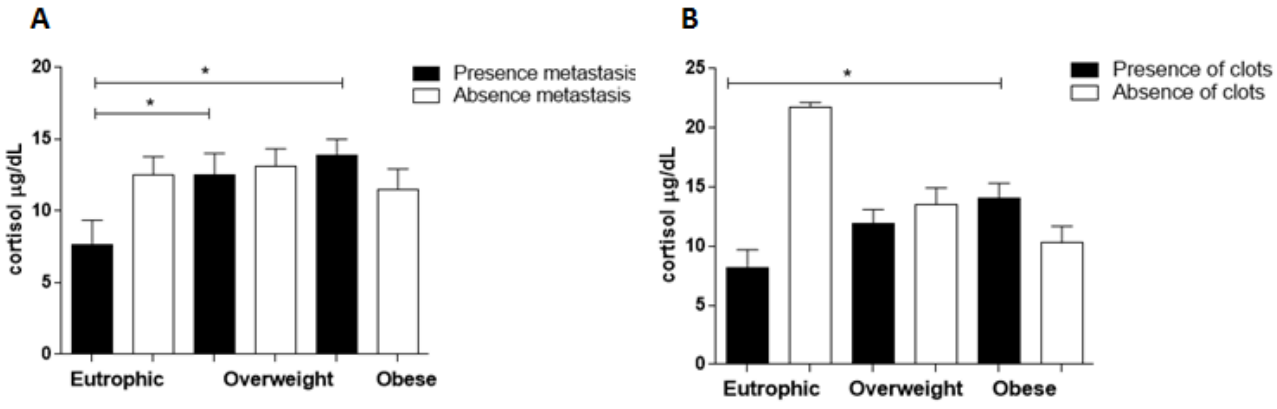

Figure 6 – Lymphonodal metastasis, clots and cortisol levels in plasma samples from patients with breast cancer according to body mass index (BMI) categories. Cortisol levels in plasma from breast cancer patients according presence or absence of lymphonodal metastasis (A) and presence or absence of clots (B). * p≤ 0.05.vs. eutrophic.

TABLE

Table 1 - Patients' clinicopathological and social data with breast cancer

Subgroups	**Eutrophic**	**Overweight**	**Obese**
Number of individuals	**N=35 (30.4%)**	**N=45 (39.1%)**	**N=35 (30.4%)**
Age at diagnosis			
≤ 50 anos	16 (45.7%)	18 (40.0%)	15 (42.9%)
> 50 anos	19 (54.3%)	27 (60.0%)	20 (57.1%)
Histological grade			
Grade I	04 (13.3%)	21 (47.7%)	11 (32.3%)
Grade II	19 (63.3%)	15 (34.1%)	11 (32.3%)
Grade III	07 (23.3%)	08 (18.2%)	12 (35.3%)
Molecular subtype			
Luminal A	05 (15.1%)	15 (35.0%)	07 (21.2%)
Luminal B	12 (36.4%)	14 (32.5%)	08 (24.2%)
HER -2	07 (21.2%)	08 (18.6%)	04 (12.1%)
Triple negative	09 (27.3%)	09 (13.9%)	14 (42.4%)
Lymph nodes metastasis			
Absence	16 (64.0%)	27 (64.3%)	18 (54.5%)
Presence	09 (36.0%)	15 (35.7%)	15 (45.5%)
Clots			
Absence	18 (64.3%)	20 (51.3%)	16 (50.0%)
Presence	10 (35.7%)	19 (48.7%)	16 (50.0%)
Menopause at diagnosis			
Absence	09 (28.1%)	17 (38.6%)	11 (32.4%)
Presence	23 (71.9%)	27 (61.4%)	23 (67.6%)

Table 2 - Correlations among cortisol levels and clinicopatological parameters in patients with breast cancer

	Eutrophic	Overweight	Obese
Age atdiagnosis	p=0.961	p=0.866	p=0.659
	R=0.420	R=0.437	R=0.423
Histological grade	p=0.606	p=0.189	**p=0.031***
	R=0.403	R=0.428	R=0.411
Molecular subtype	p=0.563	p=0.688	p=0.339
	R=0.386	R=0.412	R=0.393
Lymphnodesmetastasis	**p=0.035***	p=0.931	p=0.398
	R=0.406	R=0.433	R=0.420
Clots	p=0.128	p=0.981	**p=0.037***
	R=0.411	R=0,432	R=0.418
Menopause at diagnosis	p=0.788	p=0.978	p=0.949
	R=0.410	R=0.429	R=0.413

Data are shown as p values (p) and Pearsons Chi-Square (R). *p ≤ 0.05

Table 3 – Linear regression concerning cortisol levels and patients' clinicopathological parameters

Regression	**R**	**Beta**	**p values**
Eutrophic			
Lymph node metastasis	**0.429**	**Negative**	**0.032***
Obese			
Histological grade	**0.366**	**Negative**	**0.028***
Presence of clots	**0.368**	**Positive**	**0.035***

Data are shown as p values (p) and Pearsons Chi-Square (R). *p ≤ 0.05

CONCLUSÃO

- Mais da metade das mulheres com suspeita de neoplasia mamária atendidas pelo Hospital de Câncer de Francisco Beltrão no período estudado apresentou câncer de mama nas biópsias. Foram observadas características clinicopatológicas relacionadas ao pior prognóstico como: prevalência de subtipos de maior agressividade, presença de invasão linfonodal, presença de êmbolos angiolinfáticos e tumores indiferenciados.

- A maior parte das pacientes estudadas está exposta diretamente aos agrotóxicos, de maneira continuada.

- Pacientes portadoras de câncer de mama em menopausa quando expostas ocupacionalmente aos agrotóxicos apresentam desregulação imunoendócrina, além de variações no perfil redox quando comparadas àquelas não expostas. Além disso, a exposição gerou alterações nos perfis imunológico e redox quando as pacientes apresentavam características de pior prognóstico como: excesso de peso corporal, tumores de grau histológico avançado e presença de êmbolos angiolinfáticos.

- A exposição ocupacional aos agrotóxicos de forma continuada parece ser um fator determinante para o desenvolvimento de neoplasias mamárias de pior prognóstico, cujo mecanismo potencialmente associado seja a desregulação imunoendócrina e o desbalanço redox (Figura 1).

- Os níveis circulantes de cortisol em pacientes portadoras de câncer de mama, independente da exposição aos pesticidas, mostraram-se relacionados a parâmetros de pior prognóstico em pacientes com sobrepeso e obesidade como: presença de subtipo de maior agressividade, tumores de grau histológico avançado, presença de metástase em linfonodo e êmbolos angiolinfáticos.

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ANEXOS

ANEXO A - Parecer Consubstanciado do CEP

UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ/

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Mapeamento do câncer de mama familial no sudoeste do Paraná e estudo de associação de risco coma exposição ocupacional à agrotóxicos.

Pesquisador: CAROLINA PANIS

Área Temática:

Versão: 1

CAAE: 35524814.4.0000.0107

Instituição Proponente: UNIVERSIDADE ESTADUAL DO OESTE DO PARANA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 810.501

Data da Relatoria: 25/09/2014

Apresentação do Projeto:

Neste estudo pretende-se avaliar todas as mulheres diagnosticadas com câncer de mama, atendidas no Hospital de Câncer de Francisco Beltrão (Ceonc), em um período de 48 meses. A partir da análise de anotações em prontuários serão selecionadas para investigação dos genes de interesse aquelas mulheres com história de câncer de mama familial com ou sem exposição ocupacional à agrotóxicos. Atende aos requisitos teóricos, metodológicos e éticos.

Objetivo da Pesquisa:

Mapear os casos de câncer de mama familial na região Sudoeste do Paraná e identificar possível associação a exposição ocupacional à agrotóxicos.

Avaliação dos Riscos e Benefícios:

Não há riscos diretos aos sujeitos, uma vez que serão estudados materiais coletados durante cirurgias oncológicas.

Comentários e Considerações sobre a Pesquisa:

Relevante para a área de oncologia.

Considerações sobre os Termos de apresentação obrigatória:

Todos apresentados.

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

UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ/

Continuação do Parecer: 810.501

Recomendações:

Não há recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado. O projeto não necessita adequações.

CASCAVEL, 29 de Setembro de 2014

Assinado por:
João Fernando Christofoletti

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

ANEXO B - 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 aos agrotóxicos.*

Pesquisador responsável: Profª Drª CAROLINA PANIS – Telefones (43)99165316 e (46) 30571079

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) e um tubo de saliva (1 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:
CPF:

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

Data:

ANEXO C - Questionário para categorização de exposição aos pesticidas

IDENTIFICAÇÃO DO PACIENTE

NOME	Data de nascimento __/__/___	Sexo: (_) F ( )M
ENDEREÇO		
MUNICÍPIO	TELEFONE (__)	

IDENTIFICAÇÃO DAS PESSOAS QUE VIVEM JUNTAS

PACIENTE	IDADE						OCUPAÇÃO		
	1		0-10	7		61-70	1		Trabalhador rural
	2		11-20	8		+ 70	2		Trabalhador urbano
	3		21-30				3		Estudante
	4		31-40				4		Do lar
	5		41-50				5		Trabalhador rural aposentado
	6		51-60				6		Trabalhador urbano aposentado
FAMILIAR 1 (Nome e parentesco)	IDADE						OCUPAÇÃO		
	1		0-10	7		61-70	1		Trabalhador rural
	2		11-20	8		+ 70	2		Trabalhador urbano
	3		21-30				3		Estudante
	4		31-40				4		Do lar
	5		41-50				5		Trabalhador rural aposentado
	6		51-60				6		Trabalhador urbano aposentado
FAMILIAR 2 (Nome e parentesco)	IDADE						OCUPAÇÃO		
	1		0-10	7		61-70	1		Trabalhador rural
	2		11-20	8		+ 70	2		Trabalhador urbano
	3		21-30				3		Estudante
	4		31-40				4		Do lar
	5		41-50				5		Trabalhador rural aposentado
	6		51-60				6		Trabalhador urbano aposentado
FAMILIAR 3 (Nome e parentesco)	IDADE						OCUPAÇÃO		
	1		0-10	7		61-70	1		Trabalhador rural
	2		11-20	8		+ 70	2		Trabalhador urbano
	3		21-30				3		Estudante
	4		31-40				4		Do lar
	5		41-50				5		Trabalhador rural aposentado
	6		51-60				6		Trabalhador urbano aposentado
FAMILIAR 4 (Nome e parentesco)	IDADE						OCUPAÇÃO		
	1		0-10	7		61-70	1		Trabalhador rural
	2		11-20	8		+ 70	2		Trabalhador urbano
	3		21-30				3		Estudante
	4		31-40				4		Do lar
	5		41-50				5		Trabalhador rural aposentado
	6		51-60				6		Trabalhador urbano aposentado
FAMILIAR 5 (Nome e parentesco)	IDADE						OCUPAÇÃO		
	1		0-10	7		61-70	1		Trabalhador rural
	2		11-20	8		+ 70	2		Trabalhador urbano
	3		21-30				3		Estudante
	4		31-40				4		Do lar
	5		41-50				5		Trabalhador rural aposentado
	6		51-60				6		Trabalhador urbano aposentado

1. Em que município você mora?

1		Ampére	12		Manfrinópolis	23		Salto do Lontra
2		Barracão	13		Marmeleiro	24		Santa Izabel do Oeste
3		Bela Vista da Caroba	14		Nova Esperança do sudoeste	25		Santo Antônio do Sudoeste
4		Boa Esperança do Iguaçu	15		Nova Prata Do Iguaçu	26		São Jorge D'oeste
5		Bom Jesus do Sul	16		Pérola D'oeste	27		Verê
6		Capanema	17		Pinhal de São Bento	28		Outro do Paraná
7		Cruzeiro do Iguaçu	18		Planalto	29		Rio Grande de Sul
8		Dois Vizinhos	19		Pranchita	30		Santa Catarina
9		Éneas Marques	20		Realeza	31		Outro estado do Brasil
10		Flor da Serra Do Sul	21		Renascença			
11		Francisco Beltrão	22		Salgado Filho			

2. Onde mora?

1		Cidade	2		Rural/ interior

3. Há quantos anos mora nesse local?

1		1-5	5		21-25	9		41-45
2		6-10	6		26-30	10		46-50
3		11-15	7		31-35	11		+ de 51
4		16-20	8		36-40			

4. Tem vaca leiteira?

1		Não	2		Sim

5. Produtos que a família planta com agrotóxicos

1		Soja/Milho	5		Fumo	9		Hortaliças
2		Soja/Trigo	6		Pastagem	10		Outros
3		Milho/pastagem	7		Feijão	11		Frutas e Hortaliças
4		Milho/trigo	8		Frutas	12		Não se aplica

6. Produtos que planta e consome com agrotóxicos

1		Milho	5		Fumo
2		Feijão	6		Outros
3		Frutas	7		Frutas e Hortaliças
4		Hortaliças	8		Não se aplica

7. Atualmente você aplica algum tipo de veneno em horta, flores ou lavoura? (Se sim, preencher o QUADRO 1)

1		Não	2		Sim

8. Sua família usa veneno hoje? (Se sim, preencher o QUADRO 1)

1		Não	2		Sim

9. Quem aplica esses produtos?

1		Pai	3		Irmãos	5		Marido	7		Não sabe	9		Outros
2		Mãe	4		Avô	6		Filhos	8		Não se aplica	10		

QUADRO 1 – EXPOSIÇÃO A AGROTÓXICOS NO PRESENTE

NOME DO AGROTÓXICO	CULTIVO	PERÍODO		QUANTIDADE USADA	APLICA?
		Mês	Nº. Dias		

10. Já morou em outro município?

1		Não	2		Sim

11. Se sim, onde?

1		Ampére	12		Manfrinópolis	23		Salto do Lontra
2		Barracão	13		Marmeleiro	24		Santa Izabel do Oeste
3		Bela Vista da Caroba	14		Nova Esperança do sudoeste	25		Santo Antônio do Sudoeste
4		Boa Esperança do Iguaçu	15		Nova Prata Do Iguaçu	26		São Jorge D'oeste
5		Bom Jesus do Sul	16		Pérola D'oeste	27		Verê
6		Capanema	17		Pinhal de São Bento	28		Outro do Paraná
7		Cruzeiro do Iguaçu	18		Planalto	29		Rio Grande de Sul
8		Dois Vizinhos	19		Pranchita	30		Santa Catarina
9		Enéas Marques	20		Realeza	31		Outro estado do Brasil
10		Flor da Serra Do Sul	21		Renascença	32		Não morou
11		Francisco Beltrão	22		Salgado Filho			

12. Você já viveu no rural/campo?

1		Não	2		Sim

13. Se sim, por quantos anos?

1		1-5	5		21-25	9		41-45
2		6-10	6		26-30	10		46-50
3		11-15	7		31-35	11		+ de 51
4		16-20	8		36-40	12		Não se aplica

14. Nos lugares onde viveu, sua família usava veneno? (se sim preencher quadro 2)

1		Não	2		Sim

15. Você já aplicou veneno? (Se sim, preencher o quadro 2)

1		Não	2		Sim

QUADRO 2 – EXPOSIÇÃO A AGROTÓXICOS NO PASSADO

NOME DO AGROTÓXICO	CULTIVO	PERÍODO		QUANTIDADE USADA	APLICA?
		Mês	Nº. Dias		

16. Por quantos anos você aplicou veneno?

1		1-5	5		11-25	9		41-45
2		6-10	6		26-30	10		46-50
3		11-15	7		31-35	11		+ de 51
4		16-20	8		36-40	12		Não se aplica

17. Quem da sua família também aplicava veneno?

1		Pai	3		Irmão/irmã	5		Marido	7		Não sabe
2		Mãe	4		Avô/avó	6		Filho/filha	8		Não se aplica

18. Por quantos anos?

1		1-5	5		21-25	9		41-45
2		6-10	6		26-30	10		46-50
3		11-15	7		31-35	11		+ de 51
4		16-20	8		36-40	12		Não se aplica

19. Você lava roupas de algum familiar após aplicação de veneno?

1		Não	2		Sim	3		Não se aplica

20. Você lavava roupas de algum familiar após aplicação de veneno?

1		Não	2		Sim	3		Não se aplica

21. Você usava ou usa luvas para lavar essas roupas?

1		Não	2		Sim	3		Não se aplica

22. Você lavava ou lava essas roupas contaminadas junto com as demais roupas da família?

1		Não	2		Sim	3		Não se aplica

23. Algum vizinho seu usa veneno?

1		Não	2		Sim	3		Não sabe

24. Algum vizinho seu usava veneno?

1		Não	2		Sim	3		Não sabe

25. Você utiliza ou utilizava Equipamento de Proteção ao usar o veneno?

1		Não	2		Sim	3		Não se aplica

26. Outras pessoas da sua família utilizam ou utilizavam Equipamento de Proteção ao usar o veneno?

1		Não	2		Sim	3		Não se aplica

27. Se sim, quais?

1		TODOS	6		ÓCULOS
2		LUVAS	7		MÁSCARA
3		BOTAS	8		OUTROS
4		LUVAS/BOTAS/MÁSCARA	9		Não se aplica
5		MACACÃO			

28. Você percebia alguma mudança (cheiro, tonturas, dor de cabeça, etc.) quando tinha contato direto ou indireto com veneno?

1		Não	2		Sim	3		Não se aplica

Se sim, o que? _____

29. E seus familiares já apresentaram esses sintomas?

Se sim, o que? _____

30. Você se alimentava com o que era produzido na sua propriedade com veneno?

1		Não	2		Sim	3		Não se aplica

Se sim, quais?

1		Milho	5		Fumo
2		Feijão	6		Outros
3		Frutas	7		Frutas e Hortaliças
4		Hortaliças	8		Não se aplica

De onde vem água que bebe?

1		Poço particular	4		Rede
2		Poço da comunidade	5		Rio
3		Fonte	6		Água mineral

32. Distância desta água das áreas de lavoura.

1		Até 5metros	4		21 a 30 m
2		6 a 10 m	5		Mais de 31 m
3		11 a 20 m	6		Não se aplica

SAÚDE E INTOXICAÇÃO

33. Você já apresentou alguma intoxicação por veneno?

1		Não	2		Sim

34. Quantas vezes?

1		1 vez	4		4 vezes	7		Nunca se intoxicou
2		2 vezes	5		5 vezes			
3		3 vezes	6		+ de 5 vezes			

35. Quais sintomas apresentou?

1		Gastrointestinais	6		Não lembra
2		Alteração sensorial / neurológica	7		Outros, quais?
3		Alteração de pele	8		Gastrointestinais, alteração de pele e respiratória
4		Alteração cardiovascular	9		Não se aplica
5		Alteração respiratória			

36. Local aonde foi atendido

1		Hospital	4		Consultório Particular
2		Unidade de saúde	5		Não procurou atendimento de Saúde
3		Centro de Urgência e Emergência	6		Não se aplica

37. Alguém da sua família já apresentou intoxicação por veneno?

1		Não	2		Sim	3		Não sabe

38. Quem?

1		Pai	3		Irmão/irmã	5		Marido	7		Outros	9		Não se aplica
2		Mãe	4		Avô/avó	6		Filho/filha	8		Não sabe			

39. Quantas vezes?

1		1 vez	4		4 vezes	7		Não se aplica
2		2 vezes	5		5 vezes	8		
3		3 vezes	6		+ de 5 vezes	9		

40. Quais sintomas apresentou?

1		Gastrointestinais	6		Não lembra
2		Alteração sensorial / neurológica	7		Outros, quais?
3		Alteração de pele	8		Gastrointestinais, alteração de pele e respiratória
4		Alteração cardiovascular	9		Não se aplica
5		Alteração respiratória			

41. Local aonde foi atendido

1		Hospital	4		Consultório Particular
2		Unidade de saúde	5		Não procurou atendimento de Saúde
3		Centro de Urgência e Emergência	6		Não se aplica

42. Você possui alguma doença?

1		Não	2		Sim

43. Se sim, qual (is)?

_____

_____

_____

44. Alguém da sua família possui alguma doença? Se sim preencher QUADRO 3

1		Não	2		Sim

QUADRO 3

Membro da família	Doença	Há quanto tempo			
			0 a 1 ano		2 a 3 anos
			4 a 5 anos		+ de 5 anos
			0 a 1 ano		2 a 3 anos
			4 a 5 anos		+ de 5 anos
			0 a 1 ano		2 a 3 anos
			4 a 5 anos		+ de 5 anos

45. Você já teve câncer de mama ou outro tipo de câncer?

1		Não	2		Sim

46. Se sim, qual?_____

47. Alguém da sua família já teve câncer? Se sim preencher quadro 4

1		Não	2		Sim

QUADRO 4

Membro da família	Tipo de Câncer	Resultado
		( ) cura / ( ) sequela / ( ) morte
		( ) cura / ( ) sequela / ( ) morte
		( ) cura / ( ) sequela / ( ) morte

48. Você já teve perda de movimentos, formigamento, visão embaraçada, perda da força muscular ou alteração de sensibilidade?

1		Não	2		Sim

49. Se sim, qual problema?_____

50. Você nasceu com alguma malformação?

1	Não	2	Sim

51. Se sim, qual?_____

52. Alguém da sua família nasceu com alguma malformação? Se sim preencher quadro 5

1	Não	2	Sim	3	Não sabe

QUADRO 5

Membro da familia	Tipo de malformação

53. Você já teve aborto espontâneo?

1	Não	2	Sim

54. Alguém da sua família já teve aborto espontâneo?

1	Não	2	Sim	3	Não sabe

55. Você teve alguma dificuldade para engravidar?

1	Não	2	Sim

56. Alguém da sua família teve alguma dificuldade para engravidar?

1	Não	2	Mãe	3	Irmã	4	Filha	5	Avó	6	Outros	7	Não sabe

57. Você foi exposta ao veneno durante a gestação dos filhos (gravidez)?

1	Não	2	Sim

58. O esposo foi exposto ao veneno antes da concepção dos filhos?

1	Não	2	Sim

59. Você fez alguma vez na vida exame de sangue para avaliar a exposição ao veneno (colinesterase)?

1	Não	2	Sim

60. Alguém na sua família fez alguma vez na vida exame de sangue para avaliar a exposição ao veneno (colinesterase)?

1	Não	2	Sim

ANEXO D - Carta de submissão do artigo: Systemic cortisol and poor prognosis in overweight/obese women with breast cancer

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Title: Systemic cortisol and poor prognosis in overweight/obese women with breast cancer.
Corresponding Author: CAROLINA PANIS
Co-Authors: Aedra C Kawassaki; Elaine M Dias; Daniel Rech; Wander R PAVANELLI

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