Universidade Estadual de Londrina

WALISON AUGUSTO DA SILVA BRITO

ANÁLISE DO ENVOLVIMENTO DE MARCADORES SISTÊMICOS DE ESTRESSE OXIDATIVO E DE PROCESSO INFLAMATÓRIO NO DESENVOLVIMENTO DE MÚLTIPLOS TIPOS DE CÂNCER DE PELE NÃO-MELANOMA

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de mestre em Patologia Experimental.

Orientadora: Profa. Dra. Alessandra Lourenço Cecchini Armani
Co-orientadora: Profa. Dra. Poliana Camila Marinello

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“Aprender é a única coisa que a mente não se cansa, nunca tem medo e nunca se arrepende”

(DA VINCI, Leonardo).

BRITO, Walison Augusto da Silva. **Análise do envolvimento de marcadores sistêmicos de estresse oxidativo e de processo inflamatório no desenvolvimento de múltiplos tipos de câncer de pele não-melanoma.** 2020. 66 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2020.

RESUMO

O câncer de pele não-melanoma (CPNM) é a neoplasia mais incidente no mundo. O tipo mais comum é o carcinoma basocelular (CBC), seguido pelo carcinoma espinocelular (CEC), que por sua vez, pode originar-se de uma lesão pré-neoplásica denominada queratose actínica (QA). Sabe-se que o estresse oxidativo (EO) tem um papel importante na carcinogênese da pele, que ocorre principalmente devido à radiação ultravioleta, que também é capaz de induzir processo inflamatório, ambos relacionados com a iniciação, promoção e a progressão tumoral. Entretanto, não está claro se marcadores sistêmicos de EO e do processo inflamatório estão associados com a presença de único e múltiplos tipos de CPNM. Assim, este trabalho tem como objetivo investigar marcadores sistêmicos de EO e do processo inflamatório em pacientes com QA e CPNM que poderiam estar associados com a presença de múltiplos tipos de CPNM em um mesmo paciente. Participantes foram categorizados em 6 grupos: Controle (sem câncer, n=75), CBC (n=90), CEC (n=24), QA (n=19), CEC+CBC (n=13) e CEC+CBC+QA (n=36) (CEP-UEL n. processo 1.077.557 e n. 3.146.725). Três comparações foram feitas entre os grupos: 1) Lesões de CPNM de único tipo: controle vs CBC vs CEC; 2) Diferença entre lesão pré-maligna e CEC: controle vs QA vs CEC; 3) Lesões de múltiplos tipos de CPNM: CBC vs CEC vs CEC+CBC vs CEC+CBC+QA. Amostras de sangue foram coletadas, processadas e armazenadas para posteriores análises. Dados qualitativos foram analisados pelo teste de Qui-quadrado e pelo teste z para comparar proporções. Dados quantitativos foram analisados pelo ANOVA (one-way) ou Kruskal-Wallis depois da verificação da normalidade e homogeneidade dos dados. Todos os resultados com $p<0,1$ na análise univariada foram incluídos no modelo multinomial de regressão logística. $p<0,05$ foi considerado significativo. Na comparação 1, os pacientes com CBC e CEC apresentaram níveis menores de EO sistêmicos comparados ao controle, observado na redução da razão glutationa oxidada e reduzida ($p=0,012$ e $p=0,025$, respectivamente) e na redução da lipoperoxidação ($p=0,004$ e $p=0,002$, respectivamente). Na comparação 2, somente redução na lipoperoxidação dos pacientes com CEC comparados ao grupo controle ($p=0,037$) foi observada. Nenhum dos parâmetros avaliados diferiu entre a QA e o CEC. Na comparação 3, foi possível observar que a atividade da gama-glutamil transpeptidase (GGT) é diferente nos pacientes com um único tipo de CPNM, e também que os pacientes com ambos os tipos de CPNM (CEC+CBC), apresentaram a maior atividade da enzima. Entretanto, essa diferença é perdida quando se considera a localização da lesão. Em conclusão, pacientes com CBC e CEC apresentam baixos níveis sistêmicos de EO; a atividade da GGT sistêmica é o único parâmetro de EO, dentre os avaliados, que difere entre os dois tipos de CPNM, além de estar associada com o desenvolvimento dos dois tipos de CPNM em um mesmo paciente, entretanto, a localização do tumor interfere nessas diferenças; nenhum dos marcadores de estresse oxidativo e inflamação analisados difere sistemicamente entre quem possui a lesão pré-maligna e maligna;

os parâmetros de inflamação avaliados não diferem sistemicamente nos pacientes, independente de possuírem um ou mais tipos de CPNM.

Palavras-chave: Gama-glutamil transpeptidase. Peroxidação lipídica. Defesa antioxidante. Carcinoma basocelular. Carcinoma espinocelular.

BRITO, Walison Augusto da Silva. **Analysis of the involvement of systemic markers of oxidative stress and inflammatory process in the development of multiple types of nonmelanoma skin cancer.** 2020. 66 p. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2020.

ABSTRACT

Nonmelanoma skin cancer (NMSC) is the most incident neoplasm in the world. The most common type is basal cell carcinoma (BCC), followed by squamous cell carcinoma (SCC), which in turn may originate from a preneoplastic lesion called actinic keratosis (AK). It is known that oxidative stress (OS) plays an important role in skin carcinogenesis, which occurs mainly due to ultraviolet radiation, which is also capable of inducing inflammatory process, both related to tumor initiation, promotion and progression. However, it is not clear if systemic markers of OS and inflammatory process are associated with the presence of single and multiple NMSC. Thus, this work aims to investigate systemic markers of OS and inflammatory process in patients with AK and NMSC that could be associated to the presence of multiple types of NMSC. Participants were categorized in 6 groups: Control (without skin cancer, n=75), BCC (n=90), SCC (n=24), AK (n=19), SCC+BCC (n=13) and SCC+BCC+AK (n=36) (CEP-UEL process n. 1.077.557 and n. 3.146.725). Three comparisons were made between the groups: 1) Single type of NMSC lesions: control vs BCC vs SCC; 2) Difference between preneoplastic lesion and SCC: control vs AK vs SCC; 3) Multiple types of NMSC lesions: BCC vs SCC vs SCC+BCC vs SCC+BCC+AK. Blood samples were collected, processed and stored for further analysis. Qualitative data were analyzed by Chi-square test z test for proportion comparison. Quantitative data were analyzed by ANOVA (one-way) or Kruskal-Wallis after the verification of data normality and homogeneity. All results with $p<0.1$ in the univariate analysis were included in the multinomial logistic regression model. $p<0.05$ was considered significant. In comparison 1, patients with BCC and SCC presented lower levels of systemic OS compared to control, observed in the reduction of the oxidized and reduced glutathione ratio ($p=0.012$ and $p=0.025$, respectively) and in the reduction of lipoperoxidation ($p=0.004$ and $p=0.002$, respectively). In comparison 2, only reduction in lipoperoxidation of patients with CPB compared to the control group ($p=0.037$) was observed. None of the parameters evaluated differed between QA and SCC. In comparison 3, it was possible to observe that the activity of gamma-glutamyl transpeptidase (GGT) is different in patients with a single type of NMSC, and also that patients with both types of NMSC (SCC+BCC) had the highest enzyme activity. However, this difference is lost when considering the location of the lesion. In conclusion, patients with BCC and SCC have low systemic levels of OS; systemic GGT activity is the only OS parameter among those evaluated, which differs between the two types of NMSC, besides being associated with the development of the two types of NMSC in the same patient, however, the location of the tumor interferes with these differences; none of the markers of oxidative stress and inflammation analyzed differs systemically between those who have the premalignant and malignant lesion; the evaluated inflammation parameters do not differ systemically in patients, regardless of whether they have one or more types of NMSC.

Keywords: Gamma-glutamyl transpeptidase. Lipid peroxidation. Antioxidant defenses. Basal cell carcinoma Squamous cell carcinoma.

LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
AOPP	Produtos avançados da oxidação de proteínas (Advanced Oxidation Protein Products)
CBC	Carcinoma de células basais ou basocelular
CEC	Carcinoma de células escamosas ou espinocelular
CPNM	Câncer de pele não-melanoma
EO	Estresse oxidativo
ERO	Espécies reativas de oxigênio
GGT	Gama-glutamil transpeptidase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
H&E	Hematoxilina e Eosina
HPV	Vírus do papiloma humano (Human papiloma virus)
IL-10	Interleucina-10
INCA	Instituto Nacional do Câncer
MDA	Malondialdeído
QA	Queratose actínica
QL	Quimiluminescência
SOD	Superóxido dismutase
TGF-β1	Fator transformador do crescimento beta 1 (Transforming growth factor beta 1)
TNF-α	Fator de necrose tumoral alfa (Tumor necrosis factor alpha)
UV	Ultravioleta

SUMÁRIO

1 INTRODUÇÃO

1.1 CÂNCER DE PELE NÃO-MELANOMA

As neoplasias cutâneas podem se desenvolver a partir de células epidérmicas, de melanócitos, de células mesenquimais ou ainda de células hematolinfoides. Dentre as neoplasias cutâneas a mais comum no mundo é a de células epidérmicas. Segundo a Organização Mundial da Saúde, a incidência de cânceres de pele, não melanoma e melanoma, tem aumentado muito nas últimas décadas e isso pode ser relacionado com o aumento da população em envelhecimento, uma vez que este tipo de câncer está intimamente relacionado com a exposição solar ao longo da vida (GLOBAL BURDEN OF DISEASE CANCER COLLABORATION, 2019; WHO, 2019).

No Brasil, o câncer de pele não-melanoma (CPNM) é o mais frequente e corresponde a 30% de todos os tumores malignos registrados no país segundo o Instituto Nacional do Câncer (INCA). Dentre os tumores de pele, o CPNM é o de maior incidência, apresenta baixa mortalidade e altos percentuais de cura se diagnosticado precocemente. O INCA estima para o biênio de 2020-2021, aproximadamente 176 mil novos casos, sendo 83 mil e 93 mil casos abrangendo homens e mulheres, respectivamente (INCA, 2020). O CPNM engloba o carcinoma de células basais (CBC) e o carcinoma de células escamosas (CEC), mesmo possuindo algumas similaridades, esses dois tipos de CPNM possuem diferentes taxas de incidência e diferenças etiológicas (LUCAS et al., 2006). Ambas neoplasias possuem risco de mortalidade e de metástase baixos em comparação com o melanoma, porém, como a cura se dá por remoção cirúrgica, esse tratamento pode causar grande impacto psicológico e social, uma vez que a lesão geralmente está localizada em regiões visíveis como cabeça, pescoço e face, podendo gerar dor e desfiguração (BAILEY et al., 2019; ROTHENBERG; ELLISEN, 2012; WHO, 2019).

Os principais fatores de risco para o desenvolvimento do CPNM são a pigmentação da pele e a exposição à radiação ultravioleta (UV). A radiação UV possui diversos efeitos sobre a pele, como indução de eritema, mutação e imunossupressão, promoção de síntese de vitamina D e bronzeamento, além de possuir efeito carcinogênico, relacionando-se com o desenvolvimento de cerca de 90% dos casos de CPNM (FAJUYIGBE; YOUNG, 2016; KOH et al., 1996). A

pigmentação da pele confere fotoproteção, pois a melanina é capaz de absorver uma banda larga de radiação UV, além de ter propriedades antioxidantes, protegendo a pele do estresse oxidativo induzido pela radiação (BRENNER; HEARING, 2008). Por essa razão, a concentração de melanina pode ser inversamente relacionada com a incidência de cânceres de pele, que acomete mais facilmente indivíduos de pele clara (Classificação de Fitzpatrick I-III) (Figura 1) do que aqueles de pele escura (Classificação de Fitzpatrick IV-VI) (Figura 1). Os indivíduos que possuem pele escura tem maior presença de eumelanina, que é um tipo de melanina cuja propriedade fotoprotetora é superior ao outro tipo, a feomelanina, que está presente em maior quantidade nos indivíduos de pele mais clara (BRENNER; HEARING, 2008).

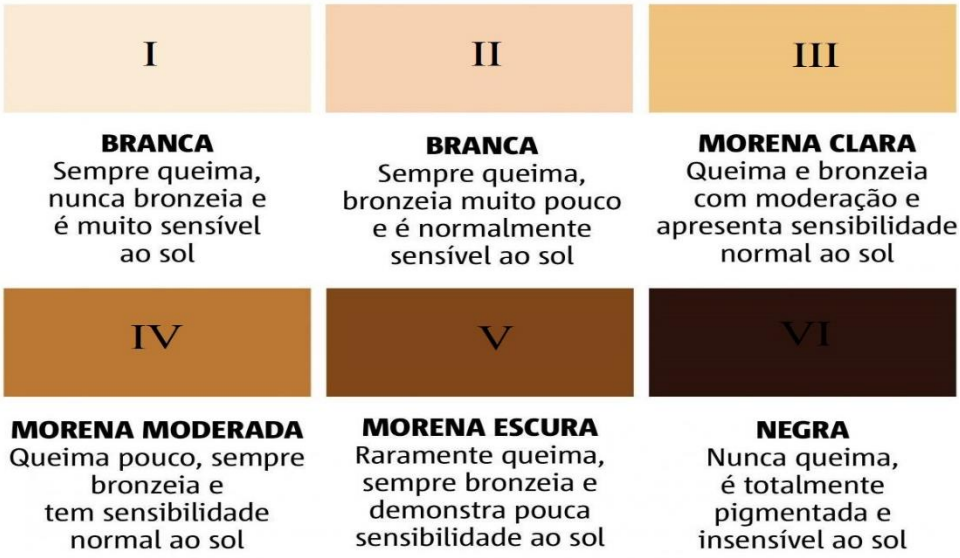

Figura 1. Classificação de Fitzpatrick dos fotótipos de pele. Adaptado de Metro Jornal (2019). Fonte: *https://www.metrojornal.com.br/estilo-vida/2019/01/12/verao-atencao-pele-cuidados-simples.html*

Além da exposição à radiação UV e a pigmentação da pele, outros fatores de risco também favorecem o desenvolvimento de CPNM, dentre eles a imunossupressão, associada ou não a transplantes de órgãos, fatores genéticos e infecção cutânea por algumas cepas do vírus do papiloma humano (HPV) são os mais importantes (BELBASIS et al., 2016; HOFBAUER; BAVINCK; EUVRARD, 2010; TESSARI; GIROLOMONI, 2012). O tabagismo é um hábito que está associado com o desenvolvimento de diferentes tipos de câncer, principalmente CEC em mucosa oral, além de indivíduos tabagistas com CPNM terem uma maior propensão a desenvolver outros tipos de câncer extracutâneos, tais como mama, cólon, esôfago, rins, pulmão, tireoide, entre outros (ROLLISON et al., 2012; SILVERBERG; RATNER, 2015).

Estudos apontam que pacientes com histórico prévio de neoplasias cutâneas apresentam maior chance de desenvolver novas lesões, sejam essas do mesmo tipo da precedente ou de tipos diferentes (BELBASIS et al., 2016; DUARTE et al., 2018; PANDEYA; OLSEN; WHITEMAN, 2017). Levando em conta que geralmente apenas a lesão primária é registrada, dados epidemiológicos sobre a existência de múltiplos tipos de CPNM são escassos, assim como existe grande dificuldade em compreender como esse processo ocorre (BELBASIS et al., 2016; DUARTE et al., 2018; PANDEYA; OLSEN; WHITEMAN, 2017). Entretanto, o melhor entendimento do desenvolvimento dessas lesões, como a participação do EO e do processo inflamatório, por exemplo, pode auxiliar na redução das taxas morbidade e mortalidade decorrentes desses tipos de câncer.

1.2 Carcinoma de células basais ou basocelular (CBC)

O CBC é o câncer mais frequentemente encontrado em humanos de pele clara, correspondendo a 75% dos casos de CPNM (BRANDT; MOORE, 2019). Apresenta crescimento lento e as metástases são raras, entretanto, sua taxa de morbidade é relativamente alta devida a sua capacidade de invasão tecidual, podendo lesionar tecidos adjacentes a pele (MARZUKA; BOOK, 2015). A exposição intensa e prolongada aos raios UV do sol está relacionada com o desenvolvimento da doença, substancialmente em indivíduos cuja ocupação se dá sob altas doses de exposição solar (SCHMITT et al., 2017).

O CBC é composto por células que se assemelham às células basais da epiderme, que se originam de células epiteliais imaturas da camada basal e mais raramente do complexo cutâneo pilo-sebáceo ou outros apêndices cutâneos. É um tumor que possui características infiltrativas nos tecidos adjacentes por expansões irregulares digitiformes (Figura 2B e 2C) (ŢÂNŢU et al., 2014). Por meio de suas características histológicas e clínicas, o CBC pode ser dividido nos seguintes subtipos: nodular, superficial, infundibulocísticos, fibroepitelial, esclerodermiforme e infiltrativo, sendo estes dois últimos os mais agressivos e o nodular e superficial os mais comuns (CAMERON et al., 2019; MARZUKA; BOOK, 2015).

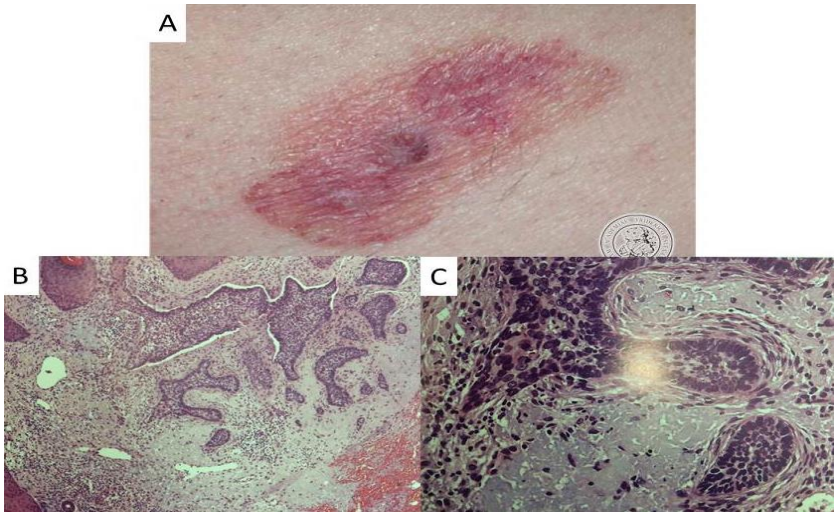

Figura 2. Carcinoma basocelular (CBC). (A) CBC em face. (B) Lâmina de CBC corado por hematoxilina e eosina (H&E) mostrando que o CBC é caracterizado histologicamente pela presença de agregados esféricos ou infiltrantes formados por células neoplásicas em azul-escuro e que se conectam com a epiderme. Artefatos de separação entre os nódulos neoplásicos e o estroma adjacente é um achado que auxilia o diagnóstico. (C) As células nos limites dos agregados neoplásicos se alinham em paliçada. O CBC apresenta um estroma fibromixóide azul peculiar, periférico aos agregados neoplásicos. Esse aspecto pode facilitar a distinção entre os nódulos neoplásicos do CBC e folículos pilosos inocentes. Fonte: Atlas de Histopatologia - Dr. Ivan Damjanov.

1.3 CARCINOMA DE CÉLULAS ESCAMOSAS OU ESPINOCELULAR (CEC)

O CEC é o segundo tipo mais frequente de CPNM, detendo aproximadamente 25% dos casos, surgindo de queratinócitos displásicos na camada espinhosa da epiderme (Figura 3) (BRANDT; MOORE, 2019).

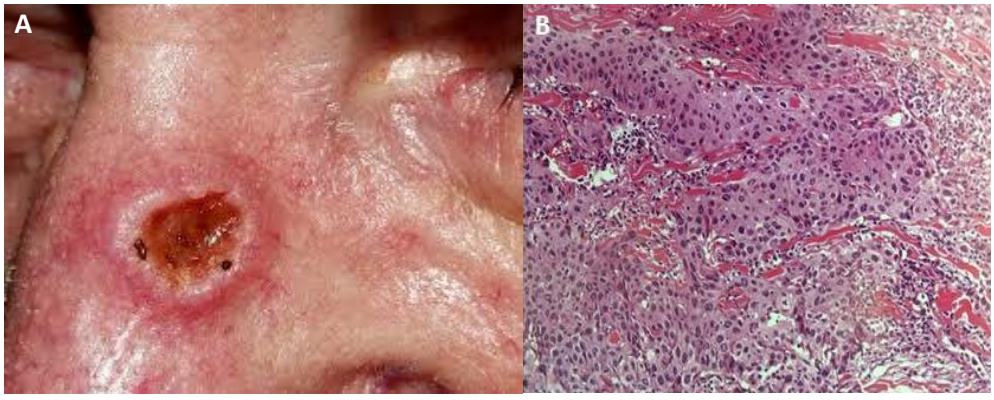

Figura 3. Carcinoma espino celular em paciente (A) e histopatologia em H&E (B). (A) Paciente com diagnóstico de carcinoma espinocelular antes da remoção cirúrgica. Fonte: Dr. Julio Bergmann. Disponível em: https://clinicabergmann.com.br/tratamento/fotos-cancer-de-pele-espinocelular-no-nariz-labio-orelhas-e-pernas-em-porto-alegre/. (B) O tumor é composto de células com núcleos grandes e pleomórficos, organizadas em agregados com formas variadas e que invadem a derme. Fonte: Atlas de Histopatologia - Dr. Ivan Damjanov.

A apresentação clínica mais frequente de CEC é a presença de placas bem diferenciadas, queratinocíticas e eritematosas. Pode ser endurecido, apresentando pápulas firmes, placas ou nódulos com hiperqueratose e ulceração (lesões bem diferenciadas), podendo também apresentar pápulas granulomatosas, carnudas e macias ou nódulos sem queratinização (lesões pouco diferenciadas). O CEC acomete principalmente as regiões do corpo mais expostas aos raios UV, como a face, cabeça, pescoço, antebraços, dorso das mãos e a parte superior do tórax (WARSZAWIK-HENDZEL et al., 2015).

A exposição crônica à radiação UV do sol com subsequente dano no DNA é o mais importante fator que predispõe o desenvolvimento do CEC. Para além deste, a idade avançada e o fotótipo de pele claro (Classificação de Fitzpatrick I-III) também estão associados (BAILEY et al., 2019; FARTASCH et al., 2012; SCHMITT; BORDEAUX, 2013). A população de maior risco inclui trabalhadores com exposição ocupacional à luz ultravioleta (FARTASCH et al., 2012; ROTHENBERG; ELLISEN, 2012). Dentre as lesões precursoras clinicamente associadas ao CEC, inclui a Queratose Actínica (QA), uma lesão pré-maligna, com alto potencial de progressão (WARSZAWIK-HENDZEL et al., 2015).

1.4 QUERATOSE ACTÍNICA (QA)

A QA é uma lesão na pele formada pela proliferação de queratinócitos displásicos em áreas com maior foto-exposição, comum em indivíduos adultos e idosos de pele clara, resultante de um longo período de exposição aos raios UV, tendo esse último como o principal mecanismo pela indução de inflamação, estresse oxidativo, imunossupressão, desregulação do ciclo celular e apoptose, além de proliferação celular e remodelamento tecidual (REINEHR; BAKOS, 2019; SIEGEL; KORGAVKAR; WEINSTOCK, 2017). Os principais fatores de risco para o desenvolvimento da QA incluem a exposição cumulativa à radiação solar, idade avançada, fotótipo de pele clara (Classificação de Fitzpatrick I-III), histórico de queimaduras na infância e habitar em regiões de baixas latitudes (regiões onde há maior incidência dos raios solares) (SCHMITT; BORDEAUX, 2013; SCHMITT; MIOT, 2012).

A QA é uma lesão eritematosa que comumente aparece como múltiplas lesões (Figura 4), além de ser considerada uma lesão pré-cancerosa (tem a

capacidade de progredir para um carcinoma espinocelular, que depende do grau de displasia), que apresenta expressões fenotípicas de fotoenvelhecimento cutâneo, juntamente com perda de elasticidade da pele, atrofia e mudanças na pigmentação (SCHMITT; BORDEAUX, 2013; SCHMITT; MIOT, 2012; WARSZAWIK-HENDZEL et al., 2015). Histologicamente, a QA pode apresentar hiperqueratose que geralmente contém áreas de paraqueratose, indicando queratinização anormal e infiltrado linfocítico, que pode ter um papel na progressão da doença (FERNANDEZ FIGUERAS, 2017).

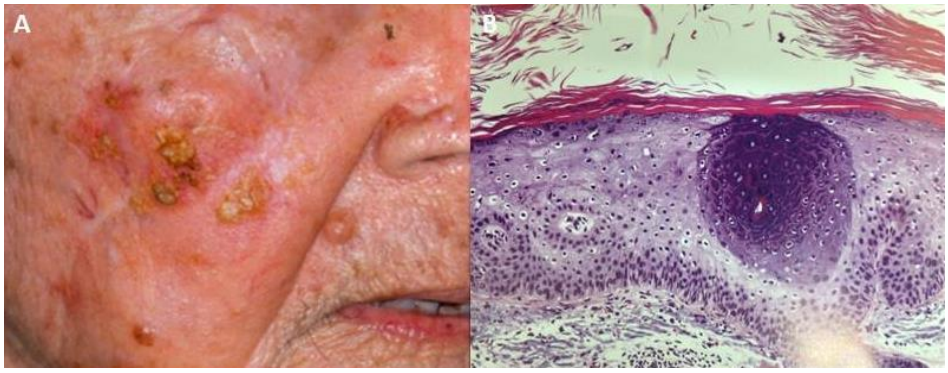

Figura 4 - Queratose actínica. A: Queratose actínica com característica elevada, esbranquiçada, áspera e escamosa em face de paciente. B: Histopatologia em H&E, a epiderme comprometida se queratiniza anormalmente com paraqueratose. Aumento de 100X. Fonte: Atlas de Histopatologia - Dr. Ivan Damjanov, 2013.

1.5 CÂNCER DE PELE NÃO-MELANOMA E MARCADORES SISTÊMICOS DE ESTRESSE OXIDATIVO E PROCESSO INFLAMATÓRIO

Sabe-se que a radiação UV é um carcinógeno ambiental que atua como iniciador e promotor do processo de carcinogênese, através da lesão direta ao DNA formando dímeros de pirimidina, causando imunossupressão, inflamação e indução de estresse oxidativo (CARR et al., 2012; DAMIANI; ULLRICH, 2016; WILLIAMS et al., 2014).

O espectro da radiação UV é subdividido em três bandas: UVA (320-400 nm), UVB (290-320 nm) e UVC (100-280 nm). A UVC é absorvida pela camada de ozônio, a radiação UVA penetra profundamente até a derme, enquanto os raios da UVB são retidos na epiderme da pele. Sabe-se que ambos os espectros UVA e UVB levam ao dano oxidativo, uma vez que são capazes de induzir e gerar espécies reativas de oxigênio (ERO) (WILLIAMS et al., 2014). Entretanto, os mecanismos da

fotocarcinogênese do CPNM induzida por estresse fotoxidativo são complexos, a constante e intensa exposição à radiação UV leva à produção elevada de ERO pelos queratinócitos com consequente redução nas defesas antioxidantes, caracterizando o EO (FEEHAN; SHANTZ, 2016). As ERO rapidamente interagem com as macromoléculas como lipídeos de membrana, proteínas e DNA, levando ao dano oxidativo (FEEHAN; SHANTZ, 2016). Em adição a isso, os danos ao DNA em sua maioria não são reparados, favorecendo a propagação de mutações em genes específicos, levando a alterações em vias de sinalização relacionadas a proliferação celular e apoptose, favorecendo o desenvolvimento de câncer (FEEHAN; SHANTZ, 2016; SCHEUER, 2017).

Foi demonstrado em estudos anteriores algumas alterações em marcadores sistêmicos de EO no CPNM. Foi demonstrado uma diminuição significativa nos antioxidantes plasmáticos, como o α-tocoferol e ácido ascórbico, e glutationa nos eritrócitos de pacientes com QA e CBC (VURAL; CANBAZ; SEKÇUKI, 1999), e aumento sérico de um subproduto da oxidação lipídica, o malondialdeído (MDA), em pacientes com CBC quando comparado a indivíduos controle (MAJIDI; DJALALI; JAVANBAKHT, 2017), demonstrando que alterações redox sistêmicas também estão presentes no CPNM. Entretanto, alguns marcadores sistêmicos de estresse oxidativo são pouco investigados tanto na queratose actínica quanto em CPNM, sejam essas lesões de um único tipo ou lesões de múltiplos tipos.

A resposta inflamatória é um componente que está muito relacionado com a carcinogênese e progressão tumoral. Isso pode ser devido à geração de espécies reativas e fatores de crescimento pelo ambiente inflamatório, que contribui com as alterações que culminam na carcinogênese (FEKECS et al., 2010; OLTEANU et al., 2012). A radiação UV é capaz de induzir resposta inflamatória e imunossupressão na pele, efeitos caracterizados pela indução da produção de mediadores inflamatórios e citocinas, alteração das respostas vasculares e de moléculas de adesão, além de recrutamento de células inflamatórias, criando um microambiente favorável para o tumor (FEEHAN; SHANTZ, 2016). A perda da capacidade apresentadora de antígenos pode contribuir com a dificuldade do sistema imunológico em eliminar as células cancerosas, e também favorece a carcinogênese na pele e sua possível progressão (FEEHAN; SHANTZ, 2016). Por conseguinte, a determinação de marcadores sistêmicos do processo inflamatório em pacientes com

CPNM, seja de um único tipo ou de múltiplos tipos é muito importante, uma vez que pode auxiliar na compreensão da resposta do organismo frente ao tumor.

Sabe-se que o estresse oxidativo é capaz de interferir em diferentes vias de sinalização intracelular e algumas delas podem relacionar-se com o desenvolvimento de neoplasias. Uma das vias que o estresse oxidativo pode alterar é a do fator transformador do crescimento beta 1 (TGF-β1), sendo o estresse oxidativo capaz de aumentar a sua expressão e ativação (KRSTIĆ et al., 2015). O TGF-β1 é um potente supressor tumoral em células pré-malignas, em contrapartida, um favorecedor da progressão e metástase em células de tumores mais avançados (MASSAGUÉ, 2012). O TGF-β1 é capaz de induzir a produção de ERO, regular sua atividade, não apenas na produção, mas também na modulação de antioxidantes enzimáticos, como a catalase e a SOD, e também não enzimáticos, como a GSH, através da inibição da expressão de genes importantes para sua formação (KRSTIĆ et al., 2015). Entretanto ainda não foi investigado a concentração sistêmica dessa citocina no CPNM, e se essa citocina a nível sistêmico exerce um papel importante no desenvolvimento de mais de um tipo de CPNM nos pacientes.

Dessa forma, sabe-se que tanto o EO quanto o processo inflamatório exercem papéis importantes no desenvolvimento do CPNM, e estudos prévios sugerem algumas particularidades com relação ao EO nos diferentes tipos de CPNM. Além disso, sabendo-se que tanto o EO quanto o processo inflamatório são capazes de interferir na ativação de vias sinalizadoras críticas para o desenvolvimento e progressão tumoral, a hipótese desse trabalho é que existem diferenças no perfil oxidativo e inflamatório sistêmico nos pacientes com queratose actínica e com CPNM, que possam estar associados com a presença de mais de um tipo de CPNM nos pacientes.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar marcadores sistêmicos de estresse oxidativo e de processo inflamatório em pacientes com queratose actínica e com câncer de pele não-melanoma e sua associação a presença de múltiplos tipos de câncer de pele não-melanoma em um mesmo paciente.

2.2 Objetivos Específicos

- Avaliar marcadores de EO sistêmico em pacientes com queratose actínica e com um ou mais tipos de CPNM;
- Quantificar parâmetros inflamatórios no sangue de pacientes com queratose actínica e com um ou mais tipos de CPNM;
- Investigar se parâmetros sistêmicos de EO e de processo inflamatório são capazes de diferenciar os dois tipos de CPNM, controlando os fatores confundidores e fatores de risco que podem influenciar os resultados obtidos;
- Analisar se os marcadores sistêmicos de EO e do processo inflamatório são capazes de diferenciar a lesão pré-cancerosa do câncer estabelecido, fazendo o controle das variáveis confundidoras e fatores de risco;
- Avaliar se os marcadores de EO e do processo inflamatório sistêmicos estão associados com a presença de múltiplos tipos de CPNM em um mesmo paciente, controlando os fatores confundidores e fatores de risco que possam estar influenciando nos resultados obtidos.

3 ARTIGO PARA PUBLICAÇÃO

O presente trabalho, realizado na Universidade Estadual de Londrina, nos laboratórios de Patologia Molecular, de Fisiopatologia e Radicais Livres, de Pesquisa em Imunologia Aplicada, e no Ambulatório de Dermatologia do Hospital Universitário da UEL e no Hospital Norte Paranaense de Arapongas, originou um artigo científico.

O artigo será submetido para publicação em revista internacional a ser definida e intitula-se “Evaluation of systemic parameters of oxidative stress and inflammation in patients with single and multiple types of nonmelanoma skin cancer”.

SYSTEMIC PARAMETERS OF OXIDATIVE STRESS AND INFLAMMATION IN SINGLE AND MULTIPLE TYPES OF NONMELANOMA SKIN CANCER

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Abstract

The research purpose is to investigate the systemic parameters of oxidative stress (OS) and inflammatory process in patients with actinic keratosis (AK) and nonmelanoma skin cancer (NMSC) and if those parameters differ between groups of patients with multiple types of NMSC and with pre-malign and malign lesions. Basal cell carcinoma (BCC; n=90), Squamous cell carcinoma (SCC; n=24), AK (n=19), SCC+BCC (n=13) and SCC+BCC+AK (n=36) groups were analyzed in which blood samples were collected immediately before surgery to resect skin lesions. From controls (without cancer, n=75) only blood was collected. Reduced (GSH) and oxidized glutathione (GSSG), catalase, superoxide dismutase, lipid hydroperoxides (CL-LOOH), total thiols, malondialdehyde, advanced oxidized protein products, TNF-α, IL-10, TGF-β1, ferritin, C-reactive protein and gamma-glutamyl transpeptidase (GGT) were evaluated. BCC and SCC presented reduced levels of GSSG/GSH ratio and CL-LOOH compared to control. GGT activity was lower in BCC than in SCC and, the higher activities were found in patients from BCC+SCC. GGT results lost statistical difference after control by confounding factors. Remaining evaluated parameters of OS and inflammation did not alter. In conclusion, single type NMSC patients present low levels of systemic OS (independently associated with NMSC); GGT activity is different in patients with single and multiple types of NMSC. However, GGT is influenced by confounding factors. Besides, others OS and inflammatory markers used are not good parameters to differ systemically between the types of NMSC, nor in patients with single and multiple types of NMSC, as well as none evaluated parameter differed between pre-malign and malign lesions.

Keywords: Gamma-glutamyl transpeptidase. Lipid peroxidation. Antioxidant defenses. Basal cell carcinoma. Squamous cell carcinoma.

Introduction

Nonmelanoma skin cancer (NMSC) is the most incident cancer worldwide. This incidence is constantly raising due to the increase in population age and sun exposure throughout life (GLOBAL BURDEN OF DISEASE CANCER COLLABORATION, 2019; WHO, 2019). NMSC is a group of cutaneous malignancies that mainly comprises keratinocytes carcinomas: basal cell carcinoma (BCC), corresponding to approximately 75% of cases, and squamous cell carcinoma (SCC), 25% of the remaining cases (BAILEY et al., 2019). NMSC presents low mortality rates and rarely metastasize, however, it presents high morbidity, since most lesions occur in visible regions of the body such as face, head and neck (BAILEY et al., 2019; WHO, 2019). According to the severity, the lesions can even cause disfigurement, generating social and psychological impact on patients (BAILEY et al., 2019; WHO, 2019). Actinic keratosis (AK) is a skin lesion formed by proliferating dysplastic keratinocytes in areas with greater photo-exposure, and is considered a precursor of SCC (REINEHR; BAKOS, 2019).

Ultraviolet (UV) radiation from the sun is the greatest environmental risk factor for developing NMSC. It is able to induce direct DNA damage, inflammation, immunosupression and formation of reactive oxygen species (ROS). Patients with fair skin and high sunlight exposure throughout life are the most affected (FEEHAN; SHANTZ, 2016). In addition to UV exposure and skin pigmentation, other risk factors can contribute to the development of NMSC, such as immunosuppression, genetic factors, infection by some HPV subtypes and smoking (BELBASIS et al., 2016; TESSARI; GIROLOMONI, 2012). Further, previously history of cutaneous neoplasms confers a greater risk of new skin lesions development, including the same type of the primary lesion or multiple types of NMSC lesions (DUARTE et al., 2018).

UV radiation-induced oxidative stress (OS) is one of the main mechanisms of skin carcinogenesis (HAY et al., 2014; OLTEANU et al., 2012). Some studies demonstrated systemic OS markers changed in patients with AK and NMSC, such as decreased antioxidant defenses (SRIVASTAVA et al., 2012; VURAL; CANBAZ; SEKÇUKI, 1999), and increased lipid peroxidation (MAJIDI; DJALALI; JAVANBAKHT, 2017; SRIVASTAVA et al., 2012). The inflammatory process can also be modulated by exposure to UV radiation, culminating in greater cell proliferation, angiogenesis and decreased apoptosis, favoring tumor development (FEEHAN;

SHANTZ, 2016; OLTEANU et al., 2012). Some authors found differences in some serum cytokines levels in SCC patients, like increased interferon gamma (INF-γ) and transforming growth factor beta1 (TGF-β1) and decreased interleukin-6 (IL-6) in comparison with individuals without NMSC (YAMADA et al., 2016). In relation to BCC, it was reported increased serum levels of interleukin-10 (IL-10) and decreased interleukin-2 when compared to control (SOBJANEK et al., 2016), in addition decreased TGF-β1 when compared to BCC patients after photodynamic therapy (ADAMEK et al., 2005).

As observed, the literature has presenting some results indicating that besides the participation of OS and inflammation in skin carcinogenesis, they are also systemically different modulated in the different types of NMSC. However, despite the knowledge that previous story of NMSC can increase the risk for the development of other skin lesions, the involvement of OS and inflammation with the presence of multiple types of NMSC in the same patient have never been investigated. Based on this, the objective of this study was to evaluate systemic parameters of OS and inflammatory process in patients with AK and NMSC and to investigate if they could differ in patients with multiple types of NMSC and with pre-malign and malign lesions.

Methods and materials

Ethical approval

This study was approved by the Ethics in Research Committee involving human beings of the State University of Londrina (CEP/UEL), under registers CAAE: 44235015.3.0000.5231 in 20/05/2015 and CAAE: 00745118.7.0000.5231 in 14/09/2019. All the practices were approved by the institutional board and were in accordance with the ethical standards of the Brazilian National Commission on Ethics in Research (CONEP) and with the Brazilian National Health Council Resolution 466/12.

Sample collection and study design

A total of 182 patients aged between 29 and 87 years old were recruited from Dermatology outpatients of the University Hospital of Londrina, Paraná, Brazil, and from Northern Paranaense Hospital (HONPAR) of Arapongas, Paraná,

Brazil, from June 2015 to December 2019. Before surgery for removal of skin lesions, patients were invited to participate in the study and in the case of acceptance, they signed the informed consent form (ICF) after complete explanation of the study, its risks and benefits. After histopathological diagnostic of lesions, 90 patients were grouped in basal cell carcinoma (BCC) group, 24 patients were grouped in squamous cell carcinoma (SCC) group, and 19 patients were grouped in actinic keratosis (AK) group. Some patients presented more than one type of skin lesion, then, those who developed SCC and BCC were grouped in SCC+BCC group (13 patients), and those who developed SCC, BCC and AK were grouped in SCC+BCC+AK group (36 patients). The control group comprised 75 individuals and the inclusion criteria were both sex volunteers without any type of cancer history. All participants were not under antioxidant therapy and answered a questionnaire to obtain information about sex, skin phototype (according to Fitzpatrick's classification), sun exposure, location of tumor, smoking, the presence of chronic diseases and the use of medications (insulin, antihypertensive, antilipemic and antihyperglycemic drugs). Peripheral blood were collected with and without heparin and centrifuged at 1331 x G during 5 minutes at 4 ºC to obtain plasma and serum, respectively. Separated plasma and serum were frozen at -20 ºC until analysis, while erythrocytes were used fresh to the evaluation of oxidative stress parameters.

Evaluation of oxidative stress in erythrocytes

Erythrocytes were obtained from heparinized blood and washed three times with 0.9 % saline solution at 4 ºC. An aliquot of heparinized blood was used to determine total protein by Lowry technique (LOWRY; ROSEBROUGH; FARR, 1951), modified by Miller, using bovine serum albumin (Sigma Aldrich®) as standard (MILLER, 1956).

Total (GT) and reduced glutathione (GSH) levels were determined according to the method previously described by Locatelli et al (2009). The quantification of GT was based on the conversion of oxidized glutathione (GSSG) present in the medium to GSH by the action of the enzyme glutathione reductase, in the presence of NADPH (LOCATELLI et al., 2009). Erythrocytes were diluted at a ratio of 1:800 in deionized water and incubated with 5,5'-Dithiobis-(2-acid-nitrobenzoic) (DTNB). GSH reacts with DTNB forming a yellow compound (5-nitrothiobenzoate) detectable at 412 nm using a Multiskan GO® microplate reader (Thermo Fisher

Scientific®, USA). After GT and GSH quantification, the levels of GSSG were estimated taking into account the stoichiometry of the reaction, when two GSH are required to form one GSSG. Therefore, GSSG was determined by the equation: GSSG = (GT-GSH/2). The results were expressed in μM/g protein. It was also calculated GSSG/GSH ratio and the results were expressed in percentage (%).

The activity of catalase was measured using erythrocytes at a dilution of 1:80 in deionized water (PANIS et al., 2012). The method is based on the decomposition of hydrogen peroxide (H_2O_2) by catalase, directly related to its absorption at 240 nm (AEBI, 1984). The enzymatic kinetics was monitored using a UV-1650 PC UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The results were expressed in velocity of absorbance decreased in 1 min per miligram of total protein (Vabs/min/mg protein).

Superoxide dismutase (SOD) activity was determined using different volumes of diluted erythrocytes at a ratio of 1:20 in deionized water (MARKLUND; MARKLUND, 1974). This method is based on the inhibition of pyrogallol autoxidation in aqueous solution by SOD, which catalyzes the dismutation of superoxide anion to hydrogen peroxide. This autoxidation inhibition was monitored at 420 nm in a UV-1650 PC UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) during 6 minutes. Autoxidation of pyrogallol alone was used as control. The amount of SOD capable of inhibit in 50 % pyrogallol autoxidation was defined as a unit of enzymatic activity (U). Results were expressed in USOD/g total protein.

Lipid peroxidation in erythrocytes was evaluated by chemiluminescence stimulated by tert-butyl hydroperoxide (CL-LOOH) (GONZALEZ FLECHA; LLESUY; BOVERIS, 1991). For this method, erythrocytes were diluted at a ratio of 1:1200 in 10 mM phosphate buffer and the reaction was assessed in a luminometer Lumat^3 LB 9508 (Berthold Technologies Bioanalytic) during 40 minutes. After measurement, the results were plotted and area under curve (AUC) were calculated.

Oxidative stress parameters in plasma and serum

Total thiols levels in plasma were quantified by the method based on the reaction between sulfhydryl group (thiol) and DTNB, resulting in a spectrophotometrically detectable color alteration at 412 nm (HU, 1994). Total thiol

group was calculated using a calibration curve prepared with GSH. The results were expressed in μM.

Plasma malondialdehyde (MDA) was measured by high performance liquid chromatography (HPLC) in a LC-20AT-HPLC system (Shimadzu, Kyoto, Japan) (VICTORINO et al., 2013). The readings were made during 11 minutes, at a flow rate of 0.8 mL/min at 35 ºC. The results were expressed in nM of MDA.

Serum concentrations of ferritin and the activity of gamma-glutamyl transpeptidase (GGT) were evaluated using a biochemical auto-analyzer Dimension RxL® (Dade Behring/ Siemens Healthcare Diagnostics, Dade Behring, Deerfield, IL, USA).

Plasma advanced oxidation protein products (AOPP) levels were measured in a microplate reader. AOPP results from oxidation of protein residues of tyrosine by inflammatory response, leading to the formation of dityrosine-containing protein cross-liking products detected by spectrophotometry at 340 nm (WITKO-SARSAT et al., 1996). AOPP concentrations were expressed as μmol/L of chloramine-T equivalents.

Inflammatory parameters

Plasma cytokine levels of tumor necrosis factor alpha (TNF-α), IL-10 and TGF-β1 were measured by a sandwich enzyme-linked immunosorbent assay (ELISA), following the manufacturers' instructions (eBioscience®, San Diego, CA, USA). TNF-α and IL-10 concentrations were expressed as pg/mL, and TGF-β1 concentrations were expressed as ng/mL.

Serum C-reactive protein (CRP) levels were evaluated using a high sensitivity turbidimetry assay (Architect C8000, Abbott Laboratory, Abbott Park, IL, USA), with limit of detection of 0.175 mg/L (hsCRP).

Statistical analysis

In this study, three comparisons were made between the groups: 1) Single type of NMSC lesions: control group vs BCC group vs SCC group; 2) Pre-malign lesion and malign lesion: control group vs AK group vs SCC group; 3) Single and multiple types of NMSC lesions: BCC group vs SCC group vs SCC+BCC group vs SCC+BCC+AK group. The chi-square test and z test for proportion comparison were

applied in categorical variables, and were expressed as absolute (*n*) and relative (*%*) numbers. The normality of data distribution was assessed using Shapiro-Wilk test, and when the variables were not normally distributed, the natural logarithmic (ln) of continuous data were used. Univariate analyses of continuous data were performed by ANOVA (one-way or two-way) with Tukey's post-hoc test, and expressed as mean ± standard error of mean (SEM) when data presented normal distribution. When data present nonparametric distribution, the univariated analysis was performed by Kruskal-Wallis with Dunn's post-hoc test, and results were expressed as median with interquartile range. Multinomial logistic regression analysis was made to evaluate whether the variables that presented $p < 0.1$ in the first analysis were independently associated in all comparisons performed. The results were considered statistically significant when $p<0.05$. All the statistical analyses were performed in SPSS version 24.0 (SPSS, Chicago, IL, USA).

Results

Single type of NMSC lesions comparison

The characteristics of the 114 patients and 75 controls are presented in supplementary table 1. We found that BCC and SCC patients were older than controls ($p<0.001$). There were no differences between groups in relation to sex ($p=0.471$), smoking ($p=0.063$), and chronic diseases ($p=0.927$). The most reported chronic diseases were systemic arterial hypertension, diabetes mellitus, and hypothyroidism, followed by neurological diseases such as depression, epilepsy, and chronic renal insufficiency. Regarding the use of medications: insulin ($p=0.403$), antihyperglycemic ($p=0.340$) and antihypertensive ($p=0.869$) drugs did not shown any difference between groups, however, BCC and control group differed in relation to the use of antilipemic drugs ($p=0.015$). Regarding skin phototype ($p<0.001$), sun exposure ($p<0.001$) and location of tumor ($p<0.001$), known risk factors for NMSC, all of them presented significant difference between groups. All variables with $p<0.1$ (age, use of antilipemic drugs, skin phototype and sun exposure) were used in multinomial logistic regression model as confounding factors, to understand how they can be associated with the results obtained in systemic analysis.

Table 1 presents all systemic variables analyzed by univariate analysis. In erythrocytes samples, GSH levels (p=0.004) and GSSG levels (p<0.001) decreased in both NMSC groups when compared to control group. GSSG/GSH ratio, a marker known as oxidative stress index, also decreased in both NMSC groups comparing to control group (p<0.001). The activity of catalase and SOD did not shown differences between groups (p=0.213 and p=0.461, respectively). Lipid peroxidation in erythrocytes (CL-LOOH) reduced in both NMSC groups when compared to control group (p<0.001). The remaining parameters (total thiols, MDA, GGT, AOPP, ferritin, TNF-α, lL-10, TGF-β1 and CRP) evaluated in serum and plasma did not show differences between groups.

Table 2 shows multinomial logistic regression analysis with the two NMSC (BCC and SCC) groups and controls as dependent variables and systemic parameters with p < 0.01 as explanatory variables. The confounding factors obtained in the supplementary table 1 were used in different models of multinomial logistic regression to identify if the explanatory variables were independently associated with the presence of NMSC. In the first model, the explanatory variables were controlled by age, smoking and use of antilipemic drugs. In this model, patients with BCC and SCC presented reduced GSSG/GSH ratio (p=0.014 and p=0.042, respectively) in comparison to control. In addition, there was a significant decrease in CL-LOOH in BCC and SCC groups when compared to control group (p = 0.002 and p=0.010, respectively). After the addition of only skin phototype (model 2) or only sun exposure (model 3) or both (model 4) as confounding factors in the regression models, the differences in GSSG/GSH ratio in BCC and SCC group (p=0.012 and p=0.025, respectively) and in lipid peroxidation (CL-LOOH) in BCC and SCC group (p=0.004 and p=0.002, respectively) compared to control, continued. None of the explanatory variables differed between NMSC types. Because control group did not presented any tumor, the variable location of tumor was not included in any regression model in this comparison.

Pre-malign and malign lesion

Supplementary Table 2 demonstrates the characteristics of the 39 patients (SCC: n=24; AK: n=15) and 75 controls included in this comparison. SCC and AK group were older than control group (p<0.001). Characteristics such as sex, skin phototype, sun exposure, and location of tumor, presence of chronic diseases and the

use of medications (data not shown) did not alter between groups. Smoking were higher in patients with SCC when compared to AK and Control (p=0.022). Variables with p<0.1 (age, smoking and skin phototype) were used in multinomial logistic regression model as confounding factors, to understand how they can be associated with the results obtained in systemic analysis.

Table 3 shows a decrease in GSH and GSSG levels decrease in SCC and AK groups compared to control group (p=0.009 and p<0.001, respectively). GSSG/GSH ratio and lipid peroxidation (CL-LOOH) also decreased in these groups in relation to control (p =0.002 and p<0.001, respectively; Table 3). The remaining parameters evaluated (catalase, SOD, total thiols, MDA, GGT, AOPP, ferritin, TNF-α, IL-10, TGF-β1 and CRP) did not shown differences between groups.

Table 4 shows multinomial logistic regression analysis with SCC, AK and control groups as dependent variables and systemic parameters of oxidative stress and inflammation with p<0.1 as explanatory variables. The confounding factors obtained in the supplementary table 2 were used in different models of multinomial logistic regression to identify if the explanatory variables were independently associated with the presence of NMSC. In the first model, the explanatory variables were controlled by age and smoking and in the second model, the explanatory variables were controlled by model 1 and skin phototype. The multinomial logistic regression analysis shows that lipid peroxidation is lower in SCC group when compared to control in both models 1 (p=0.022) and 2 (p=0.037) of regression, indicating that this difference was independently associated with SCC. In addition, none of the other explanatory variables (GSH, GSSG, GSSG/GSH ratio, TNF-α and TGF-β1) presented differences between pre-malign lesion and malignant lesion (Table 4).

Single and multiple types of NMSC lesions

Supplementary Table 3 presents the characteristics of 163 patients that were included in this comparison (BCC; SCC; SCC+BCC and SCC+BCC+AK groups). SCC+BCC+AK group was older than the other groups (p<0.001). Sun exposure (mainly intense) and location of tumor (mainly lesions located in head/neck) were different between groups (p=0.019 and p<0.001, respectively). The other characteristics (sex, skin phototype, smoking and chronic diseases) did not shown

statistical differences in this comparison. Variables with p<0.1 (age, sun exposure and tumor location) were used in multinomial logistic regression model as confounding factors, to understand how they can be associated with the results obtained in systemic analysis.

Table 5 demonstrates the results obtained from the analysis of systemic parameters of OS and inflammation. The only parameter that altered between groups was GGT activity (p=0.009). Decreased activity of GGT was found in SCC group when compared to both SCC+BCC (p=0.001) and SCC+BCC+AK (p=0.036) groups. In addition, it was observed increased activity of GGT in SCC+BCC group compared to BCC group (p=0.016). The other parameters did not present differences between groups.

Table 6 shows multinomial logistic regression analysis with BCC, SCC, SCC+BCC and SCC+BCC+AK as dependent variables, GGT activity as explanatory variable. The confounding factors obtained in the supplementary table 3 were used in different models of multinomial logistic regression to identify if the explanatory variables were independently associated with the presence of single and multiple types of NMSC. In the first model, the explanatory variables were controlled by age. In the second model they were controlled by model 1 and sun exposure. In the third model, the control was performed by model 1 and tumor location and model 1, 2 and 3 were used in the control of explanatory variables in the fourth model. In the models 1 and 2, the activity of GGT were different in BCC when compared to SCC (p=0.015), in addition, SCC+BCC presented different activity of GGT when compared to SCC (p=0.008) and SCC+BCC+AK (p=0.043). However, when both sun exposure and location of tumor were added in regression model (models 3 and 4), the activity of GGT lost significance, indicating that this variable was not independently associated with NMSC, and probably GGT activity was associated with both risk factors.

Discussion

The main findings of the present study are that patients with single type NMSC lesions present low levels of systemic OS and that GGT activity is the only evaluated parameter that differ between patients with only BCC or SCC. Furthermore, GGT activity was also the only evaluated parameter that is different between patients

that only present SCC from those that present both SCC and BCC. These results were based on a rigorous statistical analysis, by the multinomial logistic regression model that were controlled the interference of potential confounding factors. Our findings must be highlighted since, to the best of our knowledge, we are the first to investigate if systemic parameters of OS and inflammation could be different modulated in patients with single and multiple types of NMSC lesions. Previous studies related alteration in more parameters of OS and inflammation in patients with single type NMSC and AK lesions than we found in this study (ADAMEK et al., 2005; MAJIDI; DJALALI; JAVANBAKHT, 2017; SOBJANEK et al., 2016; SRIVASTAVA et al., 2012; VURAL; CANBAZ; SEKÇUKI, 1999; YAMADA et al., 2016). However, these parameters may be suffering the interference from NMSC risk factors and lifestyle habits, since the control of these confounding factors were not performed.

All patients included in this research presented more than 60 years old, fair skin (Fitzpatrick phototype I-III), high exposure to UV radiation and developed lesions in body regions with high sun exposure. This is in accordance with the main risk factors previously described for the development of NMSC and AK (BAILEY et al., 2019; FARTASCH et al., 2012; SCHMITT; BORDEAUX, 2013). It is known that aging (ZHANG; DAVIES; FORMAN, 2015), smoking habits (PASINI et al., 2019), and use of medications such as antilipemic drugs (RASMUSSEN et al., 2016), modulate in different ways systemic OS and inflammation markers. For this reason, it is important to control the confounding factors to minimize the biases in the analysis.

OS and chronic inflammation processes were previously related to cancer initiation, promotion, progression and metastasis (REUTER et al., 2011). In our study, we observed that patients with BCC and SCC demonstrated lower levels of GSSG/GSH ratio than control volunteers, which corroborates with a previous study that observed low GSSG/GSH ratio in tissues of patients with head and neck SCC, a carcinoma that mainly affects mucosas (DEQUANTER; DOK; NUYTS, 2017). GSH is a tripeptide with great antioxidant capacity, belongs to the group of non-enzymatic antioxidants and acts capturing ROS, and also as cofactor of enzymes such as glutathione peroxidase (GPx) and glutathione S-transferase (GST), which are important enzymes in detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds (METGUD; BAJAJ, 2014; REISCHL et al., 2007). The GSH consumed can be converted to the oxidized form, GSSG, or used for protein S-glutathiolation, protecting proteins from oxidative damage (MALVEZZI et al., 2012).

The assessment of GSSG/GSH ratio can provide an estimation of intracellular OS and is a well-described marker used to analyze oxidative balance (SÁNCHEZ-RODRÍGUEZ; MENDOZA-NÚÑEZ, 2019). In addition, it is considered to be a great marker of systemic OS in different clinical conditions, such as metabolic, cardiovascular and neurological diseases and cancer (SÁNCHEZ-RODRÍGUEZ; MENDOZA-NÚÑEZ, 2019). In our study, GSSG/GSH ratio presented decreased values in both BCC and SCC patients compared to control volunteers, indicating low systemic intracellular OS. In addition, reduction in GSSG/GSH ratio was independently associated with the presence of single type NMSC lesions.

Lipids from membranes are one of the main macromolecules that suffer from oxidative damage caused by interaction with ROS, generating product derived from oxidation, such as lipid hydroperoxides and MDA (WILLIAMS et al., 2014). In the current study, patients with only one type of NMSC (BCC or SCC) showed significant decrease in lipid hydroperoxides, measured by erythrocytic chemiluminescence method (CL-LOOH), and this result were different from other studies that demonstrated increased systemic lipid peroxidation, but using other methods (MAJIDI; DJALALI; JAVANBAKHT, 2017; SRIVASTAVA et al., 2012). These studies measured thiobarbituric acid reactive species (TBARS), which is a method used to measure MDA levels that possess several interferents (GUTTERIDGE, 1986). In our study, we evaluated lipid peroxidation using two methods considered highly sensitive, the measurement of lipid hydroperoxides by chemiluminescence and MDA using HPLC, both products of lipid peroxidation formed in different stages. Besides that, these analysis were performed in two distinct biological compartments, in erythrocytes and plasma, reflecting intracellular and extracellular compartments, respectively. For this reason, our analysis allow a better understanding of the systemic importance of this process in NMSC.

We found the greatest activity of serum GGT in patients with the two types of NMSC (SCC+BCC), and observed that the activity of this enzyme differed in patients that present only BCC or only SCC. Previously, it was demonstrated that patients with esophageal SCC present increased levels of serum GGT (HUANG et al., 2017). However, after the control by risk factors by logistic regression model in our study, we observed that GGT activity was associated with tumor location in patients with SCC+BCC and by tumor location combined with sun exposure in patients with single NMSC. GGT is an enzyme present in plasma membrane of all cells, but can

also be found in serum, being considered a marker of liver diseases (NICOLAE et al., 2017; POMPELLA et al., 2006). The interference of liver diseases in our GGT results was discarded because our patients did not present hepatic alterations. This enzyme is involved in the metabolism of extracellular GSH and it conjugates and provide an adequate recovery of cysteine, it also play a role in OS, generating ROS in low levels of activity and participates in leukotrienes formation (NICOLAE et al., 2017; POMPELLA et al., 2006). GSH is the main intracellular antioxidant enrolled in detoxification of ROS and lipid hydroperoxides, protecting the cells against lipid peroxidation-induced damage, however GSH can also be found in extracellular medium (POMPELLA et al., 2006). During tumor cell proliferation, cysteine needs to be recovered in intracellular compartment, thereby, GGT present in plasma membrane of cells catalyzes the hydrolysis of extracellular GSH resulting in posterior transport of cysteine into the cell (POMPELLA et al., 2006). The activity of GGT is influenced by the stability of membranes; in equilibrium GGT initiates GSH metabolism culminating in release of cysteine, but low activity of this enzyme leads to OS through generation of pro-oxidant molecules (NICOLAE et al., 2017). In our study, patients presented low GGT activity and low levels of systemic OS. However, we could not exclude the possibility of this enzyme interfere in OS in tumor tissue.

Interestingly none of systemic markers of inflammatory process analyzed presented any differences between groups in all comparisons performed in our study, and these results were different from others studies (ADAMEK et al., 2005; SOBJANEK et al., 2016; YAMADA et al., 2016). However, although the methodological procedure to measure the inflammatory mediators was similar, the experimental design was different, since we analyzed the role clinical history of patients, ensuring that patients allocated in single type of NMSC groups (SCC and BCC groups) did not present other type of skin lesions. This kind of analysis is not specified in most of studies. Our results indicate that TNF-α, IL-10, TGF-β1 and C-reactive protein are not good parameters to evaluate systemically the inflammatory process in patients with single and multiple NMSC lesions.

The main limitation of the present study is the small number of individuals that present both NMSC (SCC+BCC group). However, this limitation is a consequence of the rigor adopted in the experimental design, which was fundamental to bring a new approach on NMSC. For the first time, systemic differences between

OS and inflammatory parameters have been investigated in patients who have one or more types of NMSC.

In conclusion, the analysis of the results allow us to conclude that patients with single type of NMSC present low systemic oxidative stress. Furthermore, GGT activity was the only evaluated parameter that is different in patients with single and multiple NMSC lesions. However, it is influenced by tumor location associated with sun exposure. The results also allow us to conclude that none of the systemic parameters evaluated were able to differentiate the pre-malign lesion (AK) from the malign lesion (SCC). Besides, the inflammatory markers evaluated in this study are not good parameters to systemically investigate inflammation in patients with single and multiple NMSC lesions. These findings enable a better comprehension of systemic alterations related with oxidative stress and inflammation in patients with single and multiple types of NMSC lesions that may contribute to the reduction of morbidity and mortality due to this type of cancer in the future.

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Table 1. Univariate analysis of systemic oxidative stress and systemic inflammatory markers in patients with basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and control group.

Systemic markers	Control (N=75)	BCC (N=90)	SCC (N=24)	p value
GSH (µM/g protein)	650.4 (513.0-820.5)[a]	564.9 (399.1-701.2)[b]	436.9 (357.8-621.7)[b]	**0.004**
GSSG (µM/g protein)	271.1 (227.4-375.2)[a]	134.1 (92.30-244.7)[b]	116.0 (81.45-297.3)[b]	**<0.001**
GSSG/GSH ratio (%)	31.56 (25.52-37.74)[a]	20.54 (14.95-30.13)[b]	23.51 (16.65-37.14)[b]	**<0.001**
Catalase activity (Vabs/min . mg protein $^{-1}$)	35.25 (29.39-41.49)	30.98 (22.61-41.56)	29.78 (18.35-41.92)	0.213
SOD activity (U/g protein)	0.102 (0.082-0.116)	0.093 (0.072-0.112)	0.097 (0.074-0.111)	0.461
Total Thiols (µM)	460.2 (386.0-551.4)	487.8 (360.0-592.9)	467.2 (331.4-608.2)	0.725
MDA (nM)	472.6 (146.9-785.2)	311.8 (107.6-764.8)	302.6 (173.4-489.7)	0.374
CL-LOOH (AUC)	177072 (115205-211164)[a]	100903 (75316-137046)[b]	91248 (64502-133374)[b]	**<0.001**
GGT activity (U/L)	34 (24-49)	36 (28-55)	27 (21-44.5)	0.148
AOPP (µM chloramine-T/L)	129.9 (96.10-180.4)	121.1 (84.5-147.0)	119.5 (79.15-192.9)	0.180
Ferritin (ng/mL)	133.1 (76.15-228.6)	161.7 (91.60-294.6)	111.3 (98.20-274.0)	0.456
TNF-α (pg/mL)	186.5 ± 58.64	43.87 ± 17.55	61.16 ± 32.40	0.160
IL-10 (pg/mL)	3.051 (2.258-4.790)	2.686 (1.916-3.670)	2.625 (1.664-5.817)	0.301
TGF-β1 (ng/mL)	4.082 ± 0.675	2.660 ± 0.242	3.252 ± 0.701	0.262
CRP (mg/L)	3.617 ± 0.588	5.777 ± 1.249	2.523 ± 0.603	0.483

Results were expressed as median with interquartile range and were statistically analyzed by Kruskal-Wallis with Dunn's post-hoc test, or were expressed as mean ± SEM (standard error of mean) and were analyzed by one-way ANOVA with Tukey's post-hoc test. Natural logarithmic transformation were used in TNF-α, TGF-β1 and CRP data. Different letters were considered statistically different between groups. GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; MDA: malondialdehyde; CL-LOOH: chemiluminescence stimulated by tert-butyl hydroperoxide; AUC: area under curve; GGT: gamma-glutamyl transpeptidase; AOPP: advanced oxidative protein products; TNF-α: tumor necrosis factor-alpha; IL-10: interleukin-10; TGF-β1: transforming growth factor-beta 1; CRP: C-reactive protein. p values < 0.05 are highlighted.

Table 2. Multinomial logistic regression analyses in patients with basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and control group as dependent variables and systemic parameters of oxidative stress as explanatory variables.

Explanatory Variables	**Group comparison**	***p* value**			
		Model 1	**Model 2**	**Model 3**	**Model 4**
GSH (μM/g protein)	Control vs BCC	0.238	0.283	0.222	0.168
	Control vs SCC	0.162	0.203	0.123	0.091
	BCC vs SCC	0.616	0.619	0.562	0.496
GSSG (μM/g protein)	Control vs BCC	0.624	0.622	0.657	0.558
	Control vs SCC	0.497	0.546	0.509	0.537
	BCC vs SCC	0.830	0.866	0.844	0.949
GSSG/GSH ratio (%)	Control vs BCC	**0.014**	**0.019**	**0.014**	**0.012**
	Control vs SCC	**0.042**	0.061	**0.032**	**0.025**
	BCC vs SCC	0.855	0.856	0.896	0.899
CL-LOOH (AUC)	Control vs BCC	**0.002**	**0.002**	**0.008**	**0.004**
	Control vs SCC	**0.010**	**0.006**	**0.015**	**0.002**
	BCC vs SCC	0.956	0.741	0.839	0.495

Model 1: controlled by age, smoking and the use of antilipemic drugs; Model 2: controlled by model 1 and skin phototype; Model 3: controlled by model 1 and sun exposure; Model 4: controlled by model 1, skin phototype and sun exposure. GSH: reduced glutathione; GSSG: oxidized glutathione; CL-LOOH: chemiluminescence stimulated by tert-butyl hydroperoxide; AUC: area under curve. p values <0.05 are highlighted.

Table 3. Univariate analysis of systemic oxidative stress and systemic inflammatory markers in patients with squamous cell carcinoma (SCC), actinic keratosis (AK) and control group.

Systemic markers	Control (N=75)	SCC (N=24)	AK (N=15)	p value
GSH (µM/g protein)	650.4 (513.0-820.5)[a]	436.9 (357.8-621.7)[b]	567.4 (450.2-706.1)[b]	**0.009**
GSSG (µM/g protein)	271.1 (227.4-375.2)[a]	116.0 (81.45-297.3)[b]	182.5 (88.80-253.0)[b]	**<0.001**
GSSG/GSH ratio (%)	31.56 (25.52-37.74)[a]	23.51 (16.65-37.14)[b]	23.24 (16.76-31.22)[b]	**0.002**
Catalase activity (Vabs/min . mg protein $^{-1}$)	33.95 ± 1.197	29.81 ± 2.608	33.33 ± 4.134	0.310
SOD activity (U/g protein)	0.102 (0.082-0.116)	0.097 (0.074-0.111)	0.089 (0.081-0.114)	0.696
Total Thiols (µM)	460.2 (386.0-551.4)	467.2 (331.4-608.2)	513.6 (395.9-724.2)	0.425
MDA (nM)	472.6 (146.9-785.2)	302.6 (173.4-489.7)	769.1 (229.2-1004)	0.149
CL-LOOH (AUC)	177072 (115205-211164)[a]	91248 (64502-133374)[b]	95249 (70687-153682)[b]	**<0.001**
GGT activity (U/L)	34 (24-49)	27 (21-44.5)	40 (25-75.5)	0.378
AOPP (µM chloramine-T/L)	144.9 ± 7.718	136.6 ± 17.35	127.5 ± 15.14	0.438
Ferritin (ng/mL)	133.1 (76.15-228.6)	111.3 (98.20-274.0)	93.51 (46.36-154.7)	0.338
TNF-α (pg/mL)	8.301 (1.994-74.94)	4.407 (0.899-86.49)	1.999 (0.887-6.160)	0.050
IL-10 (pg/mL)	3.051 (2.258-4.790)	2.625 (1.664-5.817)	2.138 (2.403-5.664)	0.708
TGF-β1 (ng/mL)	4.082 ± 0.675	3.252 ± 0.701	1.691 ± 0.224	0.061
CRP (mg/L)	3.617 ± 0.588	2.523 ± 0.603	4.450 ± 1.238	0.437

Results were expressed as median with interquartile range and were statistically analyzed by Kruskal-Wallis with Dunn's post-hoc test, or were expressed as mean ± SEM (standard error of mean) and were analyzed by one-way ANOVA with Tukey's post-hoc test. Natural logarithmic transformation were used in AOPP, TGF-β1 and CRP data. Different letters were considered statistically different between groups. GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; MDA: malondialdehyde; CL-LOOH: chemiluminescence stimulated by tert-butyl hydroperoxide; AUC: area under curve; GGT: gamma-glutamyl transpeptidase; AOPP: advanced oxidative protein products; TNF-α: tumor necrosis factor-alpha; IL-10: interleukin-10; TGF-β1: transforming growth factor-beta 1; CRP: C-reactive protein. p values < 0.05 are highlighted.

Table 4. Multinomial logistic regression analyses in patients with squamous cell carcinoma (SCC) and actinic keratosis (AK) and control group as dependent variables and systemic parameters of oxidative stress and inflammation listed as explanatory variables.

Explanatory Variables	Group comparisons	*p* value	
		Model 1	Model 2
GSH	Control vs SCC	0.267	0.474
(μM/g protein)	Control vs AK	0.154	0.275
	SCC vs AK	0.196	0.525
GSSG	Control vs SCC	0.963	0.899
(μM/g protein)	Control vs AK	0.213	0.387
	SCC vs AK	0.286	0.643
GSSG/GSH ratio (%)	Control vs SCC	0.148	0.151
	Control vs AK	0.276	0.442
	SCC vs AK	0.354	0.690
CL-LOOH (AUC)	Control vs SCC	**0.022**	**0.037**
	Control vs AK	0.413	0.610
	SCC vs AK	0.832	0.964
TNF-α (pg/mL)	Control vs SCC	0.722	0.850
	Control vs AK	0.110	0.114
	SCC vs AK	0.157	0.125
TGF-β1 (ng/mL)	Control vs SCC	0.741	0.953
	Control vs AK	0.157	0.201
	SCC vs AK	0.183	0.377

Model 1: controlled by age; model 2: controlled by model 1 and smoking. GSH: reduced glutathione; GSSG: oxidized glutathione; CL-LOOH: chemiluminescence stimulated by tert-butyl hydroperoxide; AUC: area under curve; TNF-α: tumor necrosis factor-alpha; TGF-β1: transforming growth factor-beta 1. p values <0.05 are highlighted.

Table 5. Univariate analysis of systemic oxidative stress and inflammatory markers in patients with single and multiple types of nonmelanoma skin cancer.

Systemic markers	BCC (N=90)	SCC (N=24)	SCC+BCC (N=13)	SCC+BCC+AK (N=36)	p value
GSH (µM/g protein)	564.9 (399.1-701.2)	436.9 (357.8-621.7)	472.9 (454.4-713.8)	489.8 (388.3-625.3)	0.208
GSSG (µM/g protein)	184.1 ± 15.63	202.7 ± 43.57	191.4 ± 27.46	213.6 ± 26.66	0.635
GSSG/GSH ratio (%)	20.54 (14.95-30.13)	23.51 (16.65-37.14)	22.64 (17.40-32.49)	27.84 (18.54-35.21)	0.510
Catalase activity (Vabs/min . mg protein $^{-1}$)	30.98 (22.61-41.56)	29.78 (18.35-41.92)	26.69 (12.17-37.51)	27.72 (21.82-35.16)	0.604
SOD activity (U/g protein)	0.094 ± 0.003	0.094 ± 0.006	0.079 ± 0.010	0.097 ± 0.005	0.349
Total Thiols (µM)	487.8 (360.0-592.9)	467.2 (331.4-608.2)	456.4 (393.8-558.7)	434.1 (335.1-535.0)	0.547
MDA (nM)	311.8 (107.6-764.8)	302.6 (173.4-489.7)	106.0 (51.42-490.6)	356.5 (148.5-736.5)	0.461
CL-LOOH (AUC)	100903 (75316-137046)	91248 (64502-133374)	114961 (51528-186718)	109479 (61948-190995)	0.834
GGT activity (U/L)	36 (28-55)^{ab}	27 (21-44.5)^{b}	85.50 (44.25-368.75)^{c}	50.50 (27.5-79.5)^{ac}	**0.009**
AOPP (µM chloramine-T/L)	121.1 (84.5-147.0)	119.5 (79.15-192.9)	123.3 (99.53-189.2)	124.8 (85.34-162.6)	0.868
Ferritin (ng/mL)	220.9 ± 28.81	210.4 ± 53.97	264.3 ± 90.85	236.9 ± 67.08	0.924
TNF-α (pg/mL)	4.764 (1.223-27.81)	4.407 (0.899-86.49)	6.248 (1.662-35.39)	1.768 (0.554-17.15)	0.280
IL-10 (pg/mL)	2.686 (1.916-3.670)	2.625 (1.664-5.817)	3.089 (1.661-6.093)	2.191 (1.437-3.203)	0.232
TGF-β1 (ng/mL)	2.660 ± 0.242	3.252 ± 0.701	0.594 ± 0.194	0.713 ± 0.128	0.585
CRP (mg/L)	5.777 ± 1.249	2.523 ± 0.603	5.025 ± 2.125	3.614 ± 0.898	0.810

BCC: Basal cell carcinoma; SCC: Squamous cell carcinoma; SCC+BCC: Patients with SCC and BCC; SCC+BCC+AK: Patients with BCC, SCC and actinic keratosis (AK). Results were expressed as median with interquartile range and were statistically analyzed by Kruskal-Wallis with Dunn's post-hoc test, or were expressed as mean ± SEM (standard error of mean) and were analyzed by one-way ANOVA with Tukey's post-hoc test. Natural logarithmic transformation were used in GSSG, Ferritin, TGF-β1 and CRP data. Different letters were considered statistically different between groups. GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; MDA: malondialdehyde; CL-LOOH: chemiluminescence stimulated by tert-butyl hydroperoxide; AUC: area under curve; GGT: gamma-glutamyl transpeptidase; AOPP: advanced oxidative protein products; TNF-α: tumor necrosis factor-alpha; IL-10: interleukin-10; TGF-β1: transforming growth factor-beta 1; CRP: C-reactive protein. p values < 0.05 are highlighted.

Table 6. Multinomial logistic regression analyses in patients with single and multiples types of nonmelanoma skin cancer divided into those with only basal cell carcinoma (BCC) and only squamous cells carcinoma (SCC) and patients who developed BCC and SCC (SCC+BCC) and those who developed BCC, SCC and actinic keratosis lesions (SCC+BCC+AK) as dependent variables and systemic gamma-glutamyl transpeptidase (GGT) as explanatory variable.

Explanatory Variables	**Group comparison**	***p* value**			
		Model 1	**Model 2**	**Model 3**	**Model 4**
GGT (U/L)	BCC vs SCC	**0.015**	**0.031**	0.089	0.210
	BCC vs SCC+BCC	0.091	0.099	0.404	0.382
	BCC vs SCC+BCC+AK	0.078	0.121	0.131	0.338
	SCC vs SCC+BCC	**0.008**	**0.018**	0.075	0.165
	SCC vs SCC+BCC+AK	0.224	0.287	0.364	0.691
	SCC+BCC vs SCC+BCC+AK	**0.043**	0.070	0.101	0.264

Model 1: controlled by age; Model 2: controlled by Model 1 and sun exposure; Model 3: controlled by Model 1 and location of tumor; Model 4: controlled by Model 1, sun exposure and location of tumor. p values <0.05 are highlighted.

Supplementary Table 1. Characteristics of participants with single type of nonmelanoma skin cancer and control without cancer.

Characteristics	Control (N=75)	BCC (N=90)	SCC (N=24)	p value
Age Median (25%-75%)	60 (53-70)[a]	68 (60-75)[b]	69 (60.5-75)[b]	**<0.001**
Sex				0.471
Male	37 (49.3%)	53 (58.9%)	13 (54.2%)	
Female	38 (50.7%)	37 (41.1%)	11 (45.8%)	
Skin Phototype (Fitzpatrick's Classification)				**<0.001**
Type I/II/III	59 (78.7%)[a]	88 (97.8%)[b]	22 (91.7%)[ab]	
Type IV/V/VI	16 (21.3%)[a]	2 (2.2%)[b]	2 (8.3%)[ab]	
Sun Exposure				**<0.001**
Low	33 (44.0%)[a]	11 (12.2%)[b]	9 (37.5%)[a]	
Intermediate	16 (21.3%)[a]	17 (18.9%)[a]	6 (25.0%)[a]	
Intense	26 (34.7%)[a]	62 (68.9%)[b]	9 (37.5%)[a]	
Location of tumor				**<0.001**
Head/Neck	NA	74 (82.2%)[a]	12 (50.0%)[b]	
Trunk	NA	9 (10.0%)[a]	2 (8.3%)[a]	
Upper Limbs	NA	2 (2.2%)[a]	6 (25.0%)[b]	
Lower Limbs	NA	1 (1.1%)[a]	2 (8.3%)[b]	
Associated Locations	NA	4 (4.4%)[a]	2 (8.3%)[a]	
Smoking				0.063
Yes	6 (8.0%)	9 (10.0%)	6 (25.0%)	
No	69 (92.0%)	81 (90.0%)	18 (75.0%)	
Use of insulin				0.403
Yes	5 (6.7%)	4 (4.4%)	0 (0.0%)	
No	70 (93.3%)	86 (95.6%)	24 (100%)	
Use of antihyperglycemic drugs				0.340
Yes	9 (12.0%)	18 (20.0%)	5 (20.8%)	
No	66 (88.0%)	72 (80.0%)	19 (79.2%)	
Use of antihypertensive drugs				0.869
Yes	36 (48.0%)	45 (50.0%)	13 (54.2%)	
No	39 (52.0%)	45 (50.0%)	11 (45.8%)	
Use of antilipemic drugs				**0.015**
Yes	20 (26.7%)[a]	9 (10.0%)[b]	3 (12.5%)[ab]	
No	55 (73.3%)[a]	81 (90.0%)[b]	21 (87.5%)[ab]	
Chronic diseases				0.927
Yes	53 (70.7%)	62 (68.9%)	16 (66.7%)	
No	22 (29.3%)	28 (31.1%)	8 (33.3%)	

Qualitative data were evaluated by chi-square and z test for proportion comparison, and quantitative data were evaluated by Kruskal-Wallis test with Dunn's post-hoc test. Different letters were considered statistically different between groups. BCC, Basal cell carcinoma; SCC, Squamous cell carcinoma; NA, Not applicable. p values <0.05 are highlighted.

Supplementary Table 2. Characteristics of participants with squamous cell carcinoma (SCC), actinic keratosis (AK) and control without cancer.

Characteristics	Control (N=75)	SCC (N=24)	AK (N=15)	p value
Age Median (25%-75%)	60 (53-70)[a]	69 (60.5-75)[b]	72 (68-75)[b]	**<0.001**
Sex				0.900
Male	37 (49.3%)	13 (54.2%)	8 (53.3%)	
Female	38 (50.7%)	11 (45.8%)	7 (46.7%)	
Skin Phototype (Fitzpatrick's Classification)				0.062
Type I/II/III	59 (78.7%)	22 (91.7%)	15 (100%)	
Type IV/V/VI	16 (21.3%)	2 (8.3%)	0 (0.0%)	
Sun Exposure				0.537
Low	33 (44.0%)	9 (37.5%)	3 (20.0%)	
Intermediate	16 (21.3%)	6 (25.0%)	5 (33.3%)	
Intense	26 (34.7%)	9 (51.3%)	7 (46.7%)	
Location of tumor				0.490
Head/Neck	NA	12 (50.0%)	7 (46.7%)	
Trunk	NA	2 (8.3%)	4 (26.7%)	
Upper Limbs	NA	6 (25.0%)	3 (20.0%)	
Lower Limbs	NA	2 (8.3%)	0 (0.0%)	
Associated Locations	NA	2 (8.3%)	1 (6.7%)	
Smoking				**0.022**
Yes	6 (8.0%)[a]	6 (25.0%)[b]	0 (0.0%)[a]	
No	69 (92.0%)[a]	18 (75.0%)[b]	15 (100%)[a]	
Chronic diseases				0.606
Yes	53 (70.7%)	15 (62.5%)	9 (60.0%)	
No	22 (29.3%)	9 (37.5%)	6 (40.0%)	

Qualitative data were evaluated by using a chi-square and z test for proportion comparison. Quantitative data were evaluated by using Kruskal-Wallis test with Dunn's post-hoc test. Different letters were considered statistically different between groups. SCC: Squamous cell carcinoma; AK: actinic Keratosis; NA. Not applicable. p values <0.05 are highlighted.

Supplementary Table 3. Characteristics of participants who presented single and multiple types of nonmelanoma skin cancer (NMSC).

Characteristics	BCC (N=90)	SCC (N=24)	SCC+BCC (N=13)	SCC+BCC+AK (N=36)	*p* value
Age	68	69	68	76.5	**<0.001**
Median (25%-75%)	(60-75)[a]	(60.5-75)[a]	(59.5-74)[a]	(71.25-80)[b]	
Sex					0.389
Male	53 (58.9%)	13 (54.2%)	10 (76.9%)	18 (50.0%)	
Female	37 (41.1%)	11 (45.8%)	3 (23.1%)	18 (50.0%)	
Skin Phototype (Fitzpatrick's Classification)					0.210
Type I/II/III	88 (97.8%)	22 (91.7%)	12 (92.3%)	36 (100%)	
Type IV/V/VI	2 (2.2%)	2 (8.3%)	1 (7.7%)	0 (0.0%)	
Sun Exposure					**0.019**
Low	11 (12.2%)[a]	9 (37.5%)[b]	3 (23.1%)[ab]	4 (11.1%)[a]	
Intermediate	17 (18.9%)[abc]	6 (25.0%)[b]	0 (0.0%)[c]	9 (25.0%)[ab]	
Intense	62 (68.9%)[a]	9 (37.5%)[b]	10 (76.9%)[a]	23 (63.9%)[a]	
Location of tumor					**<0.001**
Head/Neck	74 (82.2%)[a]	12 (50.0%)[b]	9 (69.2%)[ab]	22 (61.1%)[b]	
Trunk	9 (10.0%)[a]	2 (8.3%)[ab]	2 (15.4%)[a]	0 (0.0%)[b]	
Upper Limbs	2 (2.2%)[a]	6 (25.0%)[b]	0 (0.0%)[ac]	6 (16.7%)[bc]	
Lower Limbs	1 (1.1%)[a]	2 (8.3%)[b]	0 (0.0%)[ab]	0 (0.0%)[ab]	
Associated Locations	4 (4.4%)[a]	2 (8.3%)[ab]	2 (15.4%)[ab]	8 (22.2%)[b]	
Smoking					0.264
Yes	9 (10.0%)	6 (25.0%)	2 (15.4%)	4 (11.1%)	
No	81 (90.0%)	18 (75.0%)	11 (84.6%)	32 (88.9%)	
Chronic diseases					0.498
Yes	62 (68.9%)	15 (62.5%)	7 (53.8%)	27 (75.0%)	
No	28 (31.1%)	9 (37.5%)	6 (46.2%)	9 (25.0%)	

Qualitative data were evaluated by using a chi-square and z test for proportion comparison. Quantitative data were evaluated by using Kruskal-Wallis test with Dunn's post-hoc test. Different letters were considered statistically different between groups. BCC: Basal cell carcinoma; SCC: Squamous cell carcinoma; AK: Actinic keratosis. SCC+BCC: patients with BCC and SCC. SCC+BCC+AK: patients with BCC, SCC and actinic keratosis.

4 CONCLUSÃO

- Os pacientes com CBC e CEC possuem baixos níveis de EO sistêmico, observados por níveis reduzidos na razão entre glutationa oxidada e reduzida e na lipoperoxidação, e essa redução deve-se à presença do câncer, não sendo influenciada por fatores de risco como fotótipo de pele clara e grau de exposição solar;
- A atividade da GGT sistêmica é o único parâmetro de EO, dentre os avaliados, que difere entre os dois tipos de CPNM, além de estar associada com o desenvolvimento dos dois tipos de CPNM em um mesmo paciente, entretanto, a localização do tumor interfere nessas diferenças;
- Nenhum dos marcadores de estresse oxidativo e inflamação analisados difere sistemicamente entre quem possui a lesão pré-maligna (QA) e maligna (CEC);
- Os parâmetros de inflamação avaliados não diferem sistemicamente nos pacientes, independente de possuírem um ou mais tipos de CPNM.

5 CONSIDERAÇÕES FINAIS

Esse trabalho apresenta como principal limitação o número reduzido de participantes em alguns grupos. Isso se deve principalmente ao rigor adotado no delineamento experimental, que foi fundamental para trazer uma nova abordagem sobre o câncer de pele não-melanoma. Pela primeira vez, diferenças sistêmicas entre parâmetros de estresse oxidativo e de inflamação foram investigadas em pessoas que apresentam um ou mais de um tipo de câncer de pele não-melanoma. Além disso, o trabalho inovou ao considerar características que podem influenciar nos níveis de estresse oxidativo e mediadores inflamatórios sistêmicos na análise estatítica dos dados obtidos, proporcionando maior robustez e relevância aos resultados.

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ANEXOS

ANEXO A

Cópia do parecer do Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UEL - 2015

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL/ HOSPITAL REGIONAL DO NORTE DO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PERFIL OXIDATIVO SISTÊMICO E DO MICROAMBIENTE TUMORAL DE PACIENTES PORTADORES DE CÂNCER DE PELE NÃO MELANOMA

Pesquisador: Alessandra Lourenço Cecchini Armani

Área Temática:

Versão: 4

CAAE: 44235015.3.0000.5231

Instituição Proponente: Programa de PG em Patologia Experimental

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.077.557

Data da Relatoria: 20/05/2015

Apresentação do Projeto:

No documento intitulado "PB_RELATORIO_PESQUISA_ 475300.pdf", item Resumo, lê-se: Sendo assim compreender os aspectos oxidativos da doença pode adicionar novos conhecimentos sobre sua etiopatogênese e auxiliar na investigação de novas estratégias terapêuticas no seu tratamento. Com isso pretende-se caracterizar biomarcadores oxidativos no sangue e de crescimento celular no tecido tumoral de pacientes com câncer de pele não-melanoma. Pacientes com diagnóstico clínico de CPNM e com indicação cirúrgica de remoção do tumor serão recrutados de Hospitais de Londrina-PR e Região durante aproximadamente 36 meses. Será retirado sangue antes do procedimento cirúrgico e amostras da biópsia do tumor com margem serão encaminhadas a patologia para o diagnóstico histopatológico da doença e processadas para pesquisa de estresse oxidativo e nitrosativo no laboratório de Patologia Molecular da Universidade. O sangue será submetido a análises bioquímicas para -glutamiltranspeptidase, proteína C reativa, ácido úrico, TNF- e IL-1b. Para a análise de estresse oxidativo plasmático serão realizados as análises de capacidade antioxidante total, catalase, glutationa, superóxido dismutase, malondialdeído e tiol total. A análise de 3-nitrotirosina por imunohistoquímica do tecido tumoral e da margem normal do tecido para verificar a participação de espécies reativas de oxigênio in situ. Os tipos de tumores

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serão agrupados como carcinoma basocelular (CBC) e carcinoma espinocelular (CEC) e a pele normal do paciente será considerada seu próprio controle. As comparações serão feitas da seguinte forma: grupo controle versus grupo CBC; controle versus CEC; CBC versus CEC. As análises estatísticas empregadas serão as mais adequadas quanto da distribuição da normalidade dos grupos."

Objetivo da Pesquisa:

OBJETIVO PRIMÁRIO

Caracterizar biomarcadores oxidativos no sangue e de crescimento celular no tecido tumoral de pacientes com câncer de pele não-melanoma.

OBJETIVOS SECUNDÁRIOS

(1) Caracterizar o estresse oxidativo sistêmico e tumoral e parâmetros inflamatórios sanguíneos em pacientes com câncer de pele não-melanoma basocelular e espinocelular, antes da ressecção cirúrgica do tumor, com estadiamentos de I a IV estabelecida pelo Comitê Americano de Câncer.

(2) Caracterizar os marcadores de crescimento tumoral p53, Ki67, PCNA e de estresse nitrosativo - 3-nitrotirosina e oxidativo - SOD e proteína carbonílica na biópsia de pacientes submetidos a cirurgia de ressecção do câncer de pele não-melanoma basocelular e espinocelular.

(3) Caracterizar o estresse oxidativo sistêmico, marcadores plasmáticos de doença avançada (estádio IV) e os níveis de TGF- circulante em pacientes com câncer de pele espinocelular.

Avaliação dos Riscos e Benefícios:

Riscos:

O atual projeto que visa obtenção das amostras dos pacientes não trará risco adicional ao paciente. O risco ao paciente será o da própria cirurgia que este deverá ser submetido o paciente participando ou não do estudo.

Benefícios:

Quanto aos benefícios, a longo prazo este estudo beneficiará todos os pacientes que desenvolverem câncer de pele basocelular e espinocelular no que se refere às estratégias terapêuticas de escolha, quando os biomarcadores e suas correlações com o estresse oxidativo

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forem estabelecidas.

Comentários e Considerações sobre a Pesquisa:

Este estudo tem como finalidade caracterizar biomarcadores oxidativos no sangue e de crescimento celular no tecido tumoral de pacientes com câncer de pele não-melanoma em pacientes com diagnóstico clínico de CPNM e com indicação cirúrgica de remoção do tumor.

Estando o mesmo de acordo com as resoluções vigentes pelo CEP e atendendo aos preceitos éticos.

Considerações sobre os Termos de apresentação obrigatória:

Os termos estão adequados e atendem as exigências deste CEP.

Recomendações:

Não existem recomendações

Conclusões ou Pendências e Lista de Inadequações:

Não existem pendências.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

LONDRINA, 25 de Maio de 2015

Assinado por:
Paula Mariza Zedu Alliprandini
(Coordenador)

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ANEXO B

Cópia do parecer do Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UEL - 2019

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ANÁLISE DO ENVOLVIMENTO DE MARCADORES DE ESTRESSE OXIDATIVO E DE PROLIFERAÇÃO CELULAR SISTÊMICOS E NO MICROAMBIENTE TUMORAL NO DESENVOLVIMENTO DE MÚLTIPLOS TIPOS DE CÂNCER DE PELE NÃO-

Pesquisador: Alessandra Lourenço Cecchini Armani

Área Temática:

Versão: 3

CAAE: 00745118.7.0000.5231

Instituição Proponente: Programa de PG em Patologia Experimental

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.146.725

Apresentação do Projeto:

Trata-se de projeto de pós-graduação, que pretende verificar se existam alterações no perfil oxidativo e inflamatório sistêmico e em marcadores de estresse oxidativo e de proliferação celular no microambiente tumoral em pacientes com queratose actínica e com câncer de pele não-melanoma que possam relacionar com o desenvolvimento de múltiplos tipos de câncer de pele não-melanoma em um mesmo paciente.

Para isso, serão recrutados pacientes do Ambulatório de Especialidades do Hospital Universitário de Londrina-PR. A análise clínica dos pacientes será realizada por médicos especialistas durante as consultas encaminhadas a eles: Dr. André Armani, Cirurgião de Cabeça e Pescoço do Departamento de Clínica Cirúrgica e Dr. Airton dos Santos Gon, Dermatologista do Departamento de Clínica Médica, ambos docentes da Universidade Estadual de Londrina. Se, durante a consulta, houver a suspeita de se tratar de CEC, CBC ou de QA, estes pacientes serão convidados a participar do estudo e caso aceitem, deverão assinar o termo de consentimento livre e esclarecido (TCLE) após total e completa explicação sobre o projeto, seus riscos e benefícios.

Objetivo da Pesquisa:

Objetivo Primário:

Investigar a presença de biomarcadores no sangue e no tecido tumoral de pacientes com câncer de pele não-melanoma que possam relacionar-se com o desenvolvimento de mais de um tipo de

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CPNM nos pacientes.

Objetivo Secundário:

- Avaliar o estresse oxidativo sistêmico; - Quantificar parâmetros inflamatórios sanguíneos; - Identificar marcadores de proliferação celular como o

Ki67 e o PCNA no tecido tumoral e no tecido normal adjacente de pacientes submetidos a cirurgia de ressecção do CBC, CEC ou queratose actínica;- Caracterizar o estresse oxidativo através da quantificação de SOD e proteínas carboniladas e

estresse nitrosativo (3-nitrotirosina) no tecido tumoral e no tecido normal adjacente de pacientes submetidos a cirurgia de ressecção do CBC, CEC ou queratose actínica;- Determinar os níveis de p53, Nrf2 e TGF-1 no tecido tumoral e no tecido normal adjacente de pacientes submetidos a cirurgia de ressecção do CBC, CEC ou queratose

actínica;

Avaliação dos Riscos e Benefícios:

Com relação aos riscos a pesquisadora descreve que: "O risco envolvido é inerente à cirurgia a qual será submetido, participando ou não do estudo. No caso de no momento da coleta do sangue o paciente ou controle passarem mal, ele será prontamente atendido pelos médicos e enfermeiros do Hospital de forma que este possa se recuperar

prontamente."

Com relação aos benefícios relata que: "Os benefícios para o paciente serão em longo prazo cuja expectativa é de que este estudo possa fornecer ferramentas necessárias para correlacionar o estresse oxidativo com a progressão e reincidência da doença, bem como verificar se há diferenças no prognóstico do paciente com

tumor cutâneo em relação ao estresse oxidativo e se este possui alguma participação na fisiopatologia do tumor"

Comentários e Considerações sobre a Pesquisa:

Não há.

Considerações sobre os Termos de apresentação obrigatória:

Apresenta folha de rosto devidamente preenchida e assinada. Apresenta autorização do HU para realização da pesquisa. O TCLE apresentado está em acordo com a resolução.

O cronograma preve a coleta de amostras para 01/02. O orçamento na Plataforma Brasil, nas informações básicas não está detalhado, mas apresenta detalhamento no arquivo em anexo.

Continuação do Parecer: 3.146.725

Conclusões ou Pendências e Lista de Inadequações:

As pendências foram atendidas, recomenda-se aprovação.

Considerações Finais a critério do CEP:

Prezado (a) Pesquisador (a),

Este é seu parecer final de aprovação, vinculado ao Comitê de Ética em Pesquisas Envolvendo Seres Humanos da Universidade Estadual de Londrina. É sua responsabilidade imprimi-lo para apresentação aos órgãos e/ou instituições pertinentes.

Coordenação CEP/UEL.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1050988.pdf	23/12/2018 12:30:30		Aceito
Outros	Resposta_Comite_de_etica_23_12_2018.docx	23/12/2018 12:24:56	Alessandra Lourenço Cecchini Armani	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Pacientes_Controles.docx	23/12/2018 12:18:14	Alessandra Lourenço Cecchini Armani	Aceito
Orçamento	Orcamento_Dez_2018.docx	07/12/2018 08:34:10	Alessandra Lourenço Cecchini Armani	Aceito
Brochura Pesquisa	Projeto_30_11_2018.pdf	07/12/2018 08:27:57	Alessandra Lourenço Cecchini Armani	Aceito
Outros	Resposta_Comite_de_etica_28_11_2018.docx	07/12/2018 08:27:08	Alessandra Lourenço Cecchini Armani	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_30_11_2018.doc	07/12/2018 08:25:59	Alessandra Lourenço Cecchini Armani	Aceito
Outros	Parecer_HU_Favoravel.pdf	08/10/2018 12:07:29	Alessandra Lourenço Cecchini Armani	Aceito
Cronograma	CRONOGRAMA.docx	08/10/2018 12:06:23	Alessandra Lourenço Cecchini Armani	Aceito
Declaração de Instituição e Infraestrutura	INFRAESTRUTURA.docx	09/05/2018 10:59:03	Alessandra Lourenço Cecchini Armani	Aceito

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Continuação do Parecer: 3.146.725

Folha de Rosto	folhaDeRosto_Assinada.pdf	09/05/2018 10:52:18	Alessandra Lourenço Cecchini Armani	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

LONDRINA, 14 de Fevereiro de 2019

Assinado por:
Clisia M. Carreira
(Coordenador(a))

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