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ESTADUAL DE LONDRINA

LARISSA JULIANI SANCHES

**RESPOSTA OXIDATIVA IN VITRO E IN VIVO DO USO DA
METFORMINA NO TRATAMENTO DO MELANOMA MURINO
=(B16F10)**

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Orientadora: Profa. Dra. Alessandra Lourenço Cecchini Armani.

Co-orientadora: Profa. Dra. Poliana Camila Marinello

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“Não é sobre chegar no topo do mundo e saber que venceu, é sobre escalar e saber que o caminho te fortaleceu.”

- Ana Vilela

SANCHES, Larissa Juliani. **Resposta oxidativa in vitro e in vivo do uso da metformina no tratamento do melanoma murino (B16F10)**. 2019. 110 f. Tese de doutorado (Programa de Pós-Graduação em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

O melanoma, tem origem nos melanócitos e sua incidência vem crescendo nas últimas décadas, resultado da interação de fatores de risco genéticos (como mutação em BRAF) e ambientais (como exposição à radiação UV, por exemplo). Quando diagnosticado precocemente, o tratamento mais indicado é a remoção cirúrgica, porém quando encontra-se metastizado para linfonodos ou órgãos distantes é comum ser refratário aos tratamentos, levando a uma baixa sobrevida. O melanoma é resistente a grande parte dos tratamentos disponíveis até o presente momento e alguns processos estão associados a essa resistência. A resistência intrínseca envolve a regulação positiva de receptores tirosina quinase e a reativação de MAPK, enquanto a resistência adquirida tardiamente está relacionada com mutações concorrentes em RAS ou MEK, amplificação da forma mutante de BRAF ou splicing alternativo. O estresse oxidativo está intimamente relacionado aos processos de iniciação, proliferação e metástase do câncer bem como na resistência à terapia. Estudos com a metformina tem mostrado que esta pode exercer uma ação na inibição do desenvolvimento do câncer. A metformina é atualmente o medicamento mais prescrito mundialmente para o tratamento do diabetes tipo 2. No câncer a metformina atua nas vias de sinalização LKB1 – AMPK e mTOR. A inibição do mTOR perturba a síntese de proteínas, ocasionando uma ação antineoplásica direta nas células tumorais, através da inibição da progressão do ciclo celular e indução da apoptose e autofagia. Outros estudos, mostram que a metformina desencadeia a apoptose através da regulação negativa de Bcl-2, ativando Bax, p53, desencadeando apoptose através de sinalização da ERK, dependentes e independentes de caspases, inibindo o proto-oncogene c-MYC e HIF-1 α via AMPK. Com base nos efeitos promissores da metformina e na resistência do melanoma aos tratamentos disponíveis, o presente trabalho teve como objetivo estudar os efeitos da metformina em células de melanoma murino metastático B16F10 em dois modelos experimentais: in vitro e in vivo. No modelo in vitro, analisamos a participação da metformina no processo de indução de resistência à dacarbazina (DTIC). Para isso um fenótipo de resistência à DTIC foi induzido em células B16F10. A indução da resistência foi feita através da exposição das células a concentrações crescentes de DTIC. As células foram plaqueadas em garrafas de 25cm² na concentração de 1x10⁶ e expostas a DTIC (1 μ g / mL, 10 μ g / mL, 25 μ g / mL, 50 μ g / mL, 75 μ g / mL, 100 μ g / mL e 125 μ g / mL). As células foram consideradas resistentes quando a exposição a DTIC não causava sua morte. Para analisar a interferência da metformina no processo de indução de resistência, as células foram pré-tratadas com metformina (6 μ M) durante 7 passagens e então foram induzidas à resistência a DTIC, grupo denominado MET-DTIC. As células foram coletadas em todas as passagens, para a análise de marcadores de estresse oxidativo 8-OHdG, p53, Nrf2 e NF- κ B. Os resultados revelam que o fenótipo de resistência a DTIC nas células B16F10 foi induzido com o protocolo experimental utilizado e que a metformina é capaz de atenuar este processo de indução de resistência. Além de prevenir a indução de resistência a metformina atua aumentando os níveis de

antioxidantes celulares e modula vias de sinalização importantes para a quimiorresistência, aumentando a marcação nuclear de p53 e reduzindo a marcação nuclear de NFκB e Nrf2. Na avaliação do efeito da metformina no melanoma in vivo, dois objetivos distintos foram trabalhados: primeiramente foi avaliado o efeito da metformina no tratamento do melanoma metastático, bem como sua ação em conjunto com o quimioterápico DTIC, em um segundo momento, avaliamos a ação da metformina na implantação das células tumorais no pulmão. Para responder o primeiro objetivo, os animais foram inoculados com células B16F10 pelo plexo oftálmico, e divididos nos grupos: controle positivo (C+), onde os animais não foram tratados; grupo Metformina (MET), tratado com metformina na concentração de 2mg i.p. do 1o ao 14o dia; Grupo Dacarbazina (DTIC), tratado com dacarbazina concentração de 50mg/kg i.p. do 5o ao 10o dia; grupo Metformina + Dacarbazina (MET-DTIC), tratado com metformina e dacarbazina nas mesmas condições mencionadas acima; e grupo controle negativo (C-), em que os animais foram inoculados com meio de cultura (DMEM) sem soro e não receberam tratamento. Para o segundo objetivo, todos os animais foram tratados com metformina na concentração de 2mg i.p. durante 5 dias, após esse período os animais foram inoculados com células B16F10 4x10⁶ pelo plexo oftálmico, e divididos nos grupos: controle positivo PTM (C+), PTM (MET), PTM (DTIC) e PTM (MET-DTIC), seguindo os mesmos protocolos de tratamento descritos para o primeiro objetivo. Nos dois protocolos experimentais, os animais foram pesados durante todo o tempo experimental e o consumo de ração por animal em cada grupo foi calculado. Após os 14 dias de experimento, os animais foram eutanasiados e foi realizada a punção cardíaca para a análise sistêmica e a retirada cuidadosa dos pulmões. Foram realizadas as contagens de nódulos metastáticos em todos os pulmões. Foram realizadas análises de estresse oxidativo no microambiente tumoral e sistêmico. A arquitetura pulmonar e marcadores de vias de sinalização envolvidas com proliferação, estresse oxidativo e morte celular também foram analisados devido à sua relação com a resistência aos tratamentos disponíveis. Os resultados demonstram que a metformina tem um importante papel na modulação de importantes vias de sinalização, como a p53, PCNA e Nrf2, interferindo assim no estresse oxidativo e culminando em uma ação antitumoral e antimetastática. Diminuindo assim o número de nódulos metastáticos quando utilizada isoladamente e potencializando o efeito da DTIC, além de diminuir os efeitos colaterais do quimioterápico. Dessa forma pode-se concluir que a metformina tem um potencial terapêutico por apresentar atividade antitumoral em modelos in vitro e in vivo de melanoma murino, estudados com as células B16F10. Essa atividade é acompanhada pela modulação de vias importantes para a viabilidade de células tumorais como, estresse oxidativo, crescimento e morte celular responsáveis, em parte, pela resistência do melanoma a quimioterápicos atualmente disponíveis.

Palavras-chave: Melanoma. Metformina. Resistência. Estresse Oxidativo.

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ABSTRACT

Melanoma originates in melanocytes and its incidence has been increasing in recent decades, as a result of the interaction of genetic risk factors (such as BRAF mutation) and environmental risk factors (such as exposure to UV radiation, for example). When it is diagnosed early, the most indicated treatment is surgical removal, but when metastasized to lymph nodes or distant organs, it is common to be refractory to the treatments, leading to a low survival rate. Melanoma is resistant to most treatments available to date and some processes are associated with this resistance. Intrinsic resistance involves positive regulation of tyrosine kinase receptors and reactivation of MAPK, whereas late acquired resistance is related to concurrent mutations in RAS or MEK, amplification of the mutant form of BRAF or alternative splicing. Oxidative stress is closely related to the processes of initiation, proliferation and metastasis of cancer as well as resistance to therapy. Studies with metformin have shown that it may exert an action in inhibiting the development of cancer. Metformin is currently the world's most widely prescribed drug for the treatment of type 2 diabetes. In cancer metformin acts on the LKB1- AMPK and mTOR signaling pathways. Inhibition of mTOR disrupts protein synthesis, leading to direct antineoplastic action in tumor cells, by inhibiting cell cycle progression and induction of apoptosis and autophagy. Other studies show that metformin triggers apoptosis through downregulation of Bcl-2, activating Bax, p53, triggering apoptosis through ERK signaling, dependent and independent of caspases, inhibiting the proto-oncogene c-MYC and HIF-1 α via AMPK. Based on the promising effects of metformin and melanoma resistance to available treatments, the present study aimed to study the effects of metformin on B16F10 metastatic murine melanoma cells in two experimental models: in vitro and in vivo. In the in vitro model, we analyzed the participation of metformin in the induction of resistance to dacarbazine (DTIC). For this a phenotype of resistance to DTIC was induced in B16F10 cells. Induction of resistance was done by exposing the cells to increasing concentrations of DTIC. The cells were plated in 1x10⁶ 25cm² bottles and exposed to DTIC (1 μ g / mL, 10 μ g / mL, 25 μ g / mL, 50 μ g / mL, 75 μ g / mL, 100 μ g / mL and 125 μ g / mL). Cells were considered resistant when exposure to DTIC did not cause their death. To analyze the interference of metformin in the resistance induction process, the cells were pretreated with metformin (6 μ M) for 7 passages and then were induced to resistance to DTIC, a group termed MET-DTIC. Cells were collected at all passages for the analysis of 8-OH-DG, p53, Nrf2 and NF- κ B oxidative stress markers. The results show that the phenotype of DTIC resistance in B16F10 cells was induced with the experimental protocol used and that metformin is able to attenuate this induction of resistance process. In addition to preventing the induction of resistance, metformin acts by increasing cellular antioxidant levels and modulates signaling pathways important for chemoresistance, increasing p53 nuclear labeling and reducing nuclear labeling of NF κ B and Nrf2. In the evaluation of the effect of metformin on melanoma in vivo, two different objectives were worked out: first, the effect of metformin in the treatment of metastatic melanoma was evaluated, as well as its action together with the DTIC chemotherapy, in a second

moment, we evaluated the action of metformin in the implantation of tumor cells in the lung. To respond to the first objective, the animals were inoculated with B16F10 cells by the ophthalmic plexus, and divided into the groups: positive control (C +), where the animals were not treated; Metformin group (MET) treated with metformin at the concentration of 2mg i.p. from the 1st to the 14th day; Dacarbazine group (DTIC), treated with dacarbazine concentration of 50mg / kg i.p. from the 5th to the 10th day; Metformin + Dacarbazine (MET-DTIC) group, treated with metformin and dacarbazine under the same conditions as mentioned above; and negative control group (C-), in which the animals were inoculated with serum-free culture medium (DMEM) and received no treatment. For the second objective, all animals were treated with metformin at the concentration of 2mg i.p. for 5 days, after that period the animals were inoculated with 4×10^6 B16F10 cells by the ophthalmic plexus and divided into the groups: positive control PTM (C +), PTM (MET), PTM (DTIC) and PTM (MET-DTIC). Same treatment protocols described for the first objective. In both experimental protocols, the animals were weighed throughout the experimental time and the feed intake per animal in each group was calculated. After 14 days of experiment, the animals were euthanized and cardiac puncture was performed for systemic analysis and careful removal of the lungs. Metastasis nodule counts were performed in all lungs. Oxidative stress analyzes were performed in the tumor and systemic microenvironment. Pulmonary architecture and markers of signaling pathways involved with proliferation, oxidative stress and cell death were also analyzed because of their relationship to resistance to available treatments. The results demonstrate that metformin plays an important role in modulating important signaling pathways, such as p53, PCNA and Nrf2, thus interfering with oxidative stress and culminating in antitumor and antimetastatic action. This decreases the number of metastatic nodules when used alone and potentiates the effect of DTIC, in addition to decreasing the side effects of chemotherapy. In this way it can be concluded that metformin has a therapeutic potential for having antitumor activity in in vitro and in vivo models of murine melanoma, studied with B16F10 cells. This activity is accompanied by the modulation of pathways important for the viability of tumor cells such as oxidative stress, growth and cell death, which are partly due to the resistance of melanoma to currently available chemotherapeutic agents.

Key words: Melanoma. Metformin. Resistance. Oxidative Stress.

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LISTA DE ABREVIATURAS E SIGLAS

Akt	Protein kinase B – proteína quinase B.
AMPK	5' AMP-activated Protein Kinase – quinase de adenosina monofosfato.
CAT	Catalase
DTIC	Dacarbazina
ER	Espécies reativas
ERK	Extracellular signal–regulated kinases
ERN	Espécies reativas de nitrogênio.
ERO	Espécies reativas de oxigênio.
FDA	Food and Drug Administration
GPx	Glutathione peroxidase.
GSH	Glutathione reduzida.
GST	Glutathione-S-Transferase.
HER-2	Human epidermal growth factor receptor 2 – receptor para o fator de crescimento epidermal humano 2.
HIF-1 α	Fator induzível por hipóxia 1 α
IFN- α	Interferon- α .
IL-2	Interleucina-2.
IGF-1	Insulin-like growth factor-1 – fator de crescimento semelhante a insulina 1.
INCA	Instituto Nacional do Câncer.
MAPK	Mitogen-activated protein kinases – proteína quinase ativada por mitogeno.
MDA	Malondialdeído.
mTOR	Mammalian target of rapamycin – alvo mamífero da rapamicina.
mTORC1	mTOR complex 1 – complexo 1 da mTOR.
mTORC2	mTOR complex 2 – complexo 2 da mTOR.
NF-kB	Nuclear factor kB – fator nuclear kappa B.
Nrf2	Nuclear factor (erythroid-derived 2)-like 2 – fator de transcrição nuclear associado ao E2.
OMG	Organização Mundial da Saúde.
PI3K	Phosphatidylinositol 3-kinase – fosfatidilinositol 3-quinase.

p53	proteína p53.
SOD	superóxido dismutase.
TSC1	Tuberous sclerosis complex 1 – complexo da esclerose tuberosa ou tuberina.
TSC2	Tuberous sclerosis complex 2 – complexo 2 da esclerose tuberosa ou tuberina.
TMZ	Temozolomida
TNF- α	Fator de necrose tumoral.
VEGF	Vascular endothelial growth factor – fator de crescimento vascular endotelial

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1. INTRODUÇÃO

1.1. Melanoma

O melanoma é um câncer que tem origem nos melanócitos, células produtoras de melanina localizadas na camada basal da epiderme. Nas últimas décadas houve um aumento crescente na incidência e mortalidade desse tipo de câncer (BERWICK et al., 2016; RICHTIG et al., 2017). De acordo com a Organização Mundial da Saúde, mais de 230.000 novos casos de melanoma são diagnosticados anualmente e mais de 50.000 vão a óbito mundialmente (FERLAY et al., 2014).

Segundo o Instituto Nacional do Câncer (INCA) embora o câncer de pele seja o mais frequente no Brasil, correspondendo a 30% de todos os tumores malignos registrados no país, o melanoma representa apenas 3%, sendo o mais grave, devido as suas características altamente invasivas e agressivas. No país estima-se para o ano de 2018, 6.260 novos casos, 2.920 homens e 3.340 mulheres, levando ao óbito pelo menos 1.547 pessoas (INCA, 2018).

O tumor apresenta grande capacidade de metastização mesmo em fases iniciais por causa de sua característica embrionária de motilidade rápida que permanece mesmo após sua diferenciação (BANDYOPACHYAY et al., 2001). Sendo assim, a capacidade de invasão e disseminação são inatas a esse tipo de células (KIERSZEMBAUM, 2004).

A produção da melanina pelos melanócitos inicia-se durante o período fetal, onde é responsável por dar coloração aos tecidos e órgãos em desenvolvimento. Após o nascimento, a síntese de melanina é estimulada pela ação da luz ultravioleta (UV) (KIERSZEMBAUM, 2004), onde os melanócitos sintetizam e transferem os pigmentos de melanina para os queratinócitos adjacentes tendo como principal função, a proteção da pele contra esse tipo de radiação (BANDYOPADHYAY et al., 2001). Os melanócitos produzem dois tipos principais de melanina, a eumelanina (cor preta ou marrom), característica de peles escuras, e a feomelanina (cor vermelha ou amarela), presente principalmente em peles claras (HOOGDUIJN et al., 2004; ITO, WAKAMATSU, OZEKI, 2000).

Em condições fisiológicas, os melanócitos juntamente com os queratinócitos formam a “unidade epidérmica de melanina”, e esta relação é de 1 melanócito para 36 queratinócitos (FITZPATRICK, BREATHNACH, 1963; JIMBOW et al., 1976). Este equilíbrio é mantido através da indução regulada da divisão dos melanócitos (HAASS, SMALLEY, HERLYN, 2004). Para se proliferarem, os melanócitos se separam da

1 membrana basal e dos queratinócitos, retraem seus dendritos, se dividem e migram ao
2 longo da membrana basal antes de, finalmente, se reacoplarem com a matriz e com os
3 queratinócitos (HAASS et al., 2005).

4 Os queratinócitos controlam o crescimento dos melanócitos por: 1) sinalização
5 parácrina com fatores de crescimento; 2) sinalização intracelular através de transdução
6 de sinal e segundos mensageiros; e 3) sinalização intercelular com moléculas de adesão
7 célula-célula, célula-matriz e junções tipo fenda (HAASS; SMALLEY; HERLYN, 2004).
8 Uma desregulação nesse processo pode afetar o equilíbrio entre melanócitos e
9 queratinócitos e desencadear uma proliferação contínua do melanócitos, acarretando
10 no desenvolvimento do melanoma (HAASS et al., 2005).

11 Os mecanismos moleculares exatos dessa desregulação ainda são
12 desconhecidos, mas sabe-se que os melanócitos utilizam algumas estratégias para
13 escaparem do controle dos queratinócitos, como: 1) regulação negativa de receptores
14 importantes na comunicação e adesão com os queratinócitos (ex: E-caderina, P-
15 caderina e conexinas); 2) super expressão de receptores e moléculas de sinalização
16 não encontradas normalmente em melanócitos, mas importantes para promover a
17 adesão entre as próprias células e com fibroblastos e células endoteliais do estroma do
18 tumor (ex: N-caderina, Mel-CAM); 3) perda de adesão na membrana basal pela
19 expressão alterada das proteínas da família das integrinas, responsáveis pela ligação
20 com a matriz extracelular (MEYERS, BALCH, 1998; HAASS et al., 2005).

21 Estas alterações são responsáveis pela proliferação descontrolada, desarranjo
22 da diferenciação celular e morfológica, invasão e disseminação metastática para órgãos
23 distantes (HANAHAN, WEINBERG, 2000; PARK, BISSELL, BARCELLOS-HOFF,
24 2000).

25 O melanoma também pode ser o resultado da interação de fatores de risco
26 genéticos (mutações em BRAF e RAS), bem como ambientais, constituindo um modelo
27 de doença multifatorial. O exato mecanismo molecular que leva à desregulação da
28 homeostasia que envolve os melanócitos ainda é desconhecido. Estudos sugerem que
29 a exposição aguda à radiação UV na infância e/ou adolescência seja responsável por
30 cerca de 40% a 50% de risco de desenvolvimento de melanoma (MILLER, MIHM, 2006),
31 pois a irradiação ultravioleta B (UVB), pode ser absorvida pelo DNA celular resultando
32 em lesões denominadas dímeros de pirimidina. A UVB e a irradiação ultravioleta A
33 (UVA) também geram radicais livres no tecido epitelial que induzem danos em diversas
34 estruturas celulares. Caso o ambiente apresente algum processo inflamatório, mesmo
35 que inicial, a quebra do H_2O_2 gera radical hidroxila, o verdadeiro responsável pelo dano

1 oxidativo ao DNA. Na incapacidade de reparar estas lesões a célula pode fixar uma
2 mutação em um importante gene regulador de ciclo celular desencadeando o processo
3 de iniciação do câncer (ARMSTRONG, KRICKER, 2008; NARAYANAN, SALADI, FOX,
4 2010).

5 Outros fatores de risco importantes são o fenótipo da pele, sendo a pele clara
6 (tipos de pele I e II de Fitzpatrick e Path), mais susceptíveis por possuírem menos
7 melanina; nevo melanocítico displásico, uma vez que os nevos podem evoluir para o
8 melanoma; e fatores genéticos (PAEK et al., 2008; GARCIA, MCLAREN, MEYSKENS,
9 2011).

10 Nevos são neoplasmas clonais com parada no ciclo celular, iniciado por
11 mutações oncogênicas na via MAPK. Dentre os oncongenes com mutações podemos
12 destacar, NRAS (em nevos melanocíticos congênitos), HRAS (nevos de Spitz), BRAF
13 (em nevos melanocíticos adquiridos) e GNAQ e GNA11 (em nevos azuis) (BARAN,
14 DUNCAN, 2011; DAMSKY, BOSENBERG, 2017).

15 Aproximadamente 5–10% dos casos de melanoma são atribuíveis a mutações
16 genéticas, sendo portanto, hereditários, e destes, 20–40% estão associados a uma
17 mutação no gene inibidor de quinase dependente de ciclina 2A p16 (CDKN2A / p16) que
18 sinaliza a produção de proteínas inibidoras do ciclo celular, e dos genes BRAF e N-RAS,
19 envolvidos na via de sinalização das proteínas quinase ativadoras de mitose (MAPK),
20 receptoras c-KIT, quinase ERBB4, reguladora de expressão gênica TRRAP, receptoras
21 de glutamato GRIN2A e GRM3, metaloproteinase MMP8, entre outras (MILLER, MIHM,
22 2006; PAEK et al, 2008; KUNZ, 2013; SANDRU et al., 2014; HIRATA et al., 2015).

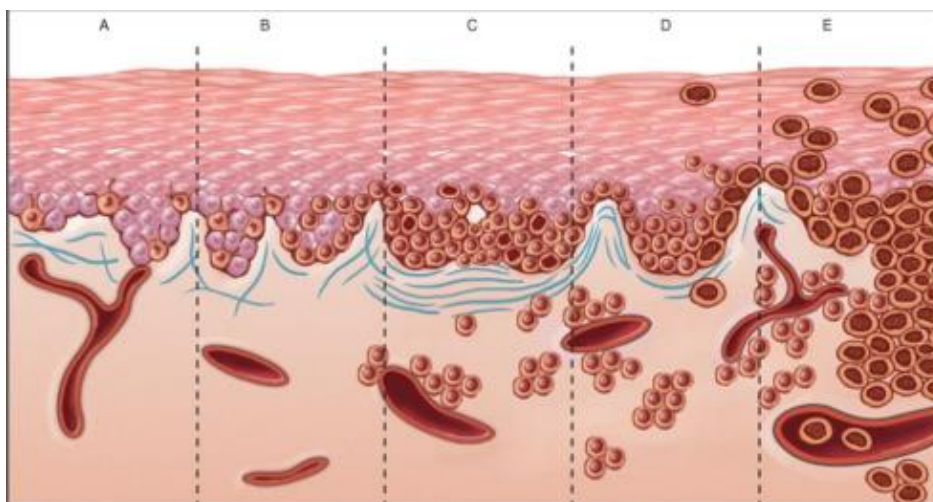
23 Geralmente a identificação do melanoma inicia-se da suspeita de uma lesão na
24 pele ou transformações em um nevo melanocítico pré-existente, onde observa-se
25 modificações com relação ao tamanho, formato e/ou cor. Durante a avaliação aplica-se
26 os critérios ABCDE, que avaliam a presença de assimetria (A), bordas irregulares ou
27 mal definidas (B), coloração mista (C), diâmetro superior a 5 mm (D) e evolução da lesão
28 com aumento de tamanho ou sangramento (E). A presença destes critérios é indicativo
29 para a realização de uma biópsia para a confirmação do diagnóstico (DOHERTY, WAY,
30 2004).

31 Durante o processo de avaliação do diagnóstico, o melanoma pode ser
32 classificado quanto às características macroscópicas e microscópicas do tumor
33 primário. Clinicamente o melanoma é dividido em quatro tipos de acordo com a

1 localização anatômica e o padrão de crescimento do tumor (LEDERMAN, SOBER,
2 1985).

- 3 (i) Melanoma expansivo superficial (MES), é o mais frequente, aparece
4 principalmente entre a quarta e quinta décadas de vida, preferencialmente
5 no tronco dos homens e membros inferiores das mulheres, o que sugere
6 que esta forma clínica esteja associada à exposição solar. Aparece junto a
7 um nevo pré-existente, e tende a apresentar um crescimento radial, com
8 invasão da derme e metástases mais tardias.
- 9 (ii) Melanoma nodular (MN) é o segundo mais comum, ocorre frequentemente
10 entre 50 e 60 anos de idade, na proporção de 2:1 para o sexo masculino,
11 apresenta-se como lesão papulosa ou nodular elevada, de cor castanha,
12 negra ou azulada, com crescimento vertical, metástase precoce, sendo
13 frequentes os processos de ulceração e sangramento.
- 14 (iii) Melanoma lentiginoso acral (MLA), ocorre nas regiões palmoplantares,
15 extremidades digitais, mais freqüente em indivíduos não-caucasianos na
16 faixa etária dos 70 anos de idade. Nas extremidades digitais, o MLA
17 apresenta-se como uma lesão tumoral acastanhada subungueal,
18 melanoníquia estriada, fragmentação longitudinal da lâmina ungueal, além
19 de paroníquia crônica e persistente.
- 20 (iv) Melanoma lentigo maligno (MLM), é considerado o menos frequente, ocorre
21 geralmente em idosos; surge em áreas de foto exposição crônica,
22 apresenta-se como mancha acastanhada ou enegrecida de limites nítidos
23 e irregulares, alcançando vários centímetros de diâmetro, localizada na
24 face (90% dos casos), em mãos e membros inferiores (10% dos casos)

25



26

1 **Figura 1.** Progressão do melanoma. Estágios da progressão tumoral em nevos
2 displásicos. A) Hiperplasia melanocítica lentiginosa. B) Nevo juncional lentiginoso. C) Nevo
3 composto lentiginoso com características citológicas, e arquiteturas anormais (nevo dispásico).
4 D) Melanoma inicial, ou melanoma em fase de crescimento radial (grandes células escuras na
5 epiderme). E) Melanoma avançado (fase de crescimento vertical), com acometimento para
6 dentro da derme e vasos sanguíneos. Robbins & Cotran (2005)

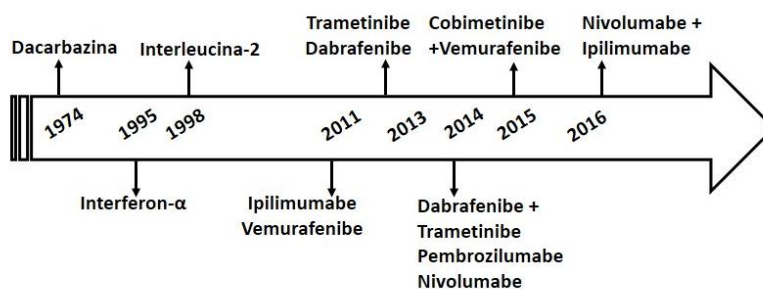
7 O diagnóstico precoce do melanoma é fundamental para o aumento da sobrevida
8 do paciente, devido à correlação existente entre espessura do tumor e mortalidade. Para
9 avaliar o grau de disseminação faz-se o estadiamento do tumor, que reflete a taxa de
10 crescimento, extensão da doença, tipo de tumor e sua relação com o hospedeiro,
11 através de parâmetros histológicos e clínicos (FREEDBERG et al., 2003; TSAO,
12 ATKINS, SOBER, 2004; THOMPSON; SCOLYER; KEFFORD, 2005).

13 O melanoma pode metastizar através de três rotas: (I) contínua: com
14 crescimento tumoral se estendendo do tumor primário; (II) linfogênica: em linfonodos
15 regionais; e (III) hematogênica: em órgãos distantes, incluindo os pulmões e cérebro.
16 Normalmente, metástases linfogênicas antecedem a metástase hematogênica. Vários
17 mecanismos são importantes no desenvolvimento de metástases, dentre eles
18 destacam-se a indução da proliferação de células tumorais, inibição da apoptose,
19 mobilização e invasão de células tumorais, bem como a angiogênese (VALERO et al.,
20 2010).

21

22 1.2. Tratamentos do Melanoma

23 Quando o melanoma é diagnosticado no início, ainda sem evidência de
24 metástase, o tratamento preconizado é a remoção cirúrgica. Porém, quando encontra-
25 se disseminado para linfonodos ou há presença de metástases distantes, o tratamento
26 envolve processos cirúrgicos associados à quimioterapia, radioterapia e/ou
27 imunoterapia (EMMETT, DEWING, PRITCHARD-JONES, 2007; KORN et al., 2008).
28 Nos últimos anos, várias terapias foram aprovadas pela Food and Drug Administration
29 (FDA), como mostra a figura abaixo.



1

2 **Figura 2.** Ordem cronológica de drogas aprovadas pela FDA para o tratamento de melanoma. A
 3 dacarbazina foi o primeiro medicamento aprovado (1974), seguido pelo interferon α -2b e
 4 interleucina-2 nos anos 90. Entre 2011 e 2015 foram aprovadas terapias com inibidores seletivos,
 5 anticorpos e terapias direcionadas combinadas, Adaptado por Domingues et al., (2018).
 6

7 A quimioterapia foi a primeira opção de tratamento não cirúrgico para melanoma
 8 metastático, e não apresentou uma melhora significativa no aumento da sobrevivência do
 9 paciente, em média em torno de 6 meses. Isso é devido a resistência das células de
 10 melanoma de entrarem em apoptose, que é o principal mecanismo de ação dos
 11 quimioterápicos (WILSON, SCHUCHTER, 2016).

12 A dacarbazina (DTIC), foi aprovada em 1974 pela FDA, como medicamento
 13 quimioterápico padrão para o tratamento melanoma metastático. A DTIC, é considerada
 14 um dos quimioterápicos mais eficazes para o melanoma metastático, sendo o mais
 15 utilizado no Brasil (BECHARA et al. 2013). É um agente alquilante de DNA e inibidor
 16 dos mecanismos de reparo, que tem como objetivo induzir lesões de DNA resultando
 17 na morte por apoptose das células neoplásicas (TEMERK, IBRAHIM, 2014). É
 18 administrada por via endovenosa, metabolizada no fígado pela família do citocromo
 19 P450 e eliminada por via renal, apresenta efeitos colaterais significativos como náuseas
 20 e vômitos (MARKOVIC et al., 2007; SMITH et al., 2008).

21 Temozolomida (TMZ), é um análogo da dacarbazina que pode ser administrado
 22 por via oral, a TMZ pode penetrar no fluido cerebrospinal e por esse motivo, tem
 23 potencial para o tratamento de metástases cerebrais (BHATIA, TYKODI, THOMPSON,
 24 2009; VELHO, 2012). No geral, a TMZ não mostrou qualquer vantagem sobre a DTIC,
 25 ambas produzindo taxas de resposta semelhantes, sendo a escolha entre as duas
 26 drogas feita com base na via de administração preferida, custo, disponibilidade e local
 27 de metástase (BHATIA, TYKODI, THOMPSON, 2009; TENTORI, GRAZIANI, 2009).

28 Devido a agressividade e a natureza do melanoma, os efeitos dos
 29 quimioterápicos sozinhos são insatisfatórios, a estratégia mais comumente abordada no
 30 tratamento do melanoma é a combinação de terapias. A politerapia tem como base a
 31 DTIC, que embora não apresente resultados satisfatórios frente ao combate do
 32 melanoma, ainda é o quimioterápico mais utilizado como base para o maior número de

1 regimes de tratamentos para o melanoma. Podendo ser combinada com outras drogas
2 citotóxicas, como a cisplatina (agente antitumoral que induz danos ao DNA) (SIDDIK,
3 2003), vimblastina (agente de direcionamento de microtúbulos), no entanto outros
4 agentes também são utilizados como: a temozolomida, as nitrosoureas (Carmustina,
5 Lomustina e fotemustina), os análogos da platina (2ª linha, cisplatina e a carboplatina),
6 e os taxanos, como o paclitaxel (DEMIERRE M-F et al., 2008; MANSFIELD,
7 MARKOVIC, 2009; APALLA et al. 2017; CASTLE et al., 2017).

8 A fotemustina ultrapassa a barreira hematoencefálica e tem como vantagem
9 maior eficácia em metástases cerebrais, bem como taxas de respostas objetivas
10 importantes (quase 25%). No entanto, revela uma toxicidade medular importante e não
11 aumenta a taxa de sobrevida quando comparada com a DTIC. O paclitaxel é
12 administrado por via endovenosa e interfere na despolimerização dos microtúbulos na
13 divisão celular, o tratamento com este agente pode provocar complicações, tais como
14 neuropatia periférica e neutropenia (MANSFIELD, MARKOVIC, 2009).

15 As células de melanoma através de vários mecanismos apresentam uma
16 resistência intrínseca à maioria das terapias convencionais (HELMBACH et al. 2001),
17 por esse motivo pesquisadores continuam procurando por diferentes estratégias
18 terapêuticas, principalmente no campo da imunoterapia, que tem como objetivo
19 potencializar a resposta imune do paciente contra o melanoma. A imunoterapia, tem
20 apresentado sucessos importantes, embora limitados, os maiores obstáculos consistem
21 na supressão imune induzida pela neoplasia e nos mecanismos de evasão imunológica
22 tumoral, que tornam o paciente tolerante aos antígenos tumorais (KIRKWOOD et al.,
23 2008). Os imunoestimuladores mais utilizados para tratamento melanoma são
24 interleucina (IL) -2, interferon (IFN- α) -alfa, ipilimumabe e timosina alfa 1 (CHEN et al.
25 2013).

26 O interferon-alfa (IFN- α) recombinante é uma citocina com um amplo espectro
27 de atividades biológicas, seu efeito antiproliferativo direto inibe a atividade tumoral,
28 tornando-o mais susceptível às células de defesa do próprio paciente. O IFN- α foi
29 aprovado pelo FDA em 1996 para uso em terapia adjuvante em pacientes que possuem
30 alto risco de recorrência após ressecção de melanoma (DI FRANCO et al. 2017). No
31 melanoma, o IFN- α demonstra um efeito antitumoral imunomodulador, induzindo um
32 efeito na expressão da classe I do complexo de histocompatibilidade principal em
33 células de imunidade do melanoma, sendo capaz de inibir proliferação de células de
34 melanoma, com uma dose dependente, efeito pró-apoptótico (ROH et al., 2013).

35 A interleucina-2 (IL-2), é um hormônio recombinante do sistema imunitário
36 descrita como “fator de crescimento para células T” (MARKOVIC et al., 2007). Quando

1 administrada em *bolus* e em altas doses, induz o crescimento de células T efectoras, o
2 que aumentaria a resposta imunológica do hospedeiro contra o tumor, sendo os
3 resultados mais evidentes em indivíduos com metástases linfonodais ou metástases em
4 tecidos moles. É o único agente atualmente disponível que produz remissões a longo
5 prazo em pacientes com melanoma avançado, e foi aprovada pelo FDA em 1998 para
6 o tratamento do melanoma metastático (KOON, ATKINS, 2007; KIRKWOOD et al.,
7 2008; JEN, et al., 2011).

8 A IL-2 está associada a uma toxicidade significativa, estando limitada a pacientes
9 mais jovens e sem disfunção de órgãos, pode provocar efeitos secundários como
10 sintomas gripais, toxicidade cardiovascular, efeitos gastro-intestinais, hepatotoxicidade,
11 neurotoxicidade, nefrotoxicidade, hipotireoidismo (MARKOVIC et al., 2007; KIRKWOOD
12 et al., 2008). Apesar da maioria dos efeitos adversos ser reversível, o perfil tóxico da IL-
13 2 requer monitoramento do paciente (KIRKWOOD et al., 2008). A IL-2 pode ser usada
14 em associação com outros agentes, como a DTIC, cisplatina e interferon-alfa (IFN- α) e
15 sua indicação atual é no tratamento do melanoma metastático (KOON, ATKINS, 2007).

16 O ipilimumabe é um anticorpo monoclonal que bloqueia os receptores CTLA-
17 4. Receptor proteico que funciona como um checkpoint imunológico, regulando
18 negativamente as respostas imunes. O CTLA-4 é constitutivamente expresso
19 em células T reguladoras, mas apenas regulado positivamente em células
20 T convencionais após ativação, fenômeno que só ocorre em cânceres (FRANKLIN et
21 al., 2017).

22 Ipilimumab foi aprovado pelo FDA em 2011 como tratamento de segunda linha
23 para o melanoma após Hodi e colaboradores (2010) relatarem melhora na sobrevida de
24 pacientes com melanoma tratados com ipilimumab. Foram realizados vários estudos
25 de fase I, II, III com diferentes doses de ipilimumab que resultaram em maior sobrevida
26 global mediana e taxa de resposta, estabelecendo uma correlação positiva com o
27 aumento da dose de ipilimumab (HODI et al., 2010; ROBERT et al., 2011).

28 Cerca de 70% dos pacientes com melanoma apresentam mutações nos genes
29 das principais vias de sinalizações, mutações nesses genes podem estar associadas a
30 proliferação e ao fenótipo maligno do melanoma (FLAHERTY et al., 2012). A terapia
31 alvo utiliza inibidores de pequenas moléculas ou anticorpos que afetam estas proteínas
32 mutadas, que são responsáveis para a progressão da doença.

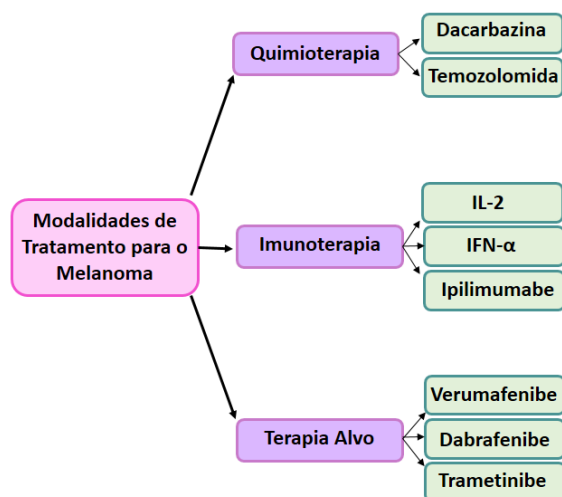
33 O BRAF é o gene com maior frequência de mutação e 50% dos melanomas
34 cutâneos sem exposição crônica ao sol apresentam mutação em BRAF. Esta mutação
35 está associada a ativação da sinalização da via da MAPK e conseqüentemente com o
36 aumento do crescimento e proliferação de células neoplásicas (CHENG, ZHANG, LI,
37 2013). A mutação mais comum de BRAF ocorre no aminoácido 600, em que a valina

1 normal é substituída pelo ácido glutâmico (BRAFFV600E), ou menos comumente por
2 lisina (BRAFFV600K) (BROSE et al.,2002).

3 O vemurafenibe, um inibidor seletivo de BRAF mutado, foi aprovado pelo FDA
4 em 2011 para o tratamento de pacientes com melanomas metastáticos portadores de
5 mutações em BRAFFV600E. Estudos relataram que pacientes que receberam o
6 vemurafenibe melhoraram a resposta clinica e apresentaram regressão tumoral
7 (CHAPMAN et al, 2011).

8 O dabrafenib também é um inibidor de BRAF, altamente potente com 100
9 vezes maior seletividade para o BRAFFV600E mutado (MANDALÀ, VOIT, 2013), foi
10 aprovado pelo FDA em 2013 para o tratamento do melanoma metastático de portadores
11 de mutações em BRAFFV600E. (RAJAKULENDRAN, ADAM, 2014). Grandes limitações
12 no uso de inibidores de BRAF são o desenvolvimento de resistência em quase todos os
13 casos e a toxicidade associada à inibição do BRAF (MENZIES, LONG, 2014).

14 Uma estratégia de superar a resistência aos inibidores de BRAF, é atacar o
15 alvo jusante a MEK (ASCIERTO et al., 2013). O trametinibe é o inibidor farmacológico
16 de MEK1/2 com atividade antitumoral aprovado pela FDA em 2013 para o tratamento
17 de melanoma maligno com mutações em BRAF e metástases (WRIGHT, Mc
18 CORMACK, 2013; LIVINGSTONE et al., 2014).



19

20 **Figura 3.** Classificação em modalidades dos tratamento do melanoma. Adaptado por Mishra et
21 al., (2018).

22

23

24

1.3. Resistência ao tratamento no melanoma

25

26

O melanoma é refratário a grande parte dos tratamentos disponíveis até o presente momento, por esse motivo é atualmente um dos principais desafios para a

1 comunidade científica, vários estudos vem sendo realizados, na tentativa de descobrir
2 novos medicamentos para seu tratamento.

3 A grande plasticidade das células de melanoma promove a sua evolução clonal,
4 emergindo fenótipos que superam a inibição de MAPK fenotipicamente e
5 metabolicamente (ROESCH, 2014; SHI et al., 2014). Dois processos estão relacionados
6 com a resistência do melanoma, (I) resistência intrínseca ou resposta tumoral adquirida,
7 como a regulação positiva do receptor tirosina quinase e a reativação de MAPK, que
8 está presente em 70% dos casos; (II) resistência adquirida tardiamente, os principais
9 mecanismos relacionados com este fenômeno são mutações concorrentes em RAS ou
10 MEK, amplificação da forma mutante de BRAF ou *splicing* alternativo (SHI et al., 2012;
11 STRUB et al., 2018).

12 Outro mecanismo de resistência que podemos citar é a expressão dos membros
13 das famílias de proteínas inibidoras da apoptose (IAPs) que têm sido associado com a
14 progressão tumoral, sendo sua expressão já observada no início da disfunção
15 melanocítica (nevus melanocíticos) (SOENGAS; LOWE, 2003). Também pode-se citar
16 a expressão da cFLIP (proteína inibidora da FLICE), um inibidor da ativação de
17 caspases (especialmente da caspase 8) (BULLANI et al., 2001), com consequente
18 inibição da apoptose.

19 Outra possível característica de quimiorresistência do melanoma pode ser devida
20 a melanogênese, pois esta pode regular a expressão do fator de transcrição, o fator
21 induzível por hipóxia 1 α (HIF-1 α), a principal molécula para a resistência das células
22 neoplásicas às terapias, diminuindo a eficácia dos tratamentos e a taxas de
23 sobrevivência durante os estágios III e IV do melanoma (SLOMINSKI et al., 2014;
24 BROŻYNA et al., 2016). Tsai e colaboradores (2014) relataram em seu trabalho que as
25 células remanescentes do melanoma desenvolveram a superexpressão do HIF-1 α ,
26 como resultado da hipóxia e do estresse oxidativo gerado pelos tratamentos, isso leva
27 ao aumento da capacidade invasiva das células tumorais.

28

29 1.4. Estresse Oxidativo e progressão do melanoma

30 Espécies reativas (ER) são moléculas altamente instáveis que podem interagir e
31 alterar diversos componentes celulares como: DNA, proteína e lipídio. Estes compostos
32 perdem elétrons tornando-se moléculas reativas, propagando danos oxidativos e
33 iniciando reações em cascata que podem causar lesões irreversíveis nas células
34 (HALLIWELL, GUTTERIDGE, 2007).

1 Essas espécies podem ser radicais livres, que possuem um número ímpar de
2 elétrons (elétrons não pareados) em seu orbital mais externo, ou também podem ser
3 não radiculares, que apesar de sua configuração estável, podem ser altamente reativos
4 (FERREIRA, MATSUBARA, 1997). As ER cujo elétron reativo encontra-se centrado nos
5 átomos de oxigênio são denominadas ERO (espécies reativas de oxigênio) e quando
6 nos átomos de nitrogênio são denominadas ERN (espécies reativas do nitrogênio)
7 (VOGT, 1995)

8 As principais ER relacionadas ao estresse oxidativo são: ($^1\text{O}_2$) oxigênio singlete:
9 forma excitada do oxigênio molecular e não possui elétrons desemparelhados em sua
10 última camada (HALLIWELL, GUTTERIDGE, 1990). ($\text{O}_2\cdot^-$) radical superóxido:
11 formado em todas as células aeróbicas, cerca de 5% do oxigênio consumido é reduzido
12 a radical ânion superóxido, sua formação é especialmente induzida durante a ativação
13 de neutrófilos, monócitos, macrófagos e eosinófilos no “burst” respiratório. (HALLIWELL,
14 GUTTERIDGE, 1985). (HO_2) peroxil ou hidroperoxila: forma protonada do superóxido,
15 sendo considerado mais reativo que o radical superóxido, por apresentar maior
16 facilidade em iniciar lesões em membranas biológicas (HALLIWELL, GUTTERIDGE,
17 1990). Além das espécies reativas de oxigênio também podemos observar entre os
18 radicais livres, as espécies reativas de nitrogênio, que incluem o óxido nítrico e o
19 peroxinitrito.

20 Quando há uma produção excessiva de ER, seja ela induzida de forma
21 endógena ou durante o metabolismo celular, como na fosforilação oxidativa em
22 mitocôndrias e na β -oxidação de ácidos graxos em peroxissomos (KNAPOWSKI,
23 WIECZOROWSKA-TOBIS, WITOWSKI, 2002), ou de forma exógena, como por
24 exposição à radiação UV e poluentes (FINKEL, HOLBROOK, 2000), proteínas, lipídeos
25 e DNA são oxidados, podendo comprometer a homeostase celular. Porém, existem
26 enzimas e compostos de baixa massa molecular que constituem o sistema de defesa
27 antioxidante. Uns podem ser sintetizados pelo próprio organismo e outros podem ser
28 adquiridos através da dieta alimentar. As defesas antioxidantes são conhecidas por
29 diminuir a quantidade de ER, limitando os danos causados por essas espécies no
30 organismo (HALLIWELL, GUTTERIDGE, 2007).

31 Para se defender da toxicidade das ER, as células apresentam alguns
32 mecanismos de defesas. O primeiro mecanismo, é a prevenção da formação de ER
33 através de mecanismos antioxidantes em sentido mais amplo, como por exemplo, o
34 transporte de oxigênio. A quelatação de metais durante transporte e armazenamento
35 evitando a ocorrência da reação de Fenton, a eficiência da cadeia de transporte de

1 elétrons na mitocôndria que mantém a formação de ER em nível controlado e a
2 organização estrutural do DNA em cromatina (SIES, 1993).

3 Outro mecanismo inclui enzimas antioxidantes como superóxido dismutases
4 (SOD), catalase (CAT), glutathione peroxidase (GPX) e pequenas moléculas como a
5 glutathione reduzida (GSH) (GADJEVA, DIMOV, GEORGIEVA, 2008). Estudos *in vitro*
6 demonstraram que células de melanoma apresentam atividade da enzima antioxidante
7 catalase (CAT) diminuída, por outro lado, apresentam aumento na atividade da enzima
8 superóxido dismutase (SOD) e elevadas concentrações de vitamina E quando
9 comparadas a melanócitos normais (SANDER et al., 2004).

10 Finalmente, existem os mecanismos de reparo quando os demais mecanismos
11 não conseguem impedir a ação das ER. No DNA, esse reparo aos danos oxidativos
12 podem ser de três formas: reparo de excisão de bases, reparo de excisão de nucleotídeo
13 e reparo de erro de pareamento (LUNEC et al., 2002).

14 O estresse oxidativo é considerado o desequilíbrio entre as espécies reativas
15 de oxigênio (e/ou de nitrogênio) e as defesas antioxidantes da células, tecido ou sistema
16 (JONES, 2006; HALLIWELL, 2007). O estresse oxidativo é um processo intimamente
17 relacionado com o câncer, está envolvido, também, no processo de iniciação, pois
18 espécies reativas podem induzir lesões no DNA e modular as respostas celulares a
19 estes danos (BARZILAI, YAMAMOTO, 2004).

20 O estresse oxidativo está envolvido na inflamação crônica, ativando diversos
21 fatores de transcrição como: NF- κ B, AP-1, p53, HIF-1 α , dentre outros, que elevam à
22 expressão de diferentes genes responsáveis por fatores de crescimento, citocinas,
23 quimiocinas e reguladores de ciclo celular. Desta forma o estresse oxidativo está
24 intimamente envolvido na proliferação celular, na resistência à quimioterapia e
25 radioterapia, na angiogênese tumoral e na invasão e metástase (GADJEVA, DIMOV,
26 GEORGIEVA, 2008; REUTER et al., 2010).

27 A participação de espécies reativas na fisiopatologia do câncer é bem relatada
28 na literatura, porém seu papel na fisiopatologia do melanoma ainda é pouco conhecido
29 (GADJEVA, DIMOV, GEORGIEVA, 2008). O melanoma é uma neoplasia com perfil
30 relativamente único quando comparado com outros tipos de câncer, apresentando
31 níveis elevados de estresse oxidativo no microambiente tumoral primário (SANDER et
32 al., 2003; FRUEHAUF, TRAPP, 2008). Pacientes com melanoma demonstraram
33 aumento do estresse oxidativo sistêmico e comprometimento do sistema antioxidante,
34 o que poderia favorecer o acúmulo de ERO e promover a carcinogênese em tecidos
35 vizinhos (GADJEVA, DIMOV, GEORGIEVA, 2008).

36 A melanina é antioxidante com função de sequestrar espécies reativas (ER), que
37 incluem o radical hidroxila, superóxido, oxigênio singlete e peróxido de hidrogênio,

1 protegendo as células dos danos causados pela radiação ultravioleta (UV). Porém, a
2 síntese da melanina leva a formação de espécies reativas de oxigênio. A produção de
3 melanina inicia-se com a oxidação L-tirosina à ortoquinona, dopaquinona, seguindo-se
4 por uma sequência de reações, até a produção do pigmento indólico (eumelanina) com
5 auxílio de três enzimas essenciais, tirosinase, TRP-2 (dopacromo tautomerase) e TRP-
6 1 (DHICA oxidase). Pode ainda gerar o pigmento feomelanina que contém subunidades
7 de benzotiazina, sendo que neste caso, somente a tirosinase é necessária (ITO,
8 WAKAMATSU, OZEKI, 2000).

9 A eumelanina tem sido associada com a fotoproteção, pois age como um filtro
10 e possui propriedades que seqüestram espécies reativas geradas por UV. Por outro
11 lado, a feomelanina parece contribuir para a carcinogênese, pois apresenta diferentes
12 propriedades, sendo menos efetiva para filtrar os raios UV e aparentemente age como
13 um fotossensibilizador endógeno, e está associada com a produção de radicais livres
14 (KADEKARO et al., 2003; MEREDITH, SARNA, 2006).

15 Devido à presença de melanossomas, estruturalmente aberrantes nas células
16 de melanoma e da grande infiltração de células do sistema imunológico, grande
17 quantidade de ER são geradas nesse microambiente tumoral primário, exercendo uma
18 pressão seletiva nas células tumorais (FRUEHAUF, TRAPP, 2008). Além disso,
19 diferentemente dos melanócitos normais, as células de melanoma possuem alta
20 capacidade antioxidante e usam essa habilidade para favorecer sua proliferação e
21 invasão/metástase (MEYSKENS, FARMER, FRUEHAUF, 2001; WITTGEN, VAN
22 KEMPEN, 2007).

23 A produção de melanina é um processo que envolve uma série de eventos
24 celulares complexos, sendo a tirosinase a enzima essencial da via. Ela catalisa as
25 etapas iniciais, sendo regulada através de controles transcricionais e mecanismos
26 traducionais (ANCANS, HOOGDUIJN, THODY, 2001). Durante essa produção ocorre
27 geração e liberação de grande quantidade de peróxido de hidrogênio (H₂O₂)
28 (MEYSKENS et al., 1999). Dessa forma, um estado pró-oxidante é estabelecido, porém,
29 não o suficiente para provocar a morte celular.

30 O estado pró-oxidante é responsável pela ativação de vias sinalizadoras
31 sensíveis ao controle redox. Como a do fator de transcrição nuclear kappa B (NF-κB),
32 que é constitutivamente mais ativada em células de melanoma em comparação a
33 melanócitos normais. Outra via sensível ao controle redox é a AP-1, que é ativada em
34 condições antioxidantes. Esta última encontra-se diminuída no melanoma (MEYSKENS
35 et al., 1999).

1 Em células neoplásicas, o NF- κ B regula a proliferação celular, controla a
2 apoptose, promove angiogênese e estimula invasão e metástase (KARIN, 2006). A
3 inibição do NF- κ B sozinho ou em combinação com agentes quimioterápicos pode levar
4 a morte de células tumorais e a inibição do crescimento tumoral (SHISHODIA,
5 AGGARWAL, 2002; SCHEIDEREIT, 2006).

6 Além disso, a ativação do NF- κ B pode mediar a quimiorresistência induzida por
7 drogas quimioterápicas. Essa indução de resistência é através do aumento da ligação
8 de Nf- κ B ao DNA e conseqüente aumento na transcrição de genes relacionados à
9 progressão tumoral, como genes do ciclo celular, genes anti-apoptóticos e proteases
10 que promovem seu fenótipo invasivo (DAS, WHITE, 1997; SCHON et al., 2008).

11 Kudugunti e colabores (2010), observaram que a depleção nos níveis
12 intracelulares de glutathiona (GSH), pode levar à morte de células tumorais de melanoma
13 humano quando tratadas com éster feniletílico do ácido caféico (CAPE). Esse efeito é
14 acentuadamente observado em células de melanoma, devido à importância da enzima
15 tirosinase na citotoxicidade mediada pelo CAPE, que é um inibidor moderado do fator
16 nuclear kappa B.

17 Produtos finais de lipoperoxidação, como o CEP (ω -2-carboxietilpirrol) são
18 capazes de aumentar a angiogênese, através de receptor toll do tipo 2 em uma via
19 dependente de MyD-88. No melanoma, quando há intensa vascularização e inflamação,
20 os níveis de CEP encontram-se aumentado e o uso de anticorpos anti-CEP diminui a
21 progressão e vascularização do tumor (WEST et al., 2010).

22 Glutathiona-S-transferases (GST), são uma família de enzimas conhecidas por
23 exercerem um importante papel na proteção contra o estresse oxidativo, incluindo o
24 estresse oxidativo causado pela radiação ultravioleta. Estudo revela, que indivíduos com
25 polimorfismo nulo para as enzimas GSTM1 e GSTT1 e que sofreram queimaduras
26 solares quando crianças possuem risco extremamente aumentado para
27 desenvolvimento de melanoma (FORTES et al., 2011).

28 29 1.5. Células B16F10

30 As células B16F10 foram obtidas por Isaias J. Fidler em 1973, selecionando as
31 células tumorais com maior capacidade de colonizar os pulmões a partir de uma variante
32 parental menos metastática. Fidler, foi o pioneiro em realizar estudos com as células
33 B16. Várias formas B16 foram isoladas, e algumas diferenças como, o potencial
34 metastático foram encontradas entre as sub-linhagens (FIDLER, NICOLSON, 1976;
35 FIDLER, BUCANA, 1977).

1 A linhagem B16F10 é amplamente utilizada nos laboratórios como modelo para
2 melanoma murino. Por ser altamente agressiva, crescer em frascos de cultura, possuir
3 a capacidade de gerar tumores subcutâneos ou metástase pulmonar de acordo com o
4 método de inoculação utilizado (HART, 1979; MENON, KUTTAN, LUTTAN, 1995).

5 6 1.6. Metformina

7 A metformina (1,1-dimetilbiguanida) é uma droga de baixo custo, segura e
8 atualmente a mais prescrita em todo o mundo para o tratamento do diabetes mellitus
9 tipo 2. Aproximadamente 120 milhões de prescrições são feitas anualmente
10 (KOURELIS, SIEGEL, 2012; TAUBES, 2012; GORMSEN et al.,2016). Metformina,
11 juntamente com seus derivados fenformin e buformina, pertencem à biguanida classe
12 de drogas antidiabéticas. Eles eram originalmente derivado da galegina, um produto
13 natural da planta *Galega officinalis* usada durante séculos na Europa como um remédio
14 herbal (SHU et al., 2007; GORMSEN et al.,2016).

15 Embora tenha sido introduzida pela primeira vez na década de 1950, seu
16 mecanismo de ação está se tornando claro somente agora. A metformina reduz a
17 glicose pela gliconeogênese no fígado, e estimula a glicogênese nos tecidos periféricos
18 insulino-dependentes, aumentando a captação de glicose mediada por insulina na
19 musculatura esquelética (PERNICOVA, KORBONITS, 2014), sem aumentar os níveis
20 plasmáticos de insulina.

21 Além disso, a metformina diminui a beta-oxidação de ácidos graxos e facilita o
22 transporte de glicose, por aumentar a translocação do transportador da glicose (GLUT4)
23 para a membrana celular no tecido adiposo e muscular (SCARPELLO, HOWLETT,
24 2008). A metformina é capaz ainda de melhorar de forma indireta a função das células
25 β pancreáticas, uma vez que ao aumentar a sensibilidade à insulina, reduz a toxicidade
26 da glicose sobre essas células (SCARPELLO, HOWLETT, 2008).

27 O exato mecanismo de ação da metformina ainda não foi completamente
28 esclarecido. Sabe-se que a metformina age dentro da célula, na mitocôndria, onde ela
29 inibe transitóriamente o complexo I da cadeia respiratória. A inibição reduz
30 transitóriamente o estado de energia celular, que altera o equilíbrio entre a produção e
31 o consumo de trifosfato de adenosina (ATP), levando a um aumento na relação
32 intracelular de monofosfato de adenosina (AMP)(EL-MIR et al., 2000). A diminuição
33 resultante na produção de ATP e aumento nos níveis de AMP provavelmente conduzirá
34 duas vias metabólicas: inibição da síntese de cAMP induzida por glucagon (MILLER et
35 al., 2013) e a ativação da proteína quinase 5'-AMP-ativada (AMPK) (ZHOU et al., 2001).

1 AMPK é uma proteína quinase que monitora o status de energia celular e
2 protege as funções celulares sob restrições de energia. Importante na regulação da
3 expressão de proteínas, necessária para a gliconeogênese, participa de vias envolvidas
4 no crescimento e proliferação celular (ZHOU et al., 2001; SCARPELLO; HOWLETT,
5 2008). A ativação da AMPK faz com que a célula mude de um estado anabólico para
6 um estado catabólico, numa tentativa de restaurar o equilíbrio energético através da
7 fosforilação de enzimas metabólicas ativação de fatores de transcrição que modulam a
8 expressão gênica (VIOLLET et al., 2009).

9 Na musculatura esquelética, a ativação da AMPK aumenta a capacidade de
10 contração muscular para estimular a captação de glicose, isso pode ocorrer devido a
11 capacidade da AMPK inibir a via da mTOR, que é ativada pela insulina e exerce uma
12 regulação por *feedback* na sinalização da insulina através da regulação negativa do IRS
13 1 (GONZALEZ-ÂNGULO, MERIC-BERNSTAM, 2010). E uma vez que o mTOR seria
14 inibida, essa regulação negativa deixaria de ocorrer. Além disso, no fígado e no tecido
15 adiposo a AMPK diminui a expressão de proteínas necessárias para a gliconeogênese,
16 diminuindo a secreção de insulina e conseqüentemente a sinalização da insulina em
17 favor do crescimento celular (GONZALEZ-ÂNGULO, MERIC-BERNSTAM, 2010).

18 Alguns estudos apontam que a metformina também pode alterar o metabolismo
19 lipídico. Diminuindo os níveis de triglicérides plasmáticos e ácidos graxos livres
20 (MARCHETTI et al., 1988), reduzindo os níveis de lipoproteínas de baixa densidade
21 (LDL), aumentando os níveis de lipoproteínas de alta densidade (HDL). Há melhoras
22 nas funções endoteliais, provocando mudanças na pressão arterial (tanto sistólica,
23 quanto diastólica) (VERMA et al., 2000), redução do peso corporal, e da resistência
24 periférica à insulina (HAUPT, KNICK, KOSCHINSKY, 1991).

25 26 1.7. Metformina no Câncer

27 O diabetes está intimamente associado ao risco de desenvolvimento de câncer
28 (GIOVANNUCCI et al., 2010). O primeiro indício de que a metformina poderia apresentar
29 efeitos benéficos na prevenção do câncer, surgiu após um estudo epidemiológico com
30 pacientes diabéticos. Onde os resultados apontaram que a administração habitual de
31 metformina em pacientes reduziu a incidência do câncer e conseqüentemente a
32 mortalidade em decorrência da doença (EVANS et al., 2005). Desde então outros
33 estudos relataram os efeitos da metformina no câncer.

34 Em um estudo realizado com 10.309 diabéticos por Bowker e colaboradores
35 (2006), observou-se que a mortalidade relacionada ao câncer foi menor nos pacientes
36 tratados com metformina, quando comparados com os tratados com sulfonilurêias e

1 insulina. Mas sem comprovação se resultado era devido a um efeito protetor da
2 metformina ou a um efeito deletérico da sulfoniluréia e da insulina. Currie e colaboradores
3 (2009), mostraram que pacientes diabéticos tratados com metformina tinham menor
4 predisposição ao desenvolvimento tumores sólidos, câncer de cólon e pâncreas do que
5 os tratados com insulina. Libby e colaboradores (2009), relataram uma incidência menor
6 de câncer em pacientes diabéticos que faziam uso de metformina (7,3% contra 11,6%)
7 e tempo do desenvolvimento maior para os diabéticos que faziam uso da metformina
8 quando comparados a diabéticos que usavam outros medicamentos (3,5 anos contra
9 2,6 anos).

10 Estudos realizados *in vitro* com células neoplásicas de mama, glioma, próstata,
11 cólon, endométrio, pulmão, ovário e pâncreas, mostram um efeito antiproliferativo da
12 metformina (ZAKIKHANI et al., 2006; BUZZAI et al., 2007; BEN SAHRA et al., 2008;
13 WANG et al., 2008; RATTAN et al., 2011; ALJADA, MOUSA, 2012). Estudos *in vivo*,
14 mostraram uma ação da metformina no crescimento de adenocarcinomas, câncer de
15 cólon, câncer pancreático, próstata e pulmão (SCHNEIDER et al., 2001; ALGIRE et al.,
16 2008; ANISIMOV et al., 2010).

17 Sabe-se pouco sobre o efeito da metformina relacionado ao câncer em pacientes
18 não diabéticos. Um estudo realizado com pacientes não diabéticos, mostrou que o
19 tratamento em curto prazo com a metformina suprimiu a formação de focos de criptas
20 aberrantes (HOSONO et al., 2010). Outro estudo revelou que a metformina reduziu o
21 marcação de proliferação celular Ki67, em amostras de biópsia obtidas de mulheres não
22 diabéticas com câncer de mama tratadas com metformina (HADAD et al., 2011).

23 Mecanismos pelos quais a metformina atenua a tumorigênese e tem
24 propriedades quimioprotetoras não são bem definidos, pois vários mecanismos
25 existentes podem explicar a associação entre a metformina e a redução do
26 desenvolvimento e a progressão do câncer. Esses mecanismos se concentram na
27 inibição de estímulos de crescimento e processos metabólicos dentro das células
28 cancerígenas, e podem ser divididos em mecanismos dependentes e independentes de
29 insulina, que alteram o crescimento de células cancerígenas.

30 O fator de crescimento semelhante a insulina IGF-1 estimula a sobrevivência
31 celular, e os receptores são expressos em diversas células cancerígenas (SACHDEV,
32 YEE, 2007). Assim, é possível que o tratamento com metformina diminua os níveis de
33 IGF-1, reduzindo assim o estímulo para o crescimento.

34 Em condições normais as células geram energia na forma de ATP,
35 primeiramente através da fosforilação oxidativa mitocondrial. No entanto, o metabolismo
36 das células cancerígenas é alterado, independente da disponibilidade do oxigênio,

1 processo que chamado de glicólise anaeróbica, o “efeito Warburg”, gerando uma
2 elevada quantidade de energia, assim como metabólitos, lactato e cetonas
3 (VANDERHEIDEN, CANTLEY, THOMPSON, 2009; SONG et al., 2012). Esta energia
4 tem como principal regulador a via LKB1-AMPK. Os efeitos antineoplásicos da
5 metformina estão em parte associado a esta via de sinalização (KUHAJDA, 2000;
6 PANDOLFI, 2004).

7 A metformina atua ativando a proteína quinase dependente de adenosina
8 monofosfato (AMPK). Quinase importante na regulação da expressão de proteínas
9 necessárias para a gliconeogênese, responsável pela manutenção da homeostasia
10 energética celular e ativação de vias de crescimento e proliferação (SCARPELLO,
11 HOWLETT, 2008; SHAW, CANTLEY, 2012).

12 Os efeitos da metformina nas vias de sinalização LKB1 – AMPK e mTOR, é
13 possivelmente o efeito antineoplásico mais potente da metformina. MTOR é uma
14 subunidade catalítica de dois complexos multiproteicos, mTORC1 e mTORC2, são
15 essenciais na regulação do crescimento celular, sinalização hormonal, detecção de
16 energia, incluindo insulina e vias de fator de crescimento semelhante a insulina 1 (IGF-
17 1), IGF-2 e AMPK. A regulação negativa dependente de AMPK de mTORC1 (mTOR
18 complexo-1), resulta da ativação dos genes supressores de tumor complexo de
19 esclerose tuberosa 1 (TSC1) e TSC2, que formam um complexo inibidor de mTOR
20 (INOKI et al, 2002). A inibição do mTOR perturba a síntese de proteínas, ocasionando
21 uma ação antineoplásica direta nas células tumorais, através da inibição da progressão
22 do ciclo celular e indução da apoptose e autofagia (ALGIRE et al., 2008; GOODWIN et
23 al., 2008).

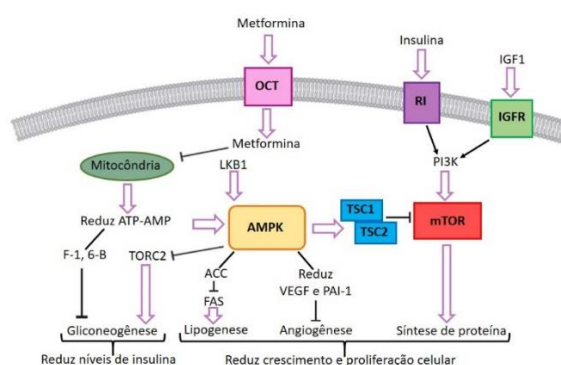
24 Há evidências de que a ativação de AMPK reduziu a expressão de FAS, e o
25 crescimento subsequente de células de câncer de próstata *in vitro* (XIANG et al., 2004).
26 Um ambiente pró inflamatório crônico, com aumento de infiltrado de citocinas como
27 leptina, interleucina -1 beta (IL-1 β), IL-6, fator de necrose tumoral alfa (TNF α), que estão
28 associados iniciação e progressão da carcinogêneses (YU, PARDOLL, JOVE, 2009;
29 BIJLAND , MANCINI, SALT, 2013). Há evidências de que a ativação da AMPK parece
30 inibir a síntese dessas citocinas pró-inflamatórias, em uma variedade de tipos celulares.
31 Sugerindo que a metformina poderia ter como alvo citocinas pró – inflamatórias dentro
32 do ambiente tumoral, inibindo assim o crescimento (YU, PARDOLL, JOVE, 2009).

33 A literatura também aponta que a metformina inibiu a proliferação neoplásica
34 parando o ciclo celular nas fases G0 / G1 ou S, em câncer de prostata (BEN SAHRA et
35 al., 2008), mama (ZHUANG, MISKIMINS, 2008; ALIMOVA et al., 2009), pancreático
36 (WANG et al., 2008), glioma (ISAKOVIC et al., 2007), endometrial (BUZZAI et al., 2007;
37 CANTRELL et al., 2010), ovário (RATTAN al., 2011).

1 Estudos mostram, que a metformina suprimiu numerosos genes relacionados
 2 com a mitose, incluindo tubulinas, histonas e auroras quinases (OLIVERAS-
 3 FERRAROS, VAZQUEZ-MARTIN, MENENDEZ, 2009). Há alguns relatos na literatura,
 4 de que a metformina pode ser acumulada na mitocôndria, induzindo a perda do potencial
 5 mitocondrial, além de promover a geração de superóxido dentro da mitocôndria.
 6 Resultando em danos a proteínas, lipídios e ácidos nucleicos, levando a morte por
 7 apoptose (BUZZAI et al., 2007).

8 Outros estudos apontam, que a metformina desencadeia a apoptose através
 9 da regulação negativa de Bcl-2 (SEGAL et al., 2011), ativando Bax, p53,
 10 desencadeando apoptose através de sinalização da ERK (MALKI, YOUSSEF, 2011),
 11 dependentes e independentes de caspases (via poli ADP-ribose ativação polimerase –
 12 PARP) (ZHUANG, MISKIMINS, 2008). Há estudos que mostram, que a metformina inibe
 13 o proto-oncogene c-MYC e o fator 1 α induzido por hipóxia (HIF-1 α) via AMPK,
 14 lembrando que a AMPK e HIF-1 α têm um papel fundamental na transformação
 15 metabólica do câncer (BLANDINO et al., 2012).

16 Porém os efeitos da metformina no metabolismo celular e no crescimento do
 17 tumor são controversos. Em células pré-neoplásicas, com um eixo AMPK intacto, a
 18 metformina pode neutralizar o efeito Warburg, ou seja, a metformina pode inibir a
 19 glicólise oxidativa (HUANG et al., 2008). No entanto, em tumores sólidos a presença de
 20 LKB1 e AMPK pode conferir uma vantagem de sobrevivência às células tumorais,
 21 protegendo-as contra o estresse energético (JEON, CHANDEL, HAY, 2012), podendo
 22 até causar aumento do fluxo glicolítico no microambiente. Portanto, os ativadores da
 23 AMPK podem ser potencialmente prejudiciais (MENDOZA et al., 2012). Por outro lado
 24 a ausência de LKB1 ou AMPK em tumores em tumores sólidos torna as células
 25 neoplásicas mais vulneráveis ao empobrecimento do ATP em decorrência da
 26 metformina, já que sua capacidade de restaurar o equilíbrio energético é prejudicada
 27 (ALGIRE et al., 2011).



28

29 **Figura 4** - Mecanismo de ação da metformina no câncer. Adaptado por Morales & Morris
 30 (2015).

1 1.8. Metformina no Melanoma

2 Pesquisas revelam os efeitos promissores da metformina frente ao melanoma.
3 Cerezo e colaboradores (2013), mostraram que células de melanoma quando tratadas
4 com metformina na concentração de 10mmol/L, eram induzidas a morte, pela a parada
5 do ciclo celular na fase G0 / G1, reduzindo a invasão e metástase. Tomic e
6 colaboradores (2011), relataram que a metformina na concentração de 10mM inibe a
7 viabilidade celular, tanto por indução a autofagia (72 horas de tratamento) quanto por
8 apoptose (96 horas de tratamento) e em diferentes linhagens de células de melanoma,
9 independente da mutação ser em BRAF ou N-Ras.

10 Os mesmos pesquisadores, relataram resultados semelhantes *in vivo*. Onde
11 os animais foram inoculados com $2,5 \times 10^6$ células A375, e cinco dias após a inoculação
12 foram tratados com metformina 2mg/ dia durante 3 semanas. A metformina reduziu o
13 desenvolvimento de tumores solidos de melanoma, induzindo as células a apoptose ou
14 autofagia.

15 No entanto, Martin e colaboradores (2012), relataram que a metformina
16 poderia aumentar o crescimento do melanoma em camundongos por aumentar a
17 angiogênese. Os animais foram inoculados com 2×10^6 células A375, tratados com uma
18 única dose de metformina, um dia antes da inoculação, na concentração de 300 mg/kg.
19 Cerezo e colaboradores (2013), com base nos resultados publicados por Janjetovic e
20 colaboradores (2011), vai na direção oposta de Martil et al., (2012), e relata que essa
21 discrepância pode estar relacionada com o tratamento e a concentração de metformina
22 utilizada, 60mg/kg durante 39 dias após a inoculação.

23 Niehr e colaboradores (2011), utilizaram a metformina na concentração de
24 10mm em combinação com o vemurafenib, um inibidor da BRAF. Os resultados foram
25 promissores para o tratamento do melanoma, a combinação apresentou um efeito
26 antitumoral sinérgico ao induzir a morte celular do melanoma (NIEHR et al., 2011). Hisch
27 e colaboradores (2013), utilizando a metformina a 0.1mmol/L associado a doxorubicina,
28 observaram a indução de morte celular, por aumento da toxicidade da droga.

29 Além disso, a metformina poderia inibir a invasão e a metastáse do melanoma
30 diminuindo seu desenvolvimento. Inibindo a ativação da metaproteinase de matriz-2
31 (MMP-2) e da metaloproteinase de matriz-9 (MMP9). Este processo depende ativação
32 de AMPK e da proteína supressora de tumor p53 (CEREZO et al., 2013). Esses
33 resultados apontam que a metformina tem um importantante papel na atuação local
34 tumoral, afetando a viabilidade das células de melanoma, e também atuando na
35 prevenção da insão e formação de metástases distantes.

36 Células iniciadoras de melanoma (MIC) podem estar envolvidas na origem da
37 metástase do melanoma. MIC são populações de células resistentes a quimioterapia,

1 que expressam marcadores específicos (ZABIEROWSKI, HERLYN, 2008). No entanto,
2 é claro que dentro de uma população de células neoplásicas, nem todas exibem o
3 mesmo potencial tumorigênico, suas características e os mecanismos que regulam a
4 transição entre MIC e a linhagem proliferativa são diversos (HOEK, GODING, 2010).
5 Um caminho para as células adquirirem propriedades da MIC, seria a ativação do fator
6 de transcrição 3 (STAT3) (OHANNA et al., 2013). Hirsch e colaboradores (2013),
7 mostraram que a metformina é capaz de inibir o NF-κB, conseqüentemente a STAT3.
8 Impedindo a transformação celular e o crescimento de células tronco neoplásicas, via
9 bloqueio de respostas ao estresse metabólico das células, inibindo a população MIC.

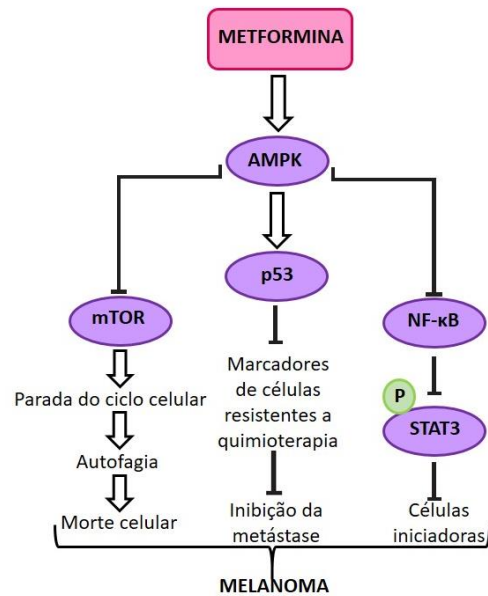
10 Com base em todos os relatos promissores encontrados na literatura sobre os
11 efeitos da metformina frente ao tratamento do melanoma, vários estudos estão sendo
12 realizados tanto *in vitro* quanto *in vivo*. Nos Estados Unidos existem ensaios clínicos em
13 andamento, onde estão recrutando pacientes para o tratamento de melanoma com
14 metformina em combinação com inibidores de BRAF (ClinicalTrials.gov, Identifier:
15 NCT01638676 e NCT02143050) e também em associação com Dacarbazina
16 (ClinicalTrials.gov, Identificador: NCT02190838) (CEREZO et al., 2015). Os resultados
17 destes ensaios clínicos serão importantes na definição do futuro da metformina para o
18 tratamento do melanoma metastático.

19 Mesmo a literatura apontando os efeitos promissores da metformina como
20 adjuvante no tratamento de vários tipos de câncer, incluindo o no melanoma. Os
21 mecanismos pelos quais a droga exerce esse efeito antiproliferativo sobre as células de
22 melanoma ainda não foram completamente elucidados. Por esse motivo, o presente
23 trabalho teve como principal objetivo estudar a atuação da metformina *in vitro* e *in vivo*
24 em células de melanoma murino B16F10.

25 No modelo *in vivo*, buscamos investigar a participação da metformina no
26 processo de indução a quimiorresistência a DTIC. Analisando sua participação no
27 estresse oxidativo ao longo desse processo, bem como a atuação de metformina em
28 vias de sinalização relacionadas com a quimiorresistência: 8-OH-DG, p53, Nrf2 e NF-
29 κB.

30 A segunda linha do trabalho foi realizada *in vivo*, teve duas vertentes. A primeira,
31 teve como questionamento a ação da metformina como um possível tratamento do
32 melanoma metastático ou como um adjuvante nesse tratamento juntamente com a
33 DTIC. Na segunda, analisamos a atuação da metformina no processo de implantação
34 das células B16F10 no tecido pulmonar, também analisamos sua ação como tratamento
35 e/ou como adjuvante a DTIC. Nos dois casos analisamos o número de nódulos
36 metastáticos, parâmetros fisiológicos dos animais, a participação do estresse oxidativo

- 1 tumoral e sistêmico, arquitetura tecidual e vias de sinalização envolvidas no crescimento
 2 tumoral, como: p53, PCNA e Nrf2.
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 5 **Figura 5.** Efeitos da metformina nas células de melanoma. Adaptado por Cerezo et al., (2015).
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1 2. OBJETIVO GERAL

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3 Investigar a participação da metformina no estresse oxidativo em células de
4 melanoma murino B16F10, em modelo *in vitro* e *in vivo*, bem como sua participação em
5 vias de sinalização envolvidas no processo de resistência a quimioterápicos.

6

7 2.1 OBJETIVOS ESPECÍFICOS

8 ARTIGO 1

9 Induzir experimentalmente a quimiorresistência a DTIC em células de melanoma
10 murino B16F10.

11 Avaliar os efeitos da metformina na indução experimental de quimiorresistência
12 a DTIC.

13 Analisar a participação no estresse oxidativo no processo de indução a
14 quimiorresistência a DTIC.

15 Estudar a atuação da metformina no estresse oxidativo durante o processo de
16 indução a quimiorresistência a DTIC.

17 Avaliar a expressão de marcadores nucleares de vias de sinalização
18 relacionadas com quimiorresistência: p53, NF-kB, Nrf2 e 8-OH-DG, no processo de
19 indução a resistência a DTIC.

20 Estudar a atuação da metformina na expressão de vias de sinalização
21 relacionadas com a quimiorresistência: p53, NF-kB, Nrf2 e 8-OH-DG no processo de
22 indução de resistência à DTIC.

23

24 ARTIGO 2

25 Investigar a atuação da metformina sobre o melanoma metastático, em um
26 modelo *in vivo*.

1 Estudar os efeitos da metformina como um possível tratamento no modelo na
2 metástase pulmonar.

3 Avaliar a atuação da metformina como adjuvante a DTIC no tratamento do
4 melanoma metastático.

5 Analisar a ação da metformina no processo de implantação da metástase
6 pulmonar.

7 Estudar os efeitos da metformina isolada e/ou associada com DTIC, no número
8 de nódulos metastáticos.

9 Analisar os efeitos da metformina isolada e/ou associada com DTIC nos
10 parâmetros fisiológicos dos animais com metástase pulmonar.

11 Estudar a participação da metformina isolada e/ou associada ao quimioterápico
12 DTIC, no estresse oxidativo sistêmico e no microambiente tumoral.

13 Estudar a atuação da metformina isolada e/ou associada com DTIC em
14 diferentes vias de sinalização envolvidas na resistência aos tratamentos quimioterápico:
15 p53, PCNA e Nrf2.

16

17 **3. RESULTADOS**

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19 **ARTIGO 1**

20 Conseguimos induzir experimentalmente a quimioresistência a DTIC em células
21 de melanoma murino B16F10. A metformina foi capaz de impedir o processo de indução
22 a DTIC, interferindo no estresse oxidativo celular, através do aumento nos níveis de tiol
23 total das células. Além disso durante o processo de indução de resistência a metformina
24 foi capaz de modular importantes vias de sinalização celular relacionadas com a
25 resistência a quimioterápico. Indicando que a metformina tem um potencial terapêutico
26 no melanoma no modelo *in vitro*, e sua ação é em várias vias de sinalização, que
27 culminam em apoptose, defesa antioxidante, invasão e metástase.

28

1 ARTIGO 2

2 No modelo *in vivo* de melanoma metastático conseguimos observar uma ação
3 antitumoral da metformina com a redução do número de nódulos, e essa ação foi maior
4 quando utilizada juntamente com a DTIC. Além de amenizar os efeitos colaterais
5 causados pelo quimioterápico. Observamos que quando a metformina era usada antes
6 da inoculação das células B16F10 o número de nódulos e o tamanho desses nódulos
7 eram menor, revelando assim uma possível ação antimetastática da droga. Nos dois
8 casos intuímos que a ação da metformina era através da interferências em vias de
9 sinalizações que geralmente são afetadas no processo de iniciação e promoção do
10 câncer, além de conferir agressividade e resistência aos tratamentos disponíveis, a
11 metformina foi capaz de mudar esse perfil que geralmente é encontrado. A metformina
12 também foi capaz de modular o estresse oxidativo no microambiente tumoral e sistêmico
13 do animal, mostrando assim mais um efeito contra o metástase pulmonar do melanoma.

14

15 **4. CONCLUSÃO**

16

17 Os resultados encontrados no presente trabalho, nos levam a intuir que a
18 metformina tem uma ação importante na participação do estresse oxidativo e em
19 diferentes vias envolvidas com a resistência a quimioterápicos, em especial a DTIC.
20 Modulando essas vias, de forma a apresentar resultados satisfatórios para diferentes
21 modelos experimentais *in vitro* e *in vivo*, como as células de melanoma murino
22 metastático B16F10.

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1 5. ARTIGOS PARA PUBLICAÇÃO

2

3 O presente trabalho originou dois artigos científicos que serão incluídos nessa
4 tese como Anexos. Ambos foram realizados na Universidade Estadual de Londrina, nos
5 laboratórios de Patologia Molecular e no laboratório de Fisiopatologia e Radicais Livres.

6

7 O primeiro trabalho intitula-se "*Metformin attenuates the induction of dacarbazine*
8 *resistance in B16F10 murine melanoma cells through the increase in p53 and the*
9 *reduction in NFkB and Nrf2 nuclear translocations*"

10 O segundo trabalho intitula-se: "*Characterization of metformin activity in vivo*
11 *murine metastatic melanoma model: the Pandora's box of oxidative stress*".

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ANEXOS

ANEXO A - Metformin attenuates the induction of dacarbazine resistance in B16F10 murine melanoma cells through the increase in p53 and the reduction in NFkB and Nrf2 nuclear translocation.

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Metformin attenuation of the induction of dacarbazine resistance in B16F10 murine melanoma cells involves the increase in p53 and the reduction in NFκB and Nrf2 nuclear translocation

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ABSTRACT

1 Melanoma has high rates of recurrence and mortality, as well as a limited response to
2 available treatments, such as dacarbazine (DTIC), due to acquired resistance
3 mechanisms. For this reason, it is necessary to discover new drugs for treatment.
4 Metformin has demonstrated antitumor effects against various types of cancer, including
5 melanoma. The present study had as main objective to study the role of metformin in the
6 induction process of resistance to DTIC. Induction of DTIC resistance in B16F10
7 metastatic murine melanoma cells was achieved by exposure to increasing
8 concentrations of DTIC. The action of metformin in the resistance induction process was
9 investigated by administering metformin for 7 passes before induction of DTIC
10 resistance. Our results showed that metformin was able to inhibit the induction process
11 of DTIC resistance by increasing cell antioxidant levels, by acting in signaling pathways
12 involved in proliferation and cell death, invasion and metastasis. Revealing a great
13 potential for metformin treatment against DTIC resistant melanoma.

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15 Key words: Melanoma, chemoresistance, dacarbazine, oxidative stress, metformin

16

17 **1 Introduction**

18 Melanoma is a malignant tumor with a strong capacity for invasion and
19 metastasis, elevated rates of recurrence and mortality, as well as a limited response to
20 currently available treatments, such as surgery, chemotherapy and radiotherapy [1,2].
21 Dacarbazine (DTIC) is the first line chemotherapeutic used to treat metastatic melanoma,
22 being the most used in Brazil. It is a DNA alkylating agent and inhibitor of repair
23 mechanisms, which aims to induce DNA damage resulting in death of neoplastic cells
24 by apoptosis. However, its efficacy is still limited due to the development of resistance
25 mechanisms in melanoma cells [3,4]. Shi et al (2012) showed that 20% of all melanoma
26 patients developed at least two different mechanisms of resistance to available
27 treatments.

1 The mechanisms of resistance are multiple: it may be intrinsic, which involves
2 the upregulation of tyrosine kinase receptors and reactivation of Mitogen-activated
3 protein kinases (MAPK) [6,7], or acquired, with mutations in RAS or MEK and
4 amplification of the mutant form of BRAF [3,8]. Resistance may also be related to the
5 increased expression of inhibitor of apoptosis protein (IAP) family members, present
6 since the onset of melanocytic dysfunction [9]. Melanogenesis maybe a resistance factor,
7 regulating the hypoxia inducible factor 1 α (HIF-1 α), the main molecule related with
8 resistance to antiangiogenic therapies [10]. Oxidative stress is also related to resistance,
9 since melanoma cells have high antioxidant capacity favoring proliferation,
10 angiogenesis, invasion and metastasis [11,12].

11 Metformin is the most widely prescribed drug for type 2 diabetes [13] and have
12 shown antitumor effects against several types of cancer [14-16] including melanoma [17-
13 19]. The mechanisms by which it attenuates tumorigenesis and have chemoprotective
14 properties may be direct or indirect. This drug acts in the AMPK-mTOR pathway,
15 inhibiting the growth and inducing apoptosis and autophagy of proteins that participates
16 in pathways involved in cell growth and proliferation [20-23]. Indirect mechanisms for the
17 effects of metformin on cancer include reductions in insulin levels, reducing the stimulus
18 for growth and bioavailability of sex hormones [24]. It is also able to inhibit NF- κ B and,
19 consequently, STAT3, preventing cell transformation and growth of neoplastic stem cells
20 by blocking responses to metabolic stress of cells [19]. Reports indicate that metformin
21 downregulates Bcl-2 [25], activates Bax and p53, [26], inhibits the c-MYC proto-
22 oncogene and the hypoxia-induced factor 1 α (HIF-1 α) via AMPK [27].

23 Considering the promisor effects of metformin in melanoma and the clinical
24 relevance of chemoresistance in the efficacy of its therapy, the present study evaluated
25 some oxidative stress-related pathways in the process of DTIC resistance induction in
26 B16F10 murine melanoma cells and the participation of metformin in this process.

1 **2 Materials and Methods**

2 2.1 Cell culture and treatment

3 The murine melanoma cell line (B16F10) was seeded in tissue culture flasks and
4 grown in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Thermo Fisher, USA),
5 supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher, USA) and 1%
6 penicillin/streptomycin mixture (Santa Cruz, Biotechnology, USA). The culture was
7 maintained in a humidified atmosphere with 5% CO₂ at 37°C (Sanyo CO₂ Incubator;
8 Sanyo, Japan).

9

10 2.1.1 Dacarbazine resistance induction

11 The DTIC resistance phenotype was achieved after exposition of B16F10 cells,
12 maintained in DMEM with 10% FBS, to increasing concentrations of DTIC (Libbs, São
13 Paulo, SP) (1µg/mL, 10µg/mL, 25µg/mL, 50µg/mL, 75µg/mL, 100µg/mL and 125µg/mL)
14 during seven sequential passages. These concentrations were chosen after a pilot study,
15 below the IC₅₀. At the beginning of the experiment, 10⁶ cells were seeded in 25-cm² cell
16 culture flasks and after 24 h, the first samples without DTIC treatment were obtained;
17 this sample was named as initial passage (P1). Next, cells were trypsinized, counted in
18 a Neubauer chamber and 10⁶ viable cells (determined by trypan blue exclusion assay)
19 were seeded in a new 25-cm² cell culture flask and cultured immediately in culture media
20 with DTIC. Cells were considered as chemoresistant when the exposition to the crescent
21 concentration of DTIC did not cause its death. The samples of the seventh passage were
22 named P7. This group was called dacarbazine resistant (DTIC) group. Throughout the
23 induction period, samples of the initial passage (P1) and the final passage (P7) were
24 collected. The same procedures were performed on untreated cells for seven
25 consecutive passages (Control group), to identify possible alterations related to cell

1 passages. The experimental design for the induction of DTIC resistance is illustrated in
2 figure 1.

3 2.1.2 Metformin treatments

4 It was investigated whether the administration of metformin could interfere with
5 the induction of DTIC resistance. To that end, a pretreatment with a non-cytotoxic
6 concentration, according to Marinello and collaborators (2016) of 6 μM metformin (Santa
7 Cruz Biotechnology, Dallas, TX) was used during seven passages and then the induction
8 of the DTIC was started. Metformin treatment was maintained during DTIC resistance
9 induction. At the beginning of the experiment, 10^6 cells were seeded in 25-cm² cell
10 culture flasks and after 24 h, we obtained the first samples, in the initial passage (P1),
11 composed of cells without DTIC and metformin treatment. Next, cells were trypsinized,
12 counted in a Neubauer chamber by trypan blue exclusion assay and 10^6 viable cells were
13 seeded in a new 25-cm² cell culture flask and kept in culture media with metformin for
14 24 h. This procedure was repeated for seven consecutive passages. After the seventh
15 passage, the induction of DTIC resistance was performed as described above. This
16 group was named metformin-treated DTIC (MET-DTIC) group and received metformin
17 for fourteen passages and DTIC for seven passages. It was also maintained a group with
18 fourteen passages of metformin administration (MET group) to identify possible
19 alterations related to metformin exposure (Figure 1).

20 2.2 Cytotoxicity assays

21 After concluded the experimental period, cellular response to DTIC was
22 evaluated by a MTT assay as described by Mosmann (1983). The cells were distributed
23 in 24 well plates (1×10^5 cells/well) and exposed during 24 hours to DTIC in the
24 concentrations of 100 $\mu\text{g}/\text{mL}$, 200 $\mu\text{g}/\text{mL}$ and 300 $\mu\text{g}/\text{mL}$. The results were expressed as
25 percentage (%) of control (P7 cells without DTIC or metformin treatment) viability.

1

2 2.3 Oxidative stress parameters

3 Total thiols levels were determined using 5,5'-dithiobis (2-nitrobenzoic acid) as
4 previously described by Hu (1994), and measured by spectrophotometry at 412nm. Total
5 thiol groups were calculated using a calibration curve prepared by glutathione (GSH)
6 (Sigma-Aldrich), and the results were expressed in μM thiol/ 10^6 cells. Malondialdehyde
7 (MDA) levels were measured as described by Victorino et al., (2012) by using a high-
8 performance liquid chromatography (HPLC) system (HPLC-Shimadzu 20AT®) equipped
9 with a LC20AT pump, an absorbance detector (UV SPDM20A diode-array), and an
10 analytical column Zorbax Eclipse XDB-C18 (4.6 \times 250 mm, 5 μm ; Agilent Technologies,
11 Santa Clara, USA). Results were expressed in nM MDA/ 10^6 cells. For both analysis it
12 was calculated the percentage of alteration in the samples in the seventh passage (P7)
13 in relation to P1 and these results were shown for each group graphically.

14

15 2.4 Immunocytochemistry labelling for p53, 8-OH-DG, Nrf2 and NF- κ B

16 During the experiment, cells samples of P1 and P7 passages (P1 to P7) were
17 cultured on circular glass coverslips for immunocytochemistry analysis, which was
18 performed using the labeled streptavidin biotin method using a LSAB KIT (DAKO,
19 Japan). The samples were incubated with 0.1% Triton X-100 solution for 15 minutes,
20 washed three times in PBS, and treated for 30 minutes at room temperature with 10%
21 BSA. The coverslips were incubated with the primary antibodies for two hours (anti-p53,
22 diluted 1:250, anti-8-OH-DG, diluted 1:1000, anti-NRF2, diluted 1:100, and anti-NF- κ B,
23 diluted 1:500, Santa Cruz Biotechnology, USA). After secondary antibody treatment (1h,
24 room temperature), horseradish peroxidase activity was visualized by treatment with
25 H_2O_2 and 3,3'-diaminobenzidine (DAB) for 1min. At the last step, the samples were
26 weakly counterstained with Harry's hematoxylin (Merck, Germany). For each case,
27 negative controls were performed by omitting the primary antibody. Intensity and
28 localization of immunoreactivities against primary antibody used were examined on all

1 coverslips using a photomicroscope (Olympus BX41, Olympus Optical Co., Ltd., Japan).
2 For the image analysis study, photomicroscopy color slides of representative areas
3 (400× magnification) were digitally acquired, and blindly analyzed. Nuclear labeling for
4 p53, 8-OH-DG, Nrf2 was determined evaluating the percentage of cells with nuclear
5 labeling. It was calculated the percentage of alteration in the P7 samples in relation to
6 P1 and these results were shown for each group graphically.

7

8 2.5 Statistical analysis

9 Data was expressed as the mean \pm standard error of the mean and were
10 analyzed using one-way analysis of variance (ANOVA). Intergroup differences were
11 assessed by Tukey post-hoc test. $p < 0.05$ was considered statistically significant. Data
12 was analyzed using GraphPad Prism (version 6; San Diego, CA, USA).

13

14 **3 Results**

15 3.1 Responsiveness profile of different groups to DTIC

16 Control group presented decreased viability after exposure to 200 μ g/mL
17 ($p < 0.005$) and 300 μ g/mL ($p < 0.0001$) of DTIC when compared to cells that did not receive
18 DTIC (omitted from graph). The concentration of 300 μ g/mL presented a greater
19 reduction in cellular viability, being statistically significant in relation to the concentration
20 of 100 μ g/mL ($p < 0.005$). In the DTIC group there was also reduction in cellular viability in
21 the concentrations of 200 μ g/mL and 300 μ g/mL ($p < 0.001$ and $p < 0.0001$, respectively) in
22 comparison to cells that did not receive DTIC (omitted from graph). The control group
23 presented more reduced viability to 300 μ g/mL of DTIC than DTIC group ($p < 0.0001$),
24 indicating that the resistance phenotype was achieved (figure 2A).

25 In the MET group, it was observed a reduction in the cellular viability in the
26 concentration of 200 μ g/mL and 300 μ g/mL of DTIC ($p < 0.05$ for both) in comparison to

1 cells that did not receive DTIC (omitted from graph). The control group presented
2 reduced viability to 100µg/mL ($p<0.005$), 200µg/mL and 300µg/mL ($p<0.0001$) (figure
3 2B). In the MET-DTIC group it was observed a significant reduction in cellular viability
4 after exposure to all concentrations of DTIC ($p<0.0001$) (figure 2C) when compared to
5 cells that did not receive DTIC (omitted from graph), when compared to the MET group.
6 The MET-DTIC group presented reduced viability to all concentrations of DTIC than the
7 DTIC group ($p<0.0001$) (figure 2D).

8

9 3.2 Oxidative stress determination

10 The MDA quantification demonstrated that DTIC group presented increased
11 levels of lipoperoxidation in relation to control ($p<0.05$) group. The MET group did not
12 differ from control and MET-DTIC group did not differ from DTIC group (figure 3A). The
13 DTIC and MET groups presented increased levels of total thiol in relation to control group
14 ($p<0.0001$) (figure 3B). The MET-DTIC group showed increased levels of total thiol levels
15 than the DTIC and MET groups ($p<0.0001$).

16

17 3.3 Analysis of nuclear labelling for 8-OH-DG, p53, NF-κB and Nrf2

18 The DTIC group presented increased nuclear labeling to 8-OH-DG when
19 compared to control ($p<0.0001$) (figure 4A). MET group did not differ from control and
20 MET-DTIC group presented reduced 8-OH-DG nuclear labeling than DTIC group (figure
21 4A). In relation to nuclear p53 labelling, DTIC and MET groups presented reduced
22 labelling in relation to control ($p<0.0001$ and $p<0.05$, respectively) (figure 4B) and MET-
23 DTIC group presented increased nuclear p53 labelling than DTIC group ($p<0.05$) (figure
24 4B).

25 The DTIC group also presented increased nuclear labelling for NF-κB in relation
26 to control group and MET did not differ (figure 5A). The MET-DTIC group showed
27 reduction in this nuclear labelling in relation to DTIC (figure 5A). The DTIC group showed

1 significant increase ($p < 0.001$) in the percentage of nuclear labelling for Nrf2 and MET
2 group showed decrease ($p < 0.005$) in this nuclear labeling in relation to control group.
3 The MET-DTIC group presented reduction labelling when compared to DTIC group
4 (figure 5B).

5

6 **4 Discussion**

7 Melanoma due to various mechanisms is resistant to the treatments available
8 so far. Because of this, the search for therapies capable of reducing this resistance,
9 making it drug-sensitive and consequently improving the patient's responsiveness to
10 treatment is a major challenge.

11 In this work, we observed that the administration of DTIC to B16F10 cells in
12 increasing concentrations induced a resistant phenotype in these cells. This was
13 accompanied by increased lipoperoxidation and total thiol levels. It seems that oxidative
14 stress contributes to resistance of melanoma cells to chemotherapy through the
15 activation of proto-oncogene pathways [31]. Corazao-Rozas et al (2013) observed that
16 different types of melanomas resistant to BRAF inhibitors developed dependence on
17 mitochondrial oxidative metabolism characterized by high levels of ROS production.

18 The DTIC resistant group also presented increased levels of nuclear labelling
19 for 8-OH-DG, which is the most indicated marker of DNA oxidative injury, caused by a
20 hydroxyl radical [33,34]. Increased levels of 8-OH-DG has been related with poor survival
21 in melanoma [35]. The increase MDA and 8-OH-DG, in DTIC group indicate that the
22 oxidative stress generated due to the DTIC exposure is responsible for DNA lesions.

23 In addition, the DTIC group presented reduced percentage nuclear labeling for
24 p53, which is a classical DNA damage sensor. P53 is involved in cellular processes,
25 such as cell apoptosis, cell cycle arrest or genome stabilization [36]. It is responsible for
26 treatment-mediated apoptosis induction in cancer cells including melanoma [37,38]. This
27 protein was previously related to chemoresistance. Martin et al., (2013) showed a

1 physiological interaction between p53 and P63, where p63 prevents the accumulation of
2 p53 in the nucleus, being a factor for the chemoresistance. Furthermore, mutation in p53
3 was related with the stimulation of the multi-drug-resistance (MDR) promoter gene and
4 ABCB1 expression in melanoma [40,41].

5 The DTIC group also presented increased nuclear labeling for NFκB. NFκB
6 signaling pathway is constitutively active in many types of tumor and have been
7 implicated in cancer cell proliferation, invasion, metastasis and angiogenesis, as well as
8 in suppression of cancer cell apoptosis [42]. Moreover, some anticancer agents may
9 induce chemoresistance of cancer cells through activation of NFκB, favoring the immune
10 escape from tumor [43,44]. Moreover, the NF-κB transcription factor is a major regulator
11 of genes involved in inflammation, immune activation and cell growth [45].

12 In addition to increase nuclear NFκB labelling, DTIC group also presented
13 increased nuclear Nrf2. In physiological conditions, Nrf2 binds to its repressor protein
14 Keap1 (Kelch ECH associating protein) and is subjected to continuous ubiquitination and
15 proteosomal degradation. Under oxidative stress conditions including chemicals, UV
16 irradiation, and heat shock, Nrf2 binding to its upstream keap1 is disrupted and leads to
17 Nrf2 nuclear translocation [46,47]. Studies showed that Nrf2 expression in melanoma is
18 related to invasion thereby worsening melanoma-specific survival [48]. Nrf2 is frequently
19 aberrantly activated and accumulated in the nucleus of different cancer types [49], being
20 related with chemoresistance of malignant tumors [50,51]. Khamari and collaborators
21 (2018), showed that Nrf2 accumulation was even higher in cells with BRAF mutation,
22 such as B16F10 cells, characterizing a resistant profile, this observation is in agreement
23 with the fact that BRAF oncogenes activate the Nrf2 antioxidant program [53].

24 Nrf2 is a transcription factor that controls the basal and inducible expression of
25 an array of antioxidant and detoxification enzymes including the proteasome, the Nrf2
26 signaling system may be most important cellular defense and survival pathway against
27 oxidative stress [54-59]. Tian and collaborators (2014) showed that ZD55-IL-24 inhibits
28 Nrf2-mediated oxidative stress response, by activating p38 signal pathway to potentiate

1 the association of Nrf2 and Keap1 and also by suppressing ERK signal pathway to
2 postpone Nrf2 nuclear translocation. Wang and collaborators (2018) showed that
3 inhibition of Nrf2 increased ROS-induced cell cycle arrest and cellular apoptosis and
4 inhibits melanoma growth by regulating the AKT-Nrf2 pathway in A375 cells. The
5 increase in total thiol levels observed in the DTIC group was probably related to the
6 increased Nrf2 labeling.

7 Treatment with metformin improved the DTIC-resistant phenotype when
8 associated with DTIC (MET-DTIC group). We observed that the drug worked mainly by
9 increasing the levels of total thiols, p53 nuclear marking and reducing the labeling for
10 NFκB and Nrf2. The literature shows that metformin action is mainly mediated by AMPK
11 activation, recent studies indicate that this activation activates p53 and that mediates, at
12 least in part, the anticancer action of metformin [62-64]. In this work we observed that
13 metformin action on DTIC resistance in B16F10 melanoma cells was also related to its
14 interference in NFκB and Nrf2 pathways, showing that oxidative stress also plays a role
15 in metformin effects on DTIC chemoresistance.

16 Our results indicate that B16F10 murine melanoma cells have become resistant
17 to DTIC, increasing levels of lipid peroxidation and reducing cellular antioxidant defense.
18 There were significant changes in pathways involved in invasion, metastasis,
19 angiogenesis, proliferation and cell death, reducing the labeling for p53 and increasing
20 nuclear translocation of NFκB and Nrf2. Metformin was able to inhibit the induction
21 process of DTIC resistance by increasing cellular antioxidant levels, interfering with
22 important signaling pathways affected in chemioresistance, reducing the labeling of
23 NFκB and Nrf2, as well as increasing the nuclear translocation of p53, responsible for
24 inducing apoptosis in cells.

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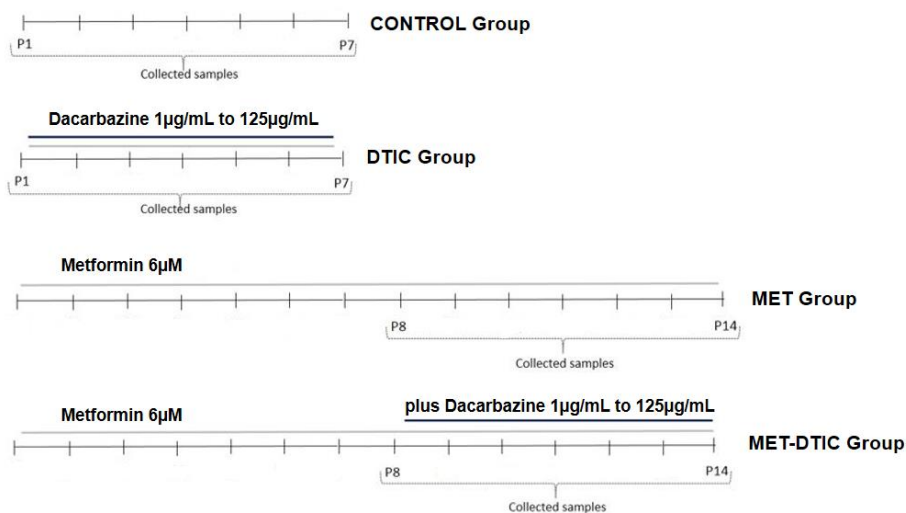
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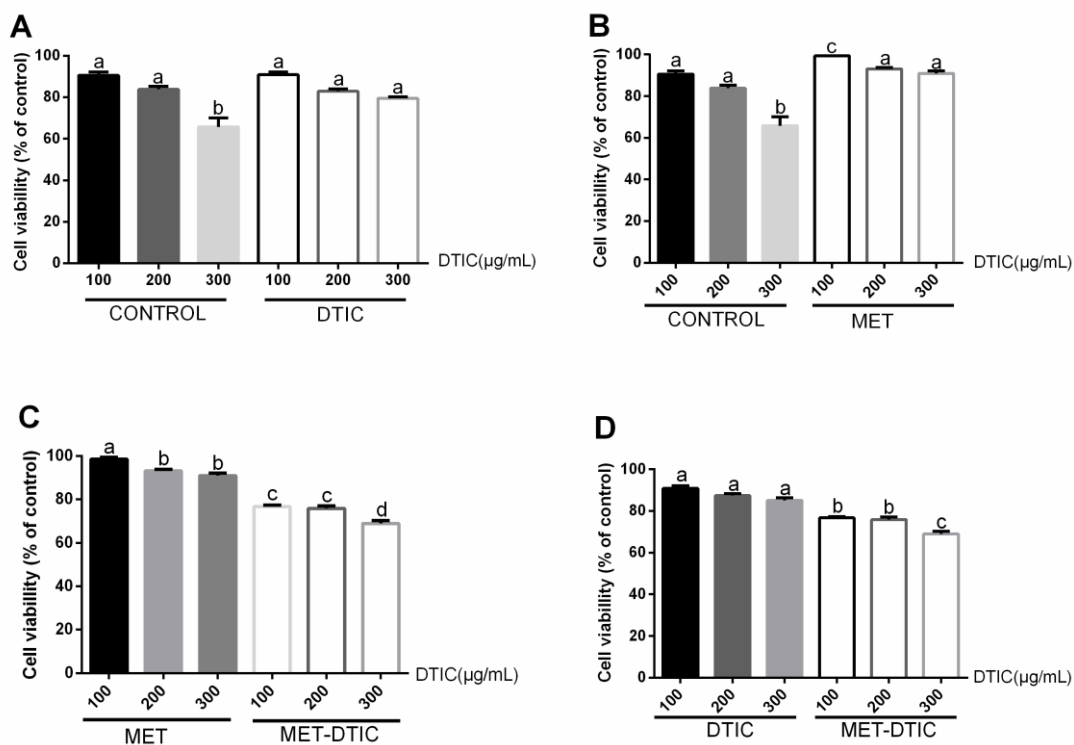
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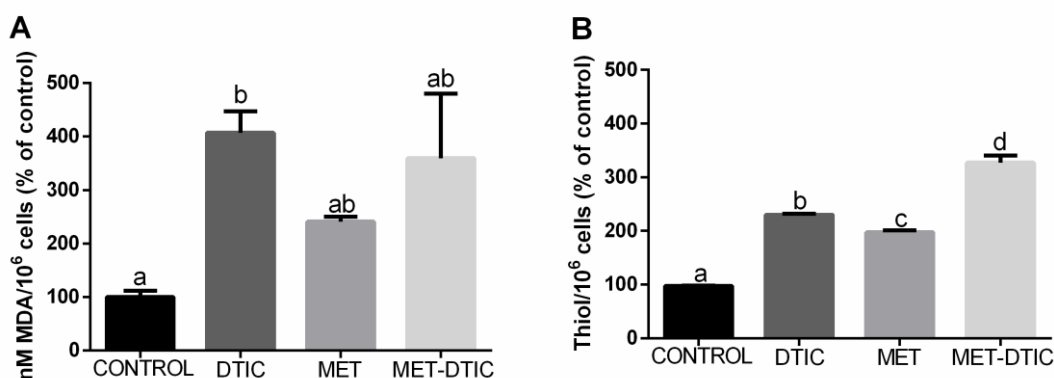


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Figure 1. Experimental design of induction of dacarbazine (DTIC) chemoresistance in B16F10 murine melanoma cells. CONTROL Group: B16F10 cells not exposed to DTIC. DTIC Group: Cells were exposed to increasing concentrations of DTIC (1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL e 125 µg/mL). MET Group: Cells were treated with 6 µM metformin during fourteen passages. MET-DTIC Group: Cells were pretreated with 6 µM metformin for seven consecutive passages before initiating the process of the induction of doxorubicin resistance. Metformin treatment was maintained during the process of chemoresistance induction.

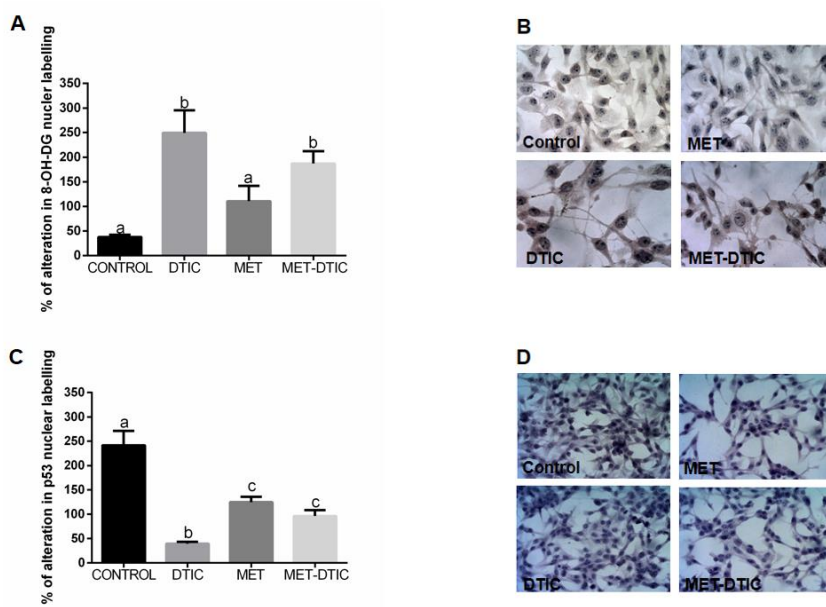


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2 **Figure 2. Metformin previne dacarbazine (DTIC) resistance in B16F10 murine melanoma**
3 **cells.** MTT assay of untreated control group compared with DTIC (A), MET (B) and MET-DTIC
4 (C) groups after 24 h of exposure to three DTIC concentrations (100, 200 and 300 µg/mL). Data
5 are expressed as mean ± standard error of mean. Statistically significant differences were
6 investigated by one-way ANOVA, followed by Tukey post-hoc and $p < 0.05$ was considered
7 significant. Letters different present statistical significant difference. DTIC: Cells experimentally
8 induced to DTIC resistance. MET: Cells were treated with 6 µM metformin during fourteen
9 passages. MET-DTIC: Cells treated with 6µM metformin and experimentally induced to DTIC
10 resistance.
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16 **Figure 3. Oxidative stress parameters in B16F10 murine melanoma cells experimentally**
17 **induced to dacarbazine (DTIC) chemoresistance exposed to metformin.** Malondialdehyde
18 (A) and thiol levels (B) of the CONTROL, DTIC, MET and MET-DTIC groups. Data are expressed
19 as mean ± standard error of mean of the percentage of the alteration in the samples in the seventh

1 passage (P7) in relation to the first passage within each group. Statistically significant differences
 2 were investigated by one-way ANOVA, followed by Tukey post-hoc and $p < 0.05$ was considered
 3 significant. Letters different present statistical significant difference. DTIC: Cells experimentally
 4 induced to DTIC resistance. MET: Cells were treated with 6 μM metformin during fourteen
 5 passages. MET-DTIC: Cells treated with 6 μM metformin and experimentally induced to DTIC
 6 resistance.



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 11 **Figure 4. Immunocytochemistry analysis of B16F10 murine melanoma cells.** Percentage of
 12 nuclear labelling for 8-OH-DG (A) and for p53 (C) in cells of the CONTROL, DTIC, MET, and
 13 MET-DTIC groups. Representative images from the 8-OH-DG (C) and for p53 (D) analysis in
 14 each experimental group. Data are expressed as mean \pm standard error of mean of the
 15 percentage of alteration in nuclear labeling in the samples in the seventh passage (P7) in relation
 16 to the first passage within each group. Statistically significant differences were investigated by
 17 one-way ANOVA, followed by Tukey post-hoc and $p < 0.05$ was considered significant. Letters
 18 different present statistical significant difference. DTIC: Cells experimentally induced to DTIC
 19 resistance. MET: Cells were treated with 6 μM metformin during fourteen passages. MET-DTIC:
 20 Cells treated with 6 μM metformin and experimentally induced to DTIC resistance.

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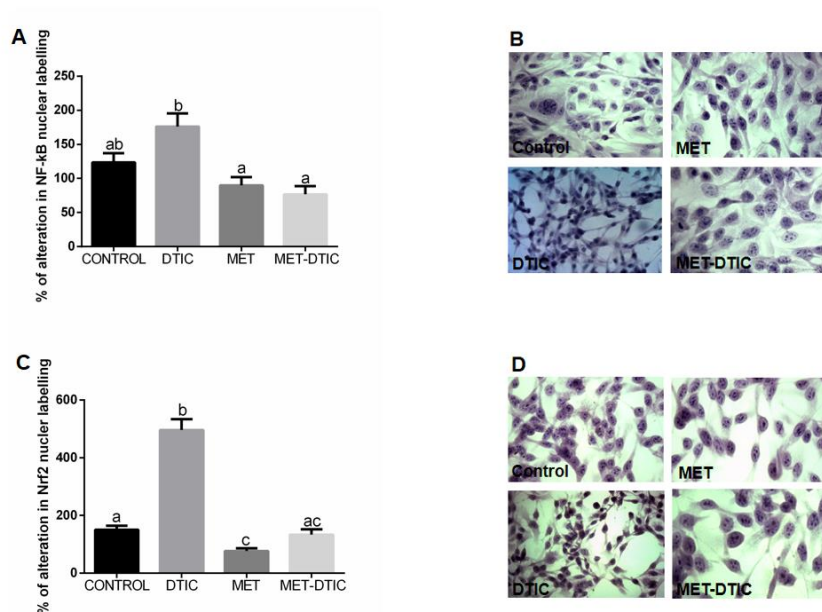
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Figure 5. Immunocytochemistry analysis of B16F10 murine melanoma cells. Percentage of nuclear labelling for NF-kB (A) and for Nrf2 (C) in cells of the CONTROL, DTIC, MET, and MET-DTIC groups. Representative images from the NF-kB (B) and for Nrf2 (D) analysis in each experimental group. Data are expressed as mean \pm standard error of mean of the percentage of alteration in nuclear labeling in the samples in the seventh passage (P7) in relation to the first passage within each group. Statistically significant differences were investigated by one-way ANOVA, followed by Tukey post-hoc and $p < 0.05$ was considered significant. Letters different present statistical significant difference. DTIC: Cells experimentally induced to DTIC resistance. MET: Cells were treated with 6 μ M metformin during fourteen passages. MET-DTIC: Cells treated with 6 μ M metformin and experimentally induced to DTIC resistance.

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1 **ANEXO B – Characterization of metformin activity *in vivo* murine metastatic**
2 **melanoma model: the Pandora’s box of oxidative stress.**

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8 **Characterization of metformin activity *in vivo* murine metastatic melanoma**
9 **model: the Pandora’s box of oxidative stress**

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1 ABSTRACT

2 Melanoma represents the most aggressive type of skin cancer, it causes the highest
3 number of skin cancer-related deaths worldwide and its incidence has been increasing
4 in recent decades. Early identification of this cancer is crucial for the success of patient
5 treatment, and once it becomes metastatic, the prognosis is very poor, metastatic
6 melanoma is refractory to most of the therapies available. Dacarbazine (DTIC), was
7 approved in 1974 by the FDA as the standard chemotherapy drug for the treatment of
8 metastatic melanoma, and is yet considered one of the most effective chemotherapeutics
9 for this disease. However, because of the aggressiveness and nature of melanoma, the
10 effects of chemotherapeutics alone are unsatisfactory, and the most commonly
11 addressed strategy in the treatment of melanoma is the combination of therapies.
12 Retrospective studies have demonstrated beneficial effects of metformin on cancer
13 prevention and treatment in diabetic patients, studies have also shown the promising
14 effects of metformin on melanoma. The aim of the present study was to analyze the
15 therapeutic potential of metformin in a metastatic murine melanoma model. For this C57
16 / BL6 mice were divided into two large groups. In the first the animals were inoculated
17 with B16F10 cells in the ophthalmic plexus for the development of pulmonary
18 metastases, and subsequently divided into groups that were treated with metformin,
19 dacarbazine or combinatory treatment with metformin and dacarbazine. In the second
20 large group, the animals were treated with metformin for 5 days and then inoculated with
21 B16F10 cells and treated as abovementioned. All animals were evaluated for
22 physiological patterns, number of pulmonary nodules, oxidative stress in the tumor and
23 systemic microenvironment, tumor histology and signaling pathways involved in
24 oxidative stress, proliferation and cell death, which confer resistance to the treatments
25 available for melanoma. Our results showed that metformin has antitumor and
26 antimetastatic potential, as it was able to reduce the number of tumor nodules, in addition
27 to potentiating the effects of dacarbazine and reducing its side effects. For this,
28 metformin interfered in some signaling pathways, such as Nrf2, and may be responsible
29 for oxidative stress in the tumor and systemic microenvironment. In addition to
30 modulating expression of PCNA, responsible for cell proliferation, and p53, apoptosis
31 signaling pathway. All these results together are responsible for the reduction of
32 implantation and size of the metastatic nodules in the tumor tissue, revealing a
33 therapeutic effect of metformin for the model of melanoma metastasis.

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35 Key words: Melanoma, dacarbazine, metformin, melanoma metastatic, oxidative stress.

1 **1.Introduction**

2

3 Melanoma arises from the occurrence of genetic mutations in melanocytes, the
4 melanoma-producing cells located in the epidermis [1]. Of the cancers that affect the
5 skin, melanoma accounts for only 4%. However, it is the most aggressive type that most
6 leads to obit [2], and its incidence has been increasing in the last decades [3]. According
7 to data from the World Health Organization, the incidence of melanoma has increased
8 over time in many countries. Around the world, more than 230,000 new cases of
9 melanoma are diagnosed and more than 50,000 people die of the disease each year [4].
10 In Brazil, it is estimated for the year of 2018, 6,260 new cases, leading to the death of
11 1,547 people [5].

12 Early identification is crucial to successful treatment. However, when it
13 becomes metastatic, the prognosis is poor [6,7]. When diagnosed in the early stage, the
14 treatment is surgical resection and the 5-year survival rate is 98%. Once the regional
15 lymph nodes are affected, the treatment is surgical removal associated with
16 chemotherapies, and the 5-year survival rate is 15.1% and when metastasized to distant
17 organs, the treatment is palliative, the survival mean overall survival is 6 to 9 months
18 [3,8]. Metastatic melanoma has historically low rates of response to currently available
19 therapies and has been one of the most difficult cancers to treat [9-11].

20 Over the past years, several therapies have been approved by the US Food and
21 Drug Administration (FDA), depending on the features of the tumor (location, stage, and
22 genetic profile), the therapeutic options may be surgical resection, chemotherapy,
23 radiotherapy, photodynamic therapy (PDT), immunotherapy, or targeted therapy [12].
24 Dacarbazine (DTIC), was approved in 1974 by the FDA as a standard chemotherapy
25 drug for the treatment of metastatic melanoma, being the most used in Brazil [13].
26 However, because of the aggressiveness and nature of melanoma, the effects of
27 chemotherapeutics alone are unsatisfactory, and the most commonly addressed
28 strategy in the treatment of melanoma is the combination of therapies.

1 Metformin is the most commonly prescribed drug for treating type 2 diabetes
2 (T2D) worldwide. Retrospective studies have demonstrated beneficial effects of
3 metformin on cancer prevention and treatment in diabetic patients [14-19]. *In vitro* studies
4 show an antiproliferative effect of metformin [20-25], *in vivo* studies have also shown a
5 role for metformin in controlling tumor growth [26-28]. Several mechanisms also exist to
6 explain an association between metformin and reduced cancer development and
7 progression. These mechanisms focus largely on inhibiting growth stimuli and metabolic
8 processes within cancer cells and can be divided into insulin-dependent and independent
9 mechanisms that alter cancer cell growth [30].

10 Oxidative stress is a process closely related to cancer, it is involved in the
11 initiation process, activation of transcription factors, cell proliferation, resistance,
12 angiogenesis, invasion and metastasis [31]. The participation of reactive species in the
13 pathophysiology of cancer is well reported in the literature, but its role in the
14 pathophysiology of melanoma is still poorly understood [32,33]. Melanoma is a neoplasm
15 with a relatively unique profile when compared to other types of cancer, presenting high
16 levels of oxidative stress in the primary tumor microenvironment [34]. Patients with
17 melanoma demonstrated an increase in systemic oxidative stress and compromised the
18 antioxidant system, which could favor the accumulation of ROS and promote
19 carcinogenesis in neighboring tissues [32].

20 Taking into account the fact that melanoma is refractory to currently available
21 therapies and that patients with metastatic melanoma inevitably develop to death, there
22 is a great need to investigate new drugs with therapeutic potential against melanoma.
23 Metformin is a drug that has shown promising results for different types of cancer, and
24 especially melanoma. For this reason, the main objective of this study was to analyze
25 the action of metformin as a treatment and as an ally with the treatment with DTIC
26 chemotherapy in the model of metastatic murine melanoma. We also analyzed the action
27 of metformin on the implantation of B16F10 melanoma cells in the above mentioned
28 model. We analyzed the participation of metformin in oxidative stress in the tumor and

1 systemic microenvironment, as well as the impact of the drug on signaling pathways, in
2 order to clarify the possible mechanics of metformin action in the metastatic murine
3 melanoma model. In all, we evaluated the joint action of metformin with DTIC.

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6 **2 Materials and Methods**

7 2.1. Cell culture

8 The murine melanoma cell line (B16F10) was seeded in tissue culture flasks and
9 grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher, USA),
10 supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher) and 1%
11 penicillin/streptomycin mixture (Santa Cruz Biotechnology, USA). The culture was
12 maintained in a humidified atmosphere with 5% CO₂ at 37°C (Sanyo CO₂ Incubator;
13 Sanyo, Japan).

14

15 2.1.2 Murine metastasis model

16 After approval by the ethics and research committee of the State University of
17 Londrina (CEUA N° 13281.2017.07), forty C57/BL6 males mice with 8 weeks of age and
18 weighing 20–25 g, were divided into five groups (Figure 1 A). In negative control group
19 (C-) the animals received 50µL of serum-free DMEM through the ophthalmic plexus; in
20 the positive control (C +) group the animals were inoculated intravenously by the
21 ophthalmic plexus with 50µL of 4x10⁶ B16F10 cells dissolved in serum-free Dulbecco's
22 modified Eagle's medium (DMEM; Gibco, Thermo Fisher, USA). In the metformin group
23 (MET) the animals were inoculated with B16F10 cells and treated from 1st to 14th days
24 with 100µL of metformin dissolved in PBS at the concentration 2mg/mice/day by
25 intraperitoneal injections as described by Souza-Neto (2017). In the dacarbazine group
26 (DTIC) the inoculated animals were treated with 100µL of dacarbazine dissolved in PBS
27 at a concentration of 50mg / kg (calculation based on the dose used in humans)

1 intraperitoneal injections from the 5th to the 10th day. In the metformin + Dacarbazine
2 group (MET-DTIC) the inoculated animals were treated with metformin and dacarbazine
3 under the same conditions mentioned above.

4 To analyze the pre-effects of metformin in our model, forty animals were pre-
5 treated with 100µL of metformin dissolved in PBS at the concentration of 2mg/mice/day
6 intraperitoneal injections for 5 consecutive days, the animals were called pre metformin
7 treated (PTM), then divided into the same groups mentioned above and treated in the
8 same way (Figure 1 B). The mice had access to chow and water *ad libitum*. The animals
9 were weighed every day during the experiment, and food intake was calculated.

10 The mice were anesthetized on 14th day with 20µL ketamine 10% and 10µL
11 xylazine 2% intraperitoneally. Heparinized blood was obtained by cardiac puncture,
12 followed by the separation of plasma and erythrocytes, which were washed three times
13 with 0.9% NaCl and stored in Alsever's solution at 4°C for posterior analysis. In
14 sequence, the mice were euthanized, the lung carefully removed and photographed with
15 standardized light conditions, position and distance of the camera for later analysis of
16 the number of metastatic nodules. The middle right lobe was fixed in bouin (5% acetic
17 acid, 9% formaldehyde and 9% picric acid) and used for histologic and
18 immunohistochemistry analyses.

19

20

21 2.2 Oxidative stress parameters

22

23 2.2.1 Glutathione (GSH) levels

24 Glutathione (GSH) levels were measured as described by Tietze et al., (1969).
25 For erythrocytes 2.5 µL of red blood cells were diluted in 2mL deionized water. For
26 pulmonary tissue, a homogenate was prepared in concentration of 25mg/mL. The assay
27 involved spectrophotometric (Multiscan Go; Thermo Scientific, USA) measurement of
28 reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) to 5-thio- 2-nitrobenzoic acid by GSH at

1 412 nm, by the formation of yellow-colored 5-thio-2-nitrobenzoic acid. Results were
2 expressed as GSH per gram ($\mu\text{M/g}$) of total protein. The total protein content was
3 determined based on the Lowry method modified by Miller (1954).

4

5

6 2.2.2 Determination catalase activity

7 Catalase activity was determined as described by Aebi, (1984). An aliquot of 100
8 μL of erythrocytes (1:80), and pulmonary homogenate (25mg/mL) was incubated in a
9 system containing 1 M TRIS buffer and 200 mM H_2O_2 solution. Absorbance
10 disappearance kinetics was monitored in a UV-1650 PC® UV-vis spectrophotometer at
11 240 nm (Shimadzu, Kyoto, Japan). The results are expressed in absorbance decreased
12 in 1 min (V abs/min) g protein.

13

14 2.2.3 Determination malondialdehyde (MDA) levels

15 Plasm and tissue malondialdehyde (MDA) measurement were performed as
16 described by Victorino et al., (2012) by using a high-performance liquid chromatography
17 (HPLC). Briefly, 160 μL of plasma and pulmonary tissue homogenate, were mixed with
18 100 μL of 0.5 M perchloric acid and incubated for 10 min on ice to precipitate the proteins,
19 and then mixed with 100 μL of 1 % thiobarbituric acid. This reaction was incubated for
20 30 min in a boiling water bath and transferred to an ice bath to stop the reaction. A 100
21 μL volume of 1 M NaH_2PO_4 (pH = 7) was added to each sample to stabilize the sample
22 pH. Samples were centrifuged for 10 min at 5000 \times g at 4 °C. The supernatants were
23 filtered and injected into the LC20AT® HPLC system (Shimadzu, Kyoto, Japan) in a
24 mobile phase consisting of 65 % 50 mM KH_2PO_4 buffer and 35 % HPLC-grade methanol
25 in a ShimPack® C18 reverse-phase column (Shimadzu, Kyoto, Japan). Acid hydrolyzed
26 1,1,3,3- tetraethoxypropane (TEP) was used as standard to obtain the linear regression
27 equation and calculate the MDA concentration of the samples. Readings were obtained

1 at 535 nm over 11 min at a flow rate of 0.8 mL/min at 35 °C. Results were expressed in
2 nanomolar of malondialdehyde as nM of MDA.

3 4 2.2.4 Chemiluminescence (CL)

5 Erythrocyte and tissue lipoperoxidation were evaluated by chemiluminescence
6 (CL) according to Panis et al., (2012). For erythrocytes 5 µL of red blood cells were added
7 to 500 µL of phosphate buffer, and 100 µL of this solution was diluted in 1200 µL of the
8 same buffer. For the lung, 880 µL of homogenate (25mg/mL) were added to 100 µL of
9 phosphate buffer. Samples were kept in water bath for 5 minutes. The chemiluminescent
10 reaction was initiated by the addition of tert-butyl (10 µl for erythrocytes and 20 µl for
11 tissue). Chemiluminescence curves were obtained in a GloMax® luminometer 20/20,
12 and the results were expressed in relative light units (RLU) g. The curve obtained was
13 used as a qualitative indicator of lipoperoxidation, and quantitative results were obtained
14 following area under curve integration using GraphPad Prism® version 6.0 (GraphPad
15 Software, San Diego, CA, USA).

16

17 2.3 Histological analyses

18 Histological analyses were performed on the right middle lobes of the lungs.
19 Samples were fixed in bouin for 48 hours and embedded in paraffin, the sections were
20 semisysed at 5µM, stained with hematoxylin and eosin. The slides were concealed for
21 analysis of the following criteria: A) tissue architecture, (-) preserved architecture (equal
22 to C-). B) Tumor implantation points: (-) absence of metastatic points, (+) 1 to 3
23 implantation points in the cut, (++) of 4 to 5 implantation points in the cut, (+++) above 6
24 metastatic points in the cut. C) Small (+) nodule size, 3-15 cells B16F10, (++) medium,
25 15 to 25 B16F10 cells, medium to large cells, 25-35 cells B16F10 (++++ large, more
26 than 35 B16F10 cells. D) melanin: (-) absence of melanin in the tumor mass, (+)
27 presence of melanin in the tumor mass. E) Inflammatory infiltrate: (-) absence of
28 inflammatory infiltrate, (+) presence of inflammatory cells. The images were obtained

1 using a high resolution camera coupled to the optical microscope, being analyzed 20
2 images per group, by two different researchers and an average per group of each
3 criterion evaluated.

4

5 2.4 Immunohistochemistry

6 Thick cuts of 3- μ m thick sections mounted on silane-coated slides (StarFrost®)
7 were deparaffinized, rehydrated, immersed in 10 mmol/L-1 citrate buffer at pH 6.0, and
8 submitted to heat-induced epitope retrieval using a vapor lock for 20 min. The slides
9 were rinsed with phosphate-buffered saline (PBS) and were immersed in 3% hydrogen
10 peroxide for 30 min to block endogenous peroxidase. Non-specific protein binding was
11 blocked with normal serum (Vectastain Elite ABC Kit; Universal, Vector Laboratories Inc.,
12 Burlingame, CA, USA) for 30 min. The sections were then incubated with monoclonal
13 primary antibodies specific for Nfr2 (1:250), p53 (1:320), and PCNA (1:250) (clone HM11;
14 Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature (25 °C) in
15 a humid chamber. Following washes in PBS, a biotinylated pan-specific universal
16 secondary antibody (Vectastain Elite ABC Kit) was applied for 30 min. Next, the slides
17 were then incubated with the avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit)
18 for 60 min and were visualized by treatment with H₂O₂ and 3,3'-diaminobenzidine (DAB)
19 for 3min. The slides were hematoxylin-coated, dehydrated and mounted with Permount
20 (Biomeda®, Foster City, CA, USA). The analyses were done blindly by 2 people in 10
21 different fields, at 400 × magnification. Percentage of nuclear labeling in cells was
22 performed.

23

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1 2.5. Statistical analysis

2 For parametric variables, groups were compared using one-way ANOVA followed
3 by Tukey's post-hoc test, all data were expressed as arithmetic means and standard
4 errors of means (SEM). Non-parametric variables were compared between groups using
5 Kruskal-Wallis test followed by Dunn's multiple comparison test. For
6 chemiluminescence curves two-way ANOVA was used. Differences were considered
7 statistically significant when $p < 0.05$. Data was analyzed using GraphPad Prism (version
8 6; San Diego, CA, USA).

9

10 **3. Results**

11 3.1. Murine metastasis model

12

13 There was a significant reduction in the number of nodules in the DTIC and
14 MET-DTIC groups of 77.78% when compared to the C + group ($p < 0.001$, $p < 0.0001$,
15 respectively) (Figure 2A). For the behavioral parameters, there was a significant increase
16 of 31% in food intake for the MET-DTIC group when compared to the C + group (p
17 < 0.0001) (Figure 3A). However, there were no significant alterations in the body weight
18 of the animals (Figure 3B).

19 When animals were pretreated with metformin for 5 days prior to inoculation
20 with the B16F10 cells, there was a significant 92.86% reduction in the number of nodules
21 for the PTM (MET) group when compared to the PTM (C +) group ($p < 0.005$), and
22 86.67% when compared to the PTM (DTIC) group ($p < 0.005$). For the PTM (MET-DTIC)
23 group the reduction in nodule numbers was 78, 58% in relation to PTM (C +) ($p < 0.005$)
24 and 60% in relation to PTM ($p < 0.005$) (Figure 10 A). For the behavioral parameters of
25 the animals, all treated groups had a significant increase in food consumption in relation
26 to the PTM (C +) ($p < 0.001$). However, there was a significant reduction in weight in all

1 groups relative to the PTM (C +) ($p < 0.005$ for PTM (MET) and PTM (DTIC), $p < 0.001$
2 for PTM (MET-DTIC) (Figure 11 A and B).

3

4 3.2. Oxidative stress parameters

5 For the oxidative stress parameters in the tumor microenvironment, GSH levels
6 were not significant (Figure 4A), there was a significant increase in the GSSG level for
7 MET-DTIC when compared to the C + group ($p < 0.001$) (Figure 4 B). There were no
8 significant changes between groups in catalase activity (Figure 5A). There was a
9 significant increase in MDA in MET when compared to C + and MET-DTIC ($p < 0.001$)
10 (Figure 5 B). C + vs DTIC ($p < 0.0001$), CTS vs DTIC ($p < 0.0001$), C + vs DTIC (p
11 < 0.0001) $p < 0.05$) and MET vs MET-DTIC ($p < 0.05$) (Figure 5 C).

12 For the parameters of systemic oxidative stress, GSH levels were not
13 significant (Figure 6A), however, there was a significant reduction in GSSG levels in the
14 MET-DTIC when compared to the C + group $p < 0.05$ (Figure 6B). There was a significant
15 increase in catalase activity in the MET-DTIC relative to C- ($p < 0.05$) (Figure 7A). The
16 MDA levels were significantly elevated in the DTIC in relation to C + ($p < 0.005$), MET (p
17 < 0.005) and MET-DTIC ($p < 0.05$) (Figure 7B). The CL curves were different between C
18 + vs MET ($p < 0.05$), MET vs MET-DTIC ($p < 0.005$), and DTIC vs MET-DTIC ($p < 0.05$)
19 (Figure 7C).

20 In animals pre-treated with metformin for 5 days prior to inoculation, oxidative
21 stress parameters in the tumor microenvironment were not significant for GSH (Figure
22 12A). However, there was a significant reduction in GSSG levels in all groups in relation
23 to PTM (C +), [PTM (C +) vs PTM (MET), $p < 0.001$; PTM (C +) vs PTM (C+), $p < 0.001$;
24 and PTM (C +) vs PTM (MET-DTIC), $p < 0.001$] (Figure 12B). The catalase activity
25 showed a significant increase of 65.48% in PTM (MET) in relation to the PTM (C +) group
26 ($p < 0.0001$), and a reduction of approximately 25% in relation to PTM (Figure 13A). The
27 CL curves were different for PTM (C +) vs PTM (MET) ($p < 0.0001$), PTM (MET) vs PTM
28 (DTIC) ($p < 0.0001$) (Figure 13C).

1 There was a significant increase in systemic GSH in animals pretreated with
2 metformin, 71% for PTM (MET-DTIC) in relation to PTM (C +) ($p < 0.5$) (Figure 14A), but
3 there was a significant reduction of 51, 34% in GSSG in PTM (MET) in relation to PTM
4 (C +) ($p < 0.005$) (Figure 14B). The catalase activity had a significant increase in PTM
5 (MET) when compared to PTM (C +) $p < 0.005$, with PTM (DTIC) $p < 0.0001$, and with
6 PTM (TEM. Figure 16A). There was a significant increase for MDA in the PTM group
7 (DTIC) when compared to PTM (C +) $p < 0.05$ (Figure 15B).

8

9 3.3. Pulmonary morphology

10 The histological analysis performed on lung tissue by staining with hematoxylin
11 and eosin was (+) for changes in lung tissue architecture for C +, MET and DTIC, and (-
12) for MET-DTIC, this showed adjacent lung tissue similar to C +, with integral septa
13 (Figure 8). For the number of metastatic points, the C + and MET presented higher
14 number of metastatic foci, above 6 (+++), but MET showed smaller tumors than C +,
15 MET (+++) and C + (+++++) (Figure 8 B and C). DTIC and MET-DTIC presented less
16 metastatic points (++) . The tumor size was also smaller (++) for DTIC and (+) for MET-
17 DTIC (Figure 8 D and E). C +, DTIC and MET-DTIC were (+) for the presence of melanin
18 in the tumor, since the MET was (-) for melanin in the tumor. All groups were (+) for
19 inflammatory infiltrate, but in MET the infiltrate was more evident (Table 1).

20 For all groups that were pretreated with metformin for 5 days there was a subtle
21 change in lung architecture (+) for PTM (C +), PTM (MET) and PTM (DTIC), while PTM
22 (MET-DTIC) retained the pulmonary architecture (Figure 16). The number of metastatic
23 implants were smaller for all groups, taking into consideration the groups of animals that
24 were not pretreated with metformin, considering (++) for PTM (C +), and (+) for PTM
25 (MET), PTM (DTIC), PTM (MET-DTIC) (Table 2). The size of the tumor nodules
26 appeared to be smaller than the animals that were not pretreated with metformin,
27 considered (++) for PTM (C +), PTM (MET), and PTM (DTIC) (Figure 16A, B and C).
28 Regarding the presence of melanin in the tumor environment, PTM (MET) was the only

1 group considered (-) (Figure 16B). There was an inflammatory infiltrate for all groups,
2 although it appeared to be smaller in relation to animals that were not pretreated with
3 metformin

4

5 3.4. Immunohistological analysis

6

7 There was a significant increase in nuclear p53 tumor staining for the MET-
8 DTIC group in relation to C + and MET group ($p < 0.0001$ for both), same significance
9 between the C + and DTIC groups, with greater staining (Figure 9 A). In the adjacent
10 region, there was a reduction of the p53 staining for the MET group in relation to the
11 other groups ($p < 0.0001$ for all). The DTIC group presented increased staining in relation
12 to the other groups ($p < 0.0001$ for C + and MET) and ($p < 0.001$ for MET-DTIC). The
13 MET-DTIC group also had a markedly greater staining in relation to C + ($p < 0.001$) and
14 MET ($p < 0.0001$) (Figure 9 B).

15 The labeling for PCNA in the tumor was significantly lower in all groups in
16 relation to C + ($p < 0.0001$ for all). Staining was weaker in the MET-DTIC group than in
17 MET ($p < 0.005$) and in DTIC ($p < 0.0001$) (Figure 9 C). In the adjunctive region, there
18 was a reduction in the staining in the MET group in relation to C + ($p < 0.005$) and p
19 < 0.0001 in relation to DTIC and MET-DTIC (Figure 9 D). Nrf2 labeling in the tumor was
20 lower for MET and MET-DTIC groups. MET was significantly lower in relation to C + (p
21 < 0.001) and DTIC ($p < 0.0001$). MET-DTIC was significantly lower in relation to DTIC (p
22 < 0.0001) (Figure 9 E). In the adjacent region the labeling was significantly lower in the
23 MET group in relation to the other groups ($p < 0.0001$ for all). On the contrary, DTIC
24 presented a significant increase in staining in relation to the other groups ($p < 0.0001$ for
25 all) (Figure 9 F).

26 For animals that were treated with metformin prior to inoculation with B16F10
27 cells, there was an increase in p53 nuclear labeling for the PTM (MET) tumor region
28 relative to PTM (C +) ($p < 0.001$) and PTM (DTIC) ($p < 0.05$). PTM (MET-DTIC) also

1 showed higher staining than PTM (C +) and PTM (DTIC) ($p < 0.0001$) (Figure 17 A).
2 However, there was a reduction in the staining of p53 in the tumor adjacent region for
3 PTM (MET-DTIC) in relation to PTM (C +) ($p < 0.0001$) and PTM (DTIC) ($p < 0.001$)
4 (Figure 17 B).

5 The PCNA staining was weaker for PTM (MET) in relation to PTM (C +) (p
6 < 0.0001), PTM (MET-DTIC) was lower than PTM (C +) ($p < 0.0001$) and PTM (DTIC) (p
7 < 0.001) (Figure 17 C). PTM (MET-DTIC), also showed weaker staining in the region
8 adjacent to the tumor in relation to PTM (C+) ($p < 0.001$), PTM (MET) ($p < 0.005$) e PTM
9 (DTIC) ($p < 0.0001$) (Figure 17 D).

10 The labeling for Nrf2 in the tumor region was significantly stronger for the
11 groups that after inoculation continued to be treated with metformin. PTM (MET) in
12 relation to PTM (C+) ($p < 0.0001$) and PTM (DTIC) ($p < 0.001$), and PTM (MET-DTIC) in
13 relation to PTM (C+) ($p < 0.0001$) and PTM (DTIC) ($p < 0.05$) (Figure 17 E). However, the
14 labeling for Nrf2 in the tissue adjacent to the tumor was significantly higher in the groups
15 receiving DTIC after inoculation of the B16F10 cells. PTM (DTIC) in relation to PTM (C+)
16 and PTM (MET) ($p < 0.005$), and PTM (MET-DTIC) in relation to PTM (C+) ($p < 0.0001$)
17 and PTM (MET) ($p < 0.001$) (Figure 17 F)

18

19 **3.Discussion**

20

21 Early diagnosis of melanoma is critical to increase patient survival, when it is
22 disseminated to the lymph nodes or to distant organs such as the lungs, liver, bones and
23 brain, treatment involves surgical procedures associated with chemotherapy, radiation
24 therapy and / or immunotherapy, and the survival rate is low, with a mean overall survival
25 of about around 6 to 9 months [41-44]. DTIC, is considered one of the most indicated
26 chemotherapeutic for metastatic melanoma [13]. It is a DNA alkylating agent and repair
27 mechanism inhibitor, which aims to induce DNA damage, resulting in death by apoptosis
28 of neoplastic cells [45]. Because of the aggressiveness and nature of melanoma, the

1 effects of DTIC alone are unsatisfactory; many treatments use combination with other
2 medications (eg, cisplatin, vinorelbine, paclitaxel) [2,46]. Metformin has shown good
3 results in relation to cancer, especially melanoma, however, there are still few studies
4 showing the mechanisms of action.

5 Our results showed that metformin was able to reduce the number of metastatic
6 nodules in lung in a murine model of metastatic melanoma. Cerezo et al. (2013), showed
7 that animals treated with 60mg / kg metformin for 39 days reduced the invasion and
8 metastasis of melanoma. Li et al. (2017) treated the animals 7 days after inoculation with
9 metformin at the concentration of 150mg / kg for 3 weeks, and observed reduction in the
10 metastatic lung nodules. Pereira et al., (2018), showed reduction in metastatic nodules
11 in animals treated with metformin at a concentration of 500mg / kg for 14 days. We may
12 note that the metformin concentration and the treatment time we used were lower than
13 those cited, showing a beneficial effect of metformin even at lower concentrations and
14 treatment time exposure.

15 We observed a higher reduction in the number of pulmonary nodules when
16 associated to the chemotherapeutic DTIC. Pereira et al., (2018), showed that metformin
17 has a greater antitumor effect in combination with other drugs such as sitagliptin. Hirsh
18 et al., (2013), observed a reduction in the volume of melanoma solid tumors when
19 metformin (2µg/mL) was associated to doxorubicin (1 mg/kg or 4 mg/kg) by doing 4
20 treatment cycles of 5 days each.

21 The DTIC, like all chemotherapeutic agents, has significant toxic effects, such
22 as nausea and vomiting [50,51] leading to weight loss. Medications that reduce or
23 eliminate these symptoms, could provide a better quality of life for the patient. In our
24 study, this was noted in behavioral parameters results, suggesting a possible action of
25 metformin against the side effects of the chemotherapeutic agent.

26 The oxidative stress is considered as the unbalance between the generation of
27 reactive oxygen species and the antioxidant defenses [52,53], and plays a role in cell
28 proliferation, resistance, angiogenesis, invasion and metastasis [34,54]. Patients with

1 melanoma demonstrated an increase in systemic oxidative stress and impairment of the
2 antioxidant system, which could favor the accumulation of ROS and promote
3 carcinogenesis [34]. The melanoma has high levels of oxidative stress in the primary
4 tumor microenvironment [32,33].

5 Reduced form of GSH glutathione is an antioxidant, considered the first line of
6 defense for cells under various conditions of oxidative stress. In our study, in the tumor
7 microenvironment, there was no change in GSH levels, but GSSG (oxidized form) levels
8 were significantly higher only in the MET-DTIC, and the inverse was observed in all
9 groups pretreated with metformin. This may be due to the fact that metformin decreases
10 gluconeogenesis by the inhibition of mitochondrial glycerophosphate dehydrogenase,
11 which alters the functions of the cytosolic and mitochondrial redox state [55].

12 At systemic level, there was an elevation in GSH levels only in animals that
13 were pre-treated with metformin. GSSG levels were significantly lower in all animals.
14 Kudugunti et al. (2010) observed that depletion of intracellular levels of glutathione
15 (GSH) could lead to the death of human melanoma tumor cells when treated with
16 phenylethyl ester of caffeic acid (CAPE).

17 MDA is commonly considered as a biomarker of oxidative stress, but is also
18 capable of inducing cellular injury, including DNA damage [57], results indicate that when
19 combined with DTIC, metformin reduces MDA levels, and when animals are pre-treated
20 with metformin prior to inoculation there is no significant difference in MDA levels.
21 Metformin-treated animals had a reduction in plasma levels of MDA compared to those
22 treated with chemotherapeutic agents. Souza-Neto et al. (2017) observed a reduction in
23 plasma levels of MDA in animals with solid tumors treated with metformin.

24 Observing the histological analysis, we were able to identify a reduction in the
25 size of the metastatic nodules in MET in relation to the C +, and in MET-DTIC in relation
26 to the DTIC, suggesting that metformin is involved in tumor reduction, this result leads to
27 that found by Li et al. al. (2017) who observed reduction of metastatic lung nodules in
28 C57BL / 6 mice inoculated with B16F10 cells when treated with metformin at 150mg / kg

1 for 21 days. Melanin confers a certain melanoma resistance to the treatments because
2 melanogenesis regulates the hypoxia-inducible transcription factor 1, which is the main
3 molecule of cell resistance to therapies (SLOMINSKI et al., 2014; BROŻYNA et al.,
4 2016), MET did not present melanin in the tumor or in the adjacent region.

5 Reinforcing the effect of metformin on the process of implantation and
6 development of pulmonary metastasis, we observed that when the animals were pre-
7 treated with metformin, the number of metastatic implants and the size of these
8 metastatic nodules were visibly lower than those of animals that were not pre-treated
9 with metformin. Suggesting a possible action of metformin during the process of
10 implantation of B16F10 cells into lung tissue. According to Li, metformin revealed
11 antitumor and anti-metastatic effects, as it was able to reduce SMAD3-mediated TRIB3
12 expression, regardless of AMPK. TRIB3 is considered a stress-adjustment switch that
13 responds to several stressors such as: inflammatory factors, insulin-like growth factor 1
14 (IGF-1) and reactive species. Its expression is considered an oncogenic marker,
15 indicating low survival [60-64].

16 Our results show that metformin was able to increase tumor staining for p53,
17 and when administered along with DTIC this effect was even more noticeable. Janjetovic
18 et al., (2011), showed that metformin was able to reduce tumor size in animals inoculated
19 with B16F10, inducing the cells to apoptosis, p53 modulation, mitochondrial damage and
20 oxidative stress independently of AMPK. Cerezo et al., (2013), showed that metformin
21 was able to decrease the invasion of melanoma and the development of metastasis,
22 inducing the cells to apoptosis, this process would be dependent on AMPK and p53. At
23 the same time, our results show that in tissue adjacent to the tumor there is a reduction
24 in p53 marking when the animals are treated with metformin, showing a possible specific
25 effect of metformin on tumor cells, besides a possible protective effect on cells adjacent
26 to the chemotherapeutic effect.

27 For labeling for p53 our results for animals pretreated with metformin were
28 similar to those not pretreated as metformin. Since the markers were increased for PTM

1 (MET) and PTM (MET-DTIC) in the tumor, and smaller in the region adjacent to the
2 tumor. Thus reinforcing a possible effect of metformin on the p53 pathway, and its
3 specificity for B16F10 tumor cells, leading to apoptosis.

4 Our results reveal that metformin was able to reduce the nuclear marking of the
5 cell proliferation antigen (PCNA) in the tumor. Similar results were observed in
6 melanoma, but with other drugs such as wedelolactone, radiotherapeutic IN- (2-
7 (diethylamino) ethyl) -5- (iodo-131I) picolinamide (131I-5-IPN), and melatonin [66,67]. A
8 study using metformin showed that it was able to reduce PCNA expression in lung cancer
9 cells *in vitro* and *in vivo* [68].

10 For the tumor region the results found for the animals that were pretreated with
11 metformin followed the same pattern as the animals that were not pretreated with
12 metformin prior to inoculation with the B16F10 cells. However, the labeling in the region
13 adjacent to the tumor was also lower for PTM (MET-DTIC). Our results are in agreement
14 with Hao and colleagues (2019), reinforcing the effect of metformin on the reduction of
15 PCNA expression.

16 Nuclear factor Nrf2 plays a critical role in carcinogenesis, Nrf2 is frequently
17 activated and accumulated aberrantly in the nucleus of different cancers [69], and is
18 related to the chemoresistance of malignant tumors [70,71]. Nrf2 is a transcription factor
19 that controls the basal and inducible expression of a number of antioxidant enzymes,
20 thus, the Nrf2 signaling system may be an important cellular defense and a pathway of
21 survival against oxidative stress [72-78]. Some studies have shown that expression of
22 Nrf2 in melanoma is related to invasion, worsening patient survival [79].

23 Wang et al. (2018) showed that inhibition of Nrf2 increased ERO-induced cell
24 cycle arrest and cell apoptosis and inhibited melanoma growth by regulating the AKT-
25 Nrf2 pathway in A375 cells. Gao et al. (2018), showed that the treatment with ionizing
26 radiation was able to stimulate the expression of Nrf2 and regulate cell viability, invasion
27 and migration of B16-F10 murine melanoma cells in animals. Our results show that
28 metformin was able to reduce nuclear Nrf2 staining in the tumor environment and tumor

1 adjacent to the C + and DTIC groups, showing a possible action of metformin on cellular
2 antioxidant activity.

3 For animals that were pretreated with metformin the labeling for Nrf2 in the
4 tumor was higher for those following metformin treatment, unlike the adjacent labeling
5 they were higher for the groups that had on treatment at DTIC. Revealing thus, a possible
6 action of metformin on tumor oxidative stress and adjacent tissue.

7 Our results reveal a possible antitumor and anti-metastatic action of metformin
8 in the metastatic murine melanoma model. When animals were treated with metformin
9 after inoculation of B16F10 murine melanoma cells, the possible antitumor effect of the
10 drug was revealed. However, when the animals were pretreated with metformin for 5
11 days prior to inoculation, we noted a possible anti-metastatic effect of the drug. In both
12 cases metformin was able to modulate important pathways in the process of resistance
13 to conventional treatments, involved with oxidative stress, growth and cell death. Thus,
14 we can infer that metformin was able to modulate the oxidative stress in the tumor and
15 systemic microenvironment. These findings indicate that metformin has a therapeutic
16 potential, combined with the reduction and size of metastatic nodules in the tumor tissue,
17 and potentiate the effects of DTIC without increasing the side effects of the
18 chemotherapeutic agent.

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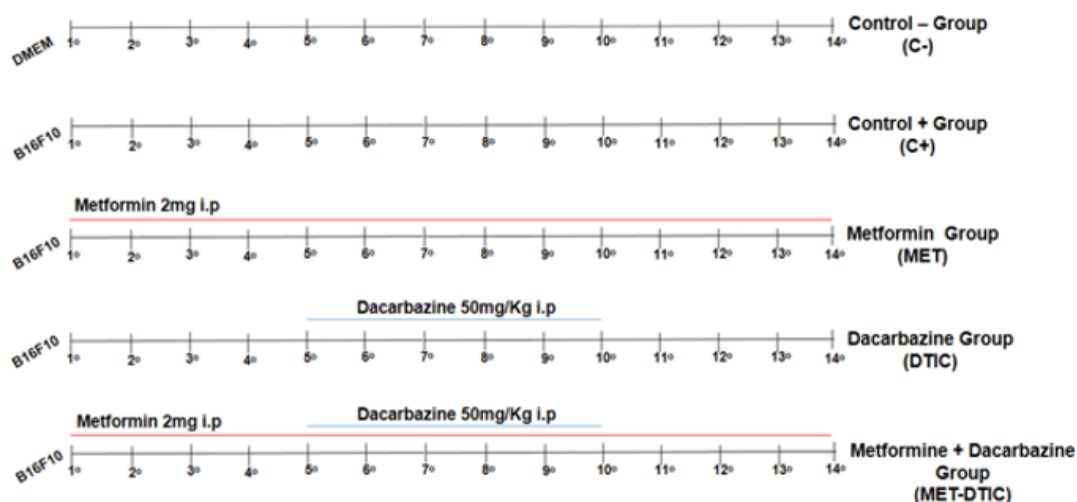
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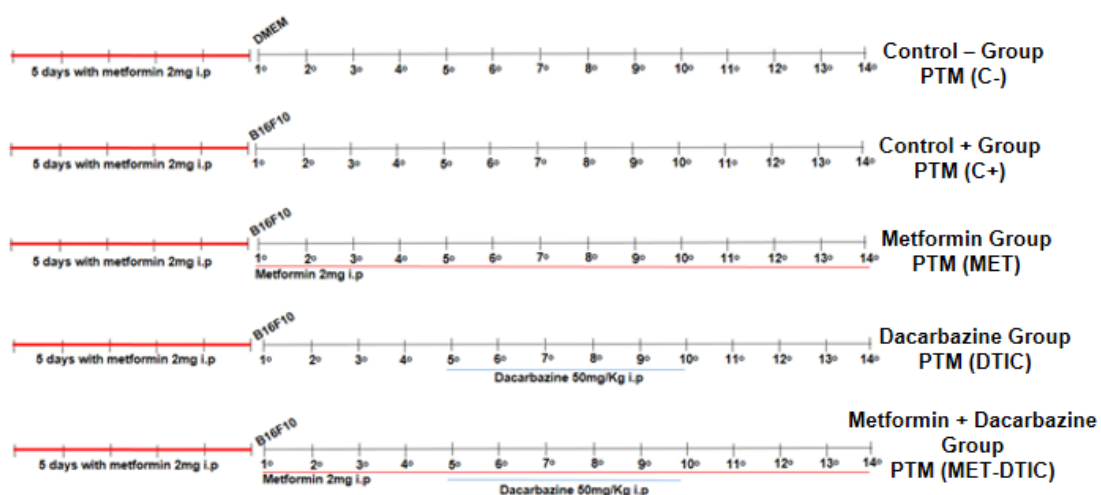
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2 **Figure 1. Experimental model of metastatic murine melanoma.** A) Control- Group (C-):
 3 inoculated with DMEM. Control+ (C+) inoculated with B16F10 cells. Metformin Group (MET):
 4 inoculated with B16F10 cells and treated with metformin 2mg/mice/day of to 1st to 14th days.
 5 Dacarbazine group (DTIC): inoculated with B16F10 cells and treated with dacarbazine 50mg/kg
 6 of 5th to 10th day. The Metformin + Dacarbazine group (MET-DTIC): inoculated with B16F10 cells
 7 and treated with metformin and dacarbazine under the same conditions mentioned above. B) The
 8 animals were treated with metformin for 5 days prior to inoculation and then the fewer treatment
 9 groups mentioned above were performed. PTM (C-), PTM (C+), PTM (MET), PTM (DTIC) and
 10 PTM (MET-DTIC).

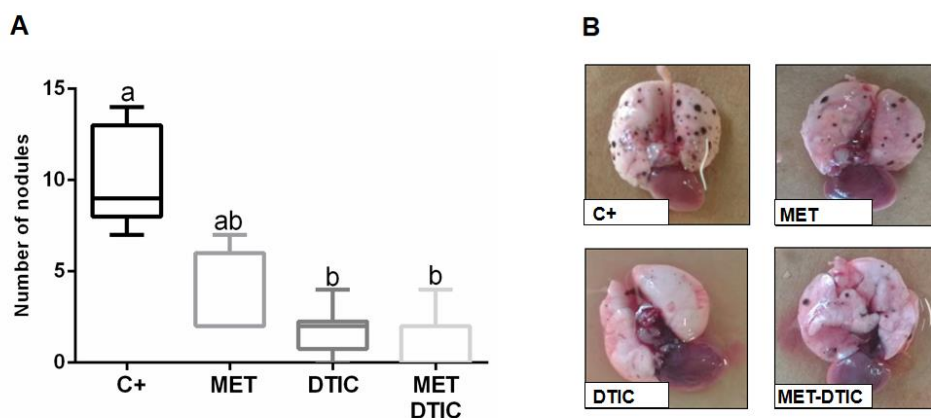
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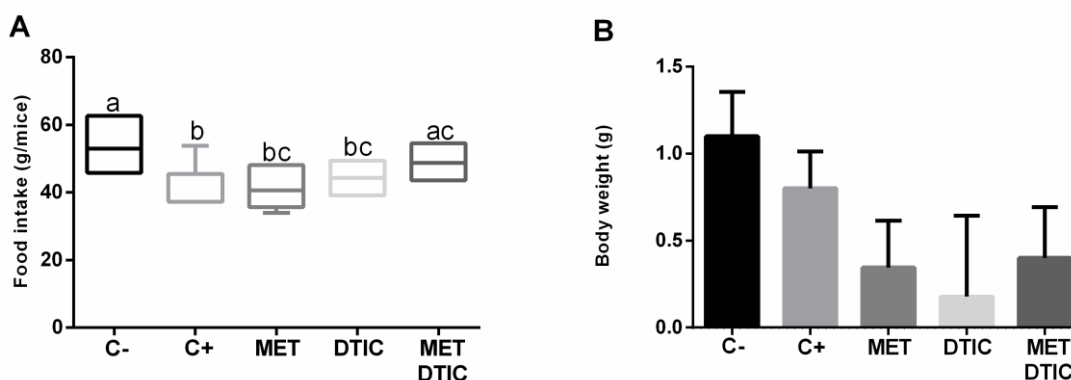
3 **Figure 2. Metastatic nodules after intravenous inoculation with B16F10 cells.** A) Number of
 4 nodules in different groups. B) Macroscopic lung of metastatic nodules. The statistical significance
 5 of the differences was analyzed by Kruskal-Wallis analysis of variance (ANOVA) followed by
 6 Dunn's post-hoc test and $p < 0.05$ was considered significant. Letters different present statistical
 7 significant difference. C+: mice with untreated pulmonary metastasis. MET: mice with pulmonary
 8 metastasis treated with metformin. DTIC: mice with pulmonary metastasis treated with
 9 dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with metformin more
 10 dacarbazine.

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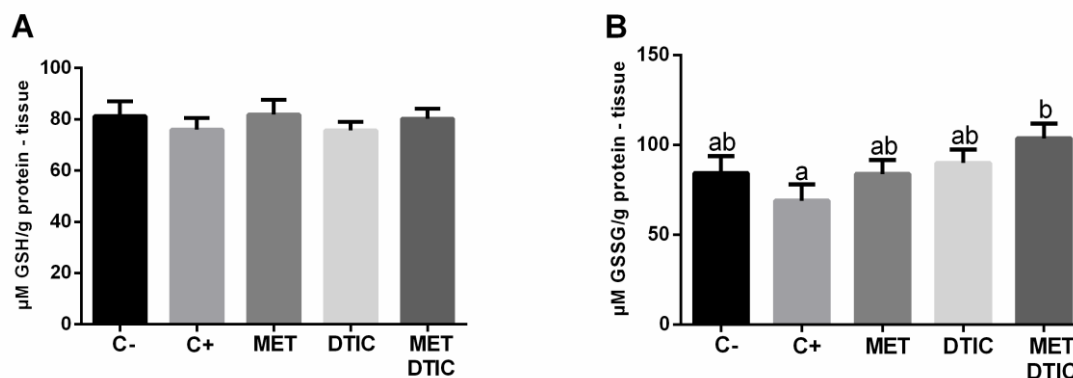
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16 **Figure 3. Behavioral parameters.** A) Consumption of feed intake per animal (in gram by mice)
 17 during experimental time. B) Analysis of weight at the end of experimental time (last weight minus
 18 first weight). Food intake statistical significance of the differences was analyzed by Kruskal-Wallis
 19 analysis of variance (ANOVA) followed by Dunn's post-hoc test and $p < 0.05$ was considered
 20 significant. Body weight, results are expressed as mean \pm SEM. The statistical significance of the
 21 differences was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-
 22 hoc test. C-: mice without pulmonary metastasis. C+: mice with untreated pulmonary metastasis.
 23 MET: mice with pulmonary metastasis treated with metformin. DTIC: mice with pulmonary
 24 metastasis treated with dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with
 25 metformin more dacarbazine.

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Figure 4. Antioxidant parameters in the tumor microenvironment. A) Glutathione (GSH). B) GSSG. Results are expressed as mean \pm SEM. The statistical significance of the differences was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and $p < 0.05$ was considered significant. C-: mice without pulmonary metastasis. C+: mice with untreated pulmonary metastasis. MET: mice with pulmonary metastasis treated with metformin. DTIC: mice with pulmonary metastasis treated with dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with metformin more dacarbazine.

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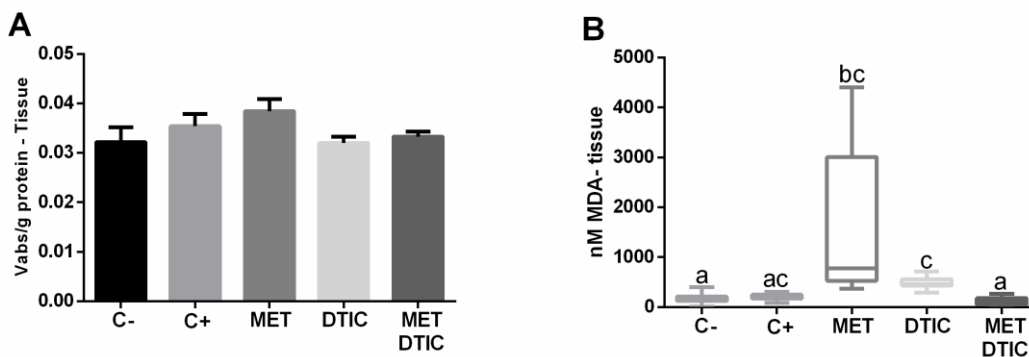
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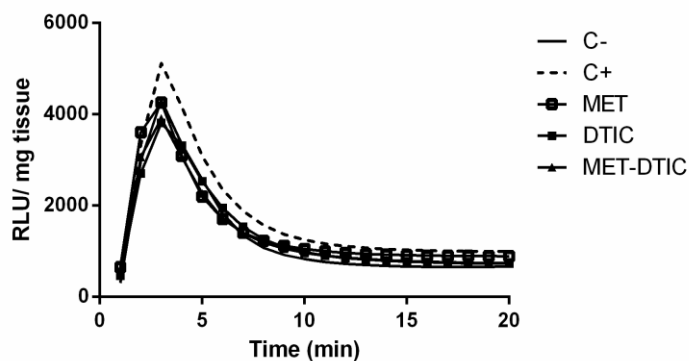
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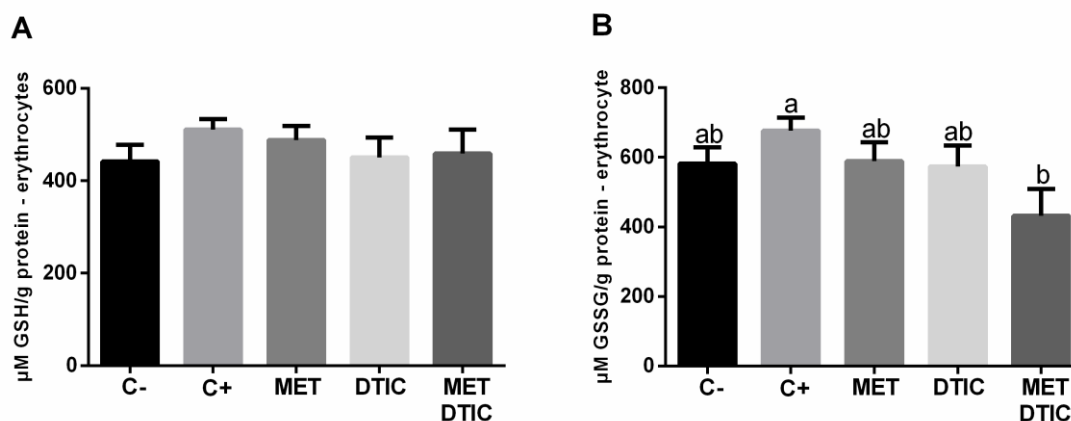


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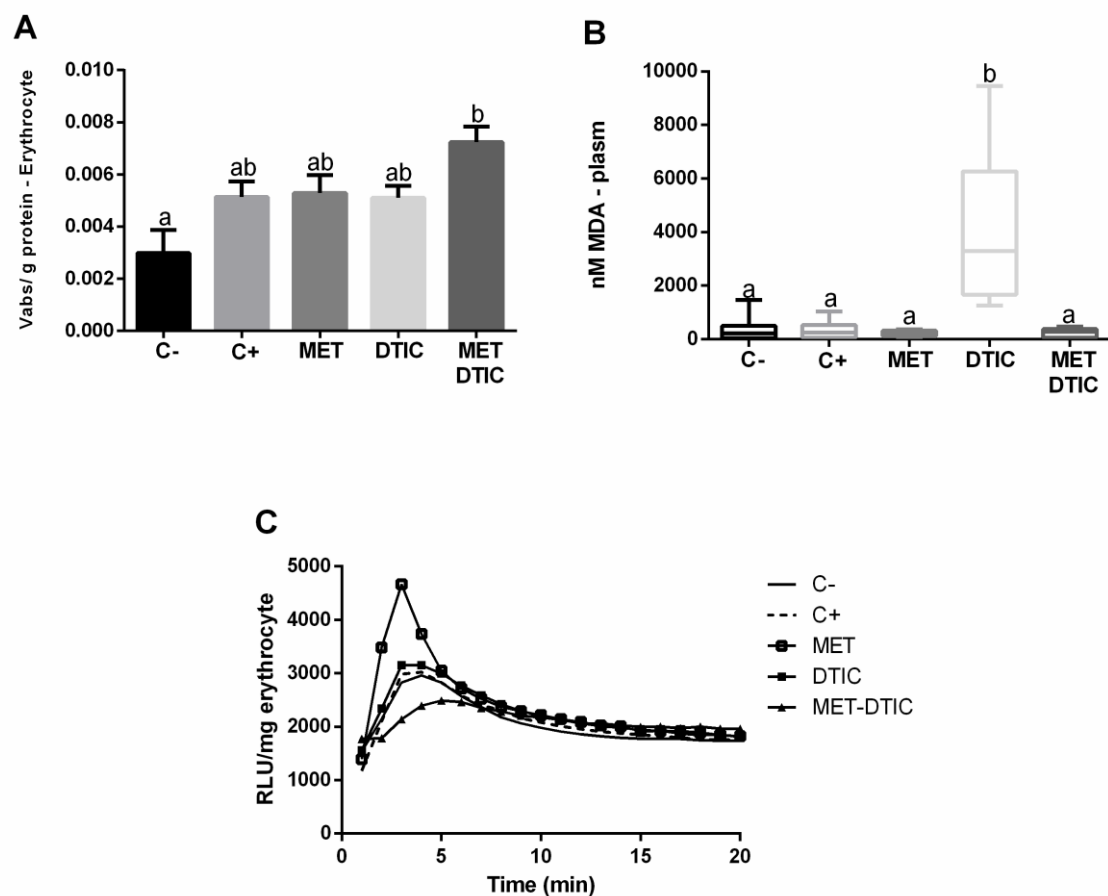


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1 **Figure 5. Oxidative stress parameters in the tumor microenvironment.** A) Catalase activity.
 2 B) Malondialdehyde levels. C) Chemiluminescence (CL). Catalase activity, results are expressed
 3 as mean \pm SEM. The statistical significance of the differences was analyzed by one-way analysis
 4 of variance (ANOVA) followed by Tukey's post-hoc test. Malondialdehyde levels, statistical
 5 significance of the differences was analyzed by Kruskal-Wallis analysis of variance (ANOVA)
 6 followed by Dunn's post-hoc test and $p < 0.05$ was considered significant. Chemiluminescence (CL),
 7 curves was used two-way ANOVA and $p < 0.05$ was considered significant. C-: mice without
 8 pulmonary metastasis. C+: mice with untreated pulmonary metastasis. MET: mice with pulmonary
 9 metastasis treated with metformin. DTIC: mice with pulmonary metastasis treated with
 10 dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with metformin more
 11 dacarbazine.
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 14 **Figure 6. Systemic antioxidant parameters.** A) Glutathione (GSH). B) GSSG. Results are
 15 expressed as mean \pm SEM. The statistical significance of the differences was analyzed by one-
 16 way analysis of variance (ANOVA) followed by Tukey's post-hoc test and $p < 0.05$ was considered
 17 significant. C-: mice without pulmonary metastasis. C+: mice with untreated pulmonary metastasis.
 18 MET: mice with pulmonary metastasis treated with metformin. DTIC: mice with pulmonary
 19 metastasis treated with dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with
 20 metformin more dacarbazine.
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Figure 7. Systemic oxidative stress parameters. A) Catalase activity. B) Malondialdehyde levels. C) Chemiluminescence (CL). Catalase activity, results are expressed as mean \pm SEM. The statistical significance of the differences was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and $p < 0.05$ was considered significant. Malondialdehyde levels, statistical significance of the differences was analyzed by Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's post-hoc test and $p < 0.05$ was considered significant. Chemiluminescence (CL), curves was used two-way ANOVA test and $p < 0.05$ was considered significant. C-: mice without pulmonary metastasis. C+: mice with untreated pulmonary metastasis. MET: mice with pulmonary metastasis treated with metformin. DTIC: mice with pulmonary metastasis treated with dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with metformin more dacarbazine.

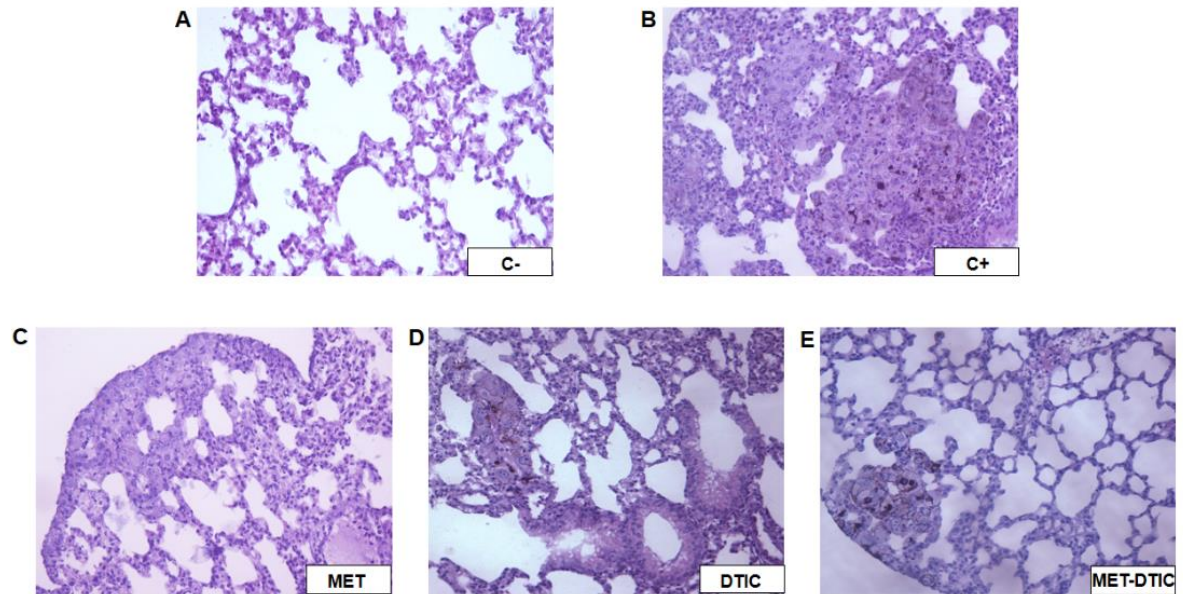
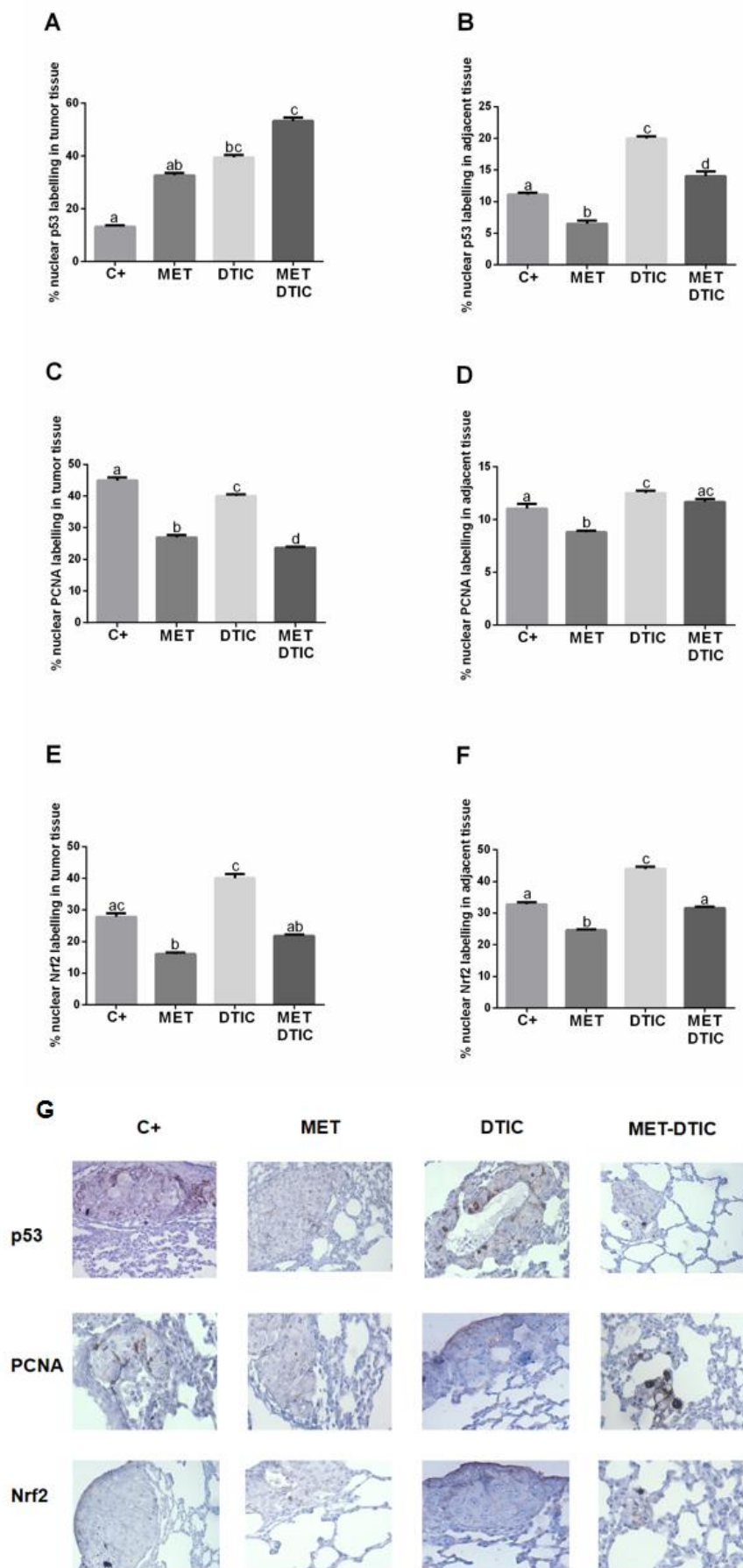
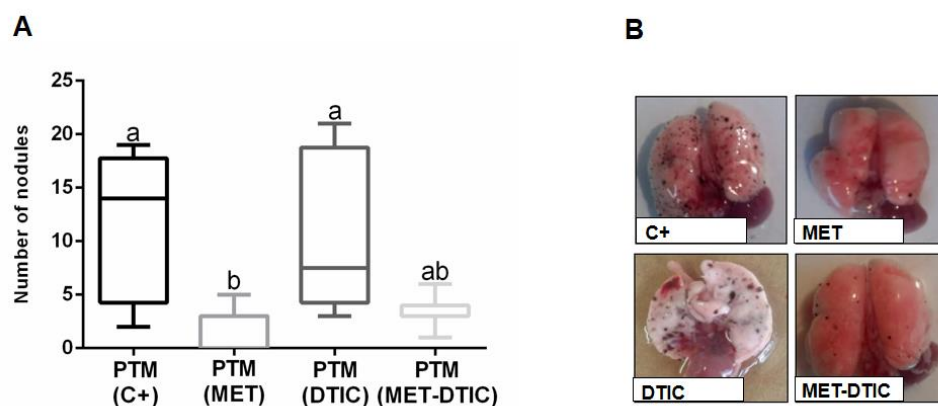


Figure 8. Metastatic lung melanoma nodules. Pulmonary tissue histology. A) C-: mice without pulmonary metastasis. B) C+: mice with untreated pulmonary metastasis. C) MET: mice with pulmonary metastasis treated with metformin. D) DTIC: mice with pulmonary metastasis treated with dacarbazine. E) MET-DTIC: mice with pulmonary metastasis treated with metformin more dacarbazine. Staining of hematoxylin and eosin. Photos representative in 200x.

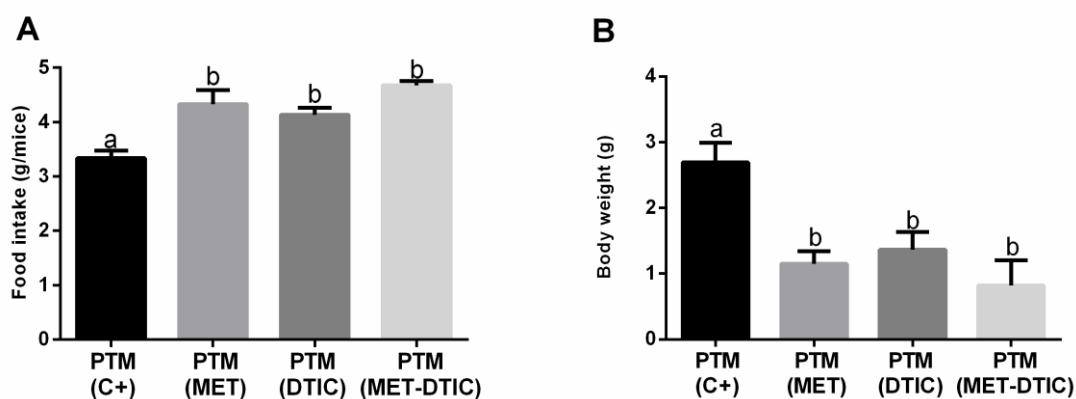
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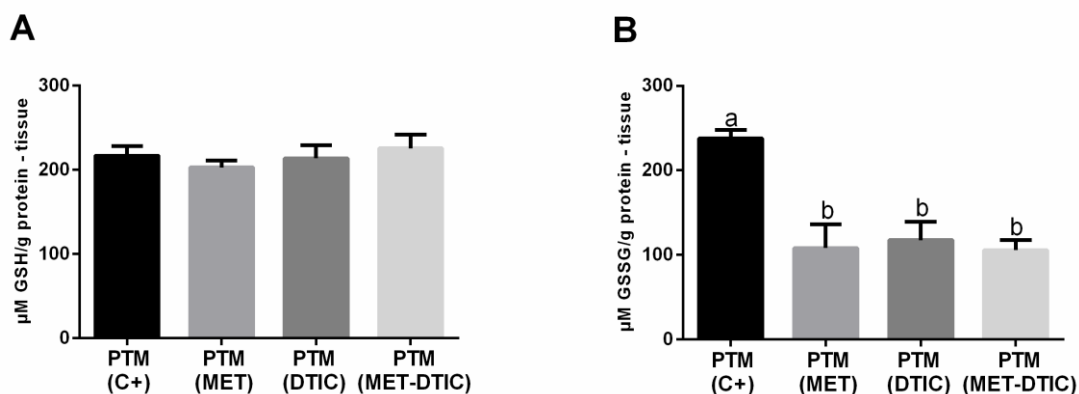
1 **Figure 9. Immunohistochemical analysis in the tumor environment and adjacent lung**
 2 **metastatic of melanoma.** Percentage of nuclear labeling for p53 in tumor A) and adjacent region
 3 B), PCNA for tumor C) and adjacent region D), and Nrf2 for tumor E) and region adjacent to tumor
 4 E). G) Images representative of the nuclear marking p53, PCNA and Nrf2 in tumor and adjacent
 5 tissue. Results are expressed as mean \pm SEM. The statistical significance of the differences was
 6 analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and $p < 0.05$
 7 was considered significant C+: mice with untreated pulmonary metastasis. MET: mice with
 8 pulmonary metastasis treated with metformin. DTIC: mice with pulmonary metastasis treated with
 9 dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with metformin more
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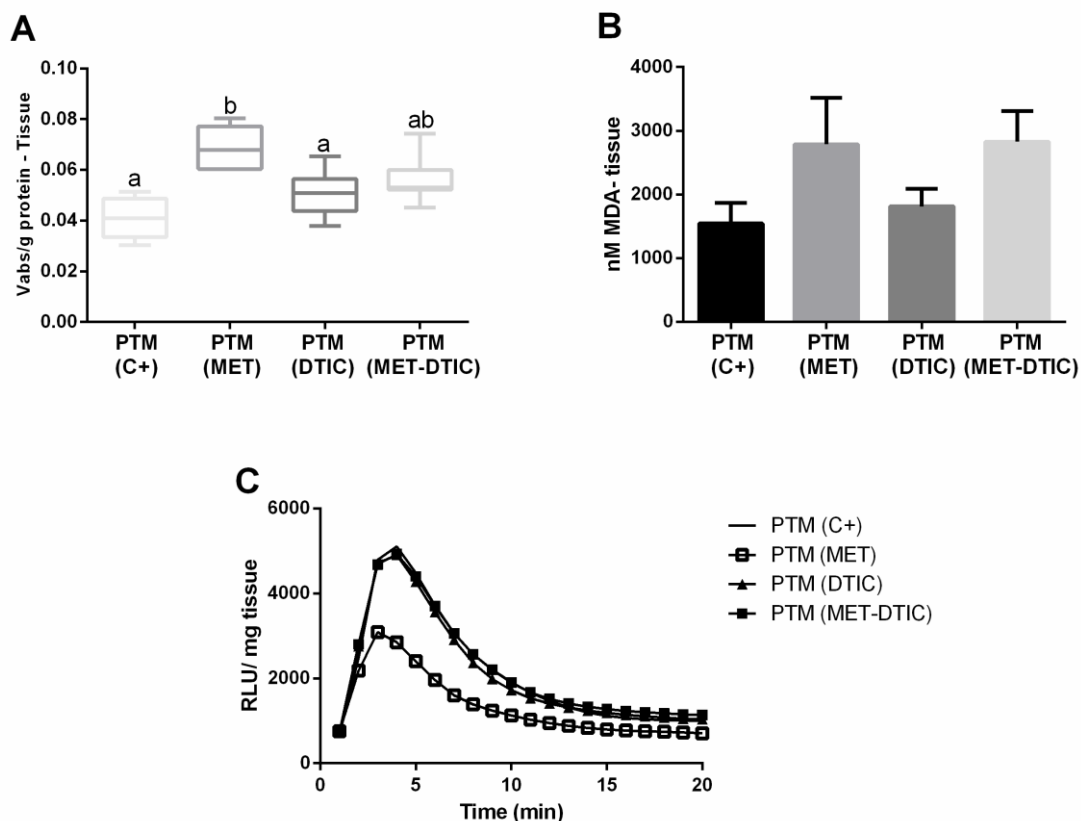
15 **Figure 10. Metastatic nodules pulmonary in mice treated with metformin during 5 days**
 16 **before inoculation with B16F10 cells and treatment.** A) Number of nodules in different groups.
 17 B) Macroscopic lung of metastatic nodules. The statistical significance of the differences was
 18 analyzed by Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's post-hoc test and
 19 $p < 0.05$ was considered significant. PTM (C+): mice with untreated pulmonary metastasis. PTM
 20 (MET): mice with pulmonary metastasis treated with metformin. PTM (DTIC): mice with pulmonary
 21 metastasis treated with dacarbazine. PTM (MET-DTIC): mice with pulmonary metastasis treated
 22 with metformin more dacarbazine.



1 **Figure 11. Behavioral parameters in animals treated with metformin during 5 days before**
 2 **inoculation with B16F10 cells and treatment.** A) Consumption of feed intake per animal (in
 3 gram by mice) during experimental time. B) Analysis of weight at the end of experimental time
 4 (last weight minus first weight). Results are expressed as mean± SEM. The statistical significance
 5 of the differences was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's
 6 post-hoc test and $p < 0.05$ was considered significant. PTM (C+): mice with untreated pulmonary
 7 metastasis. PTM (MET): mice with pulmonary metastasis treated with metformin. PTM (DTIC):
 8 mice with pulmonary metastasis treated with dacarbazine. PTM (MET-DTIC): mice with
 9 pulmonary metastasis treated with metformin more dacarbazine.
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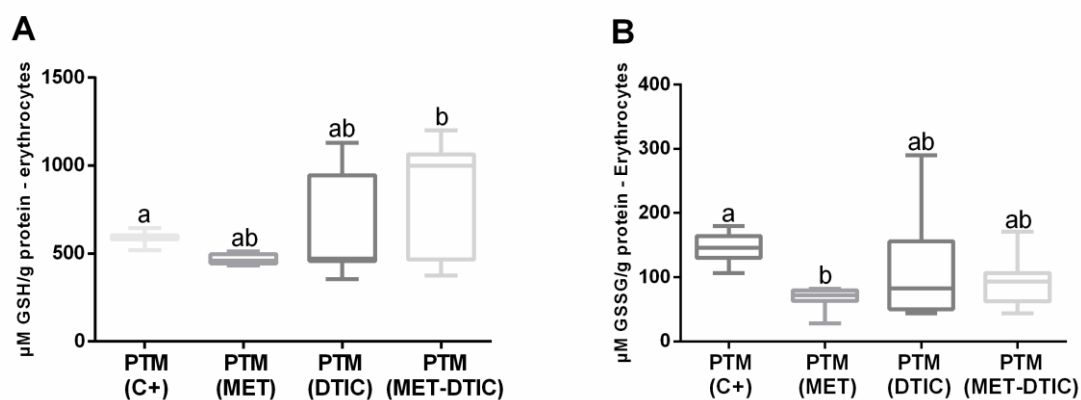


13 **Figure 12. Antioxidant parameters in the tumor microenvironment in animals treated with**
 14 **metformin during 5 days before inoculation with B16F10 cells and treatment.** A) Glutathione
 15 (GSH). B) GSSG. Results are expressed as mean± SEM. The statistical significance of the
 16 differences was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-
 17 hoc test and $p < 0.05$ was considered significant. PTM (C+): mice with untreated pulmonary
 18 metastasis. PTM (MET): mice with pulmonary metastasis treated with metformin. PTM (DTIC):
 19 mice with pulmonary metastasis treated with dacarbazine. PTM (MET-DTIC): mice with
 20 pulmonary metastasis treated with metformin more dacarbazine.
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2 **Figure 13. Oxidative stress parameters in the tumor microenvironment in animals treated with metformin during 5 days before inoculation with B16F10 cells and treatment.** A) Catalase activity. B) Malondialdehyde levels. C) Chemiluminescence (CL). Catalase activity statistical significance of the differences was analyzed by Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's post-hoc test and $p < 0.05$ was considered significant. Malondialdehyde levels results are expressed as mean \pm SEM. The statistical significance of the differences was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and $p < 0.05$ was considered significant. Chemiluminescence (CL), curves were used two-way ANOVA, test and $p < 0.05$ was considered significant. PTM (C+): mice with untreated pulmonary metastasis. PTM (MET): mice with pulmonary metastasis treated with metformin. PTM (DTIC): mice with pulmonary metastasis treated with dacarbazine. PTM (MET-DTIC): mice with pulmonary metastasis treated with metformin more dacarbazine.

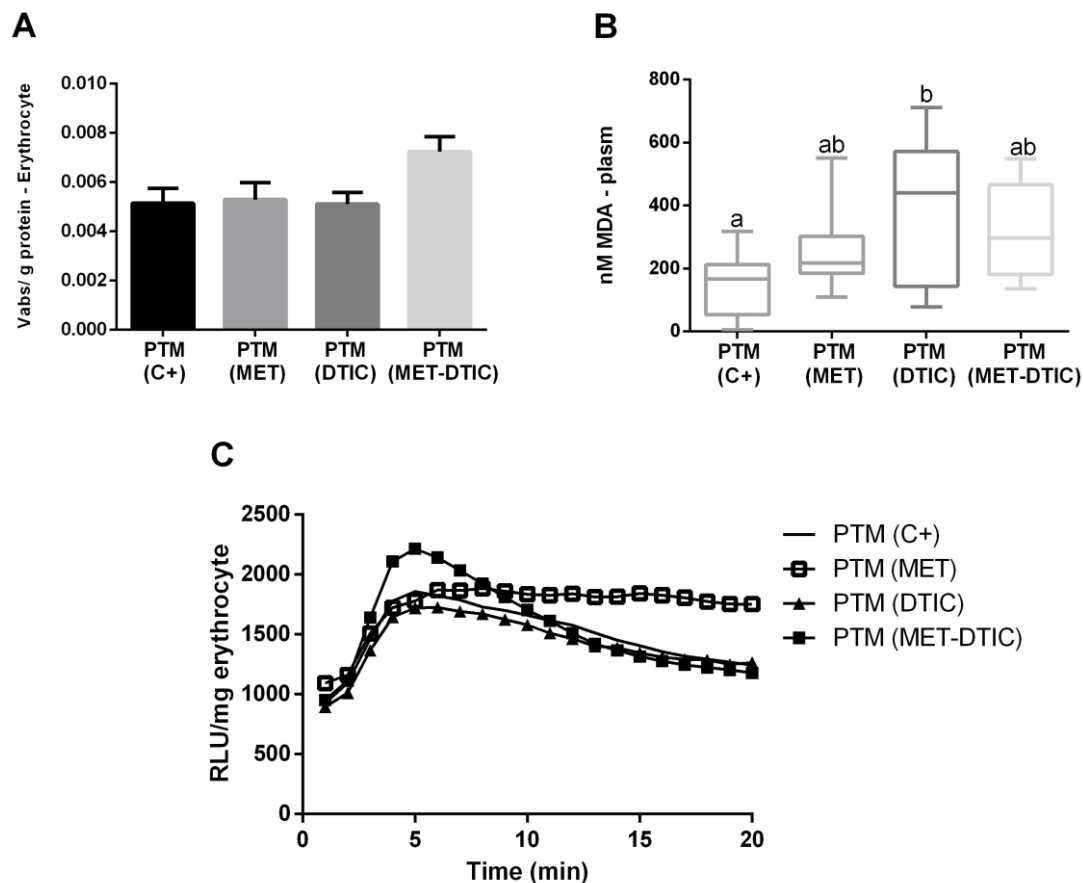
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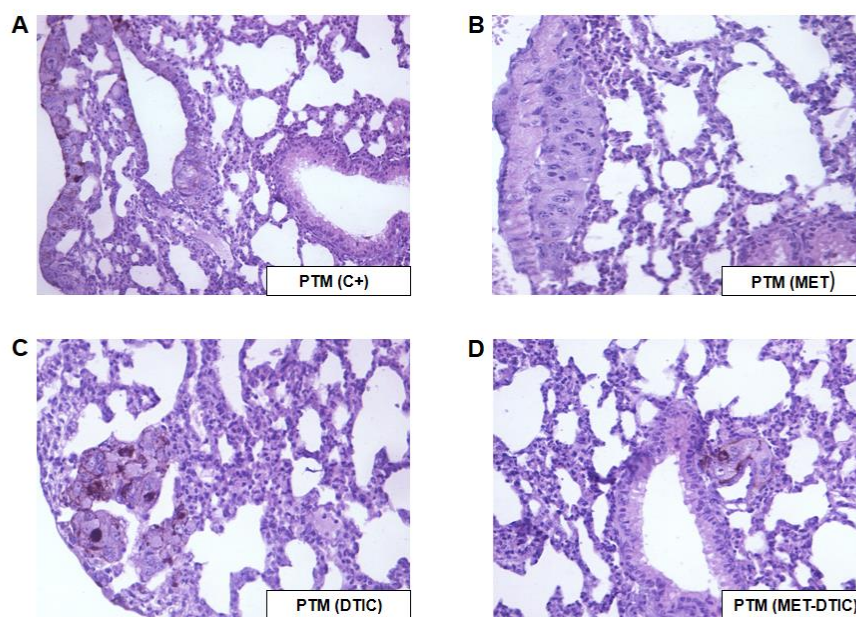
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16 **Figure 14. Systemic antioxidant parameters in animals treated with metformin during 5 days before inoculation with B16F10 cells and treatment.** A) Glutathione (GSH). B) GSSG. The statistical significance of the differences was analyzed by Kruskal-Wallis analysis of variance (ANOVA) followed by Dunn's post-hoc test and $p < 0.05$ was considered significant. PTM (C+):

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1 mice with untreated pulmonary metastasis. PTM (MET): mice with pulmonary metastasis treated
 2 with metformin. PTM (DTIC): mice with pulmonary metastasis treated with dacarbazine. PTM
 3 (MET-DTIC): mice with pulmonary metastasis treated with metformin more dacarbazine.
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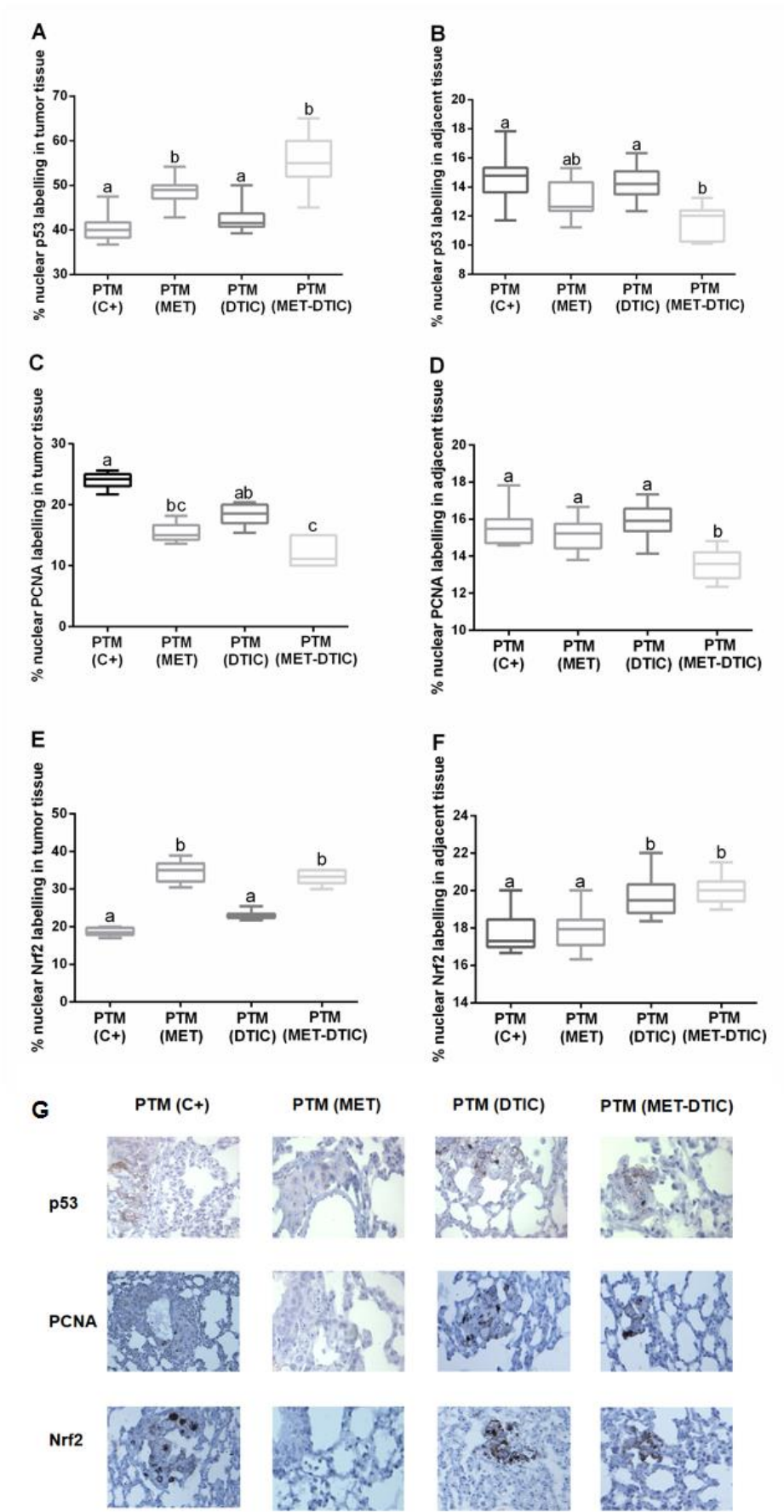


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 8 **Figure 15. Systemic oxidative stress parameters in animals treated with metformin during**
 9 **5 days before inoculation with B16F10 cells and treatment.** A) Catalase activity. B)
 10 Malondialdehyde levels. C) Chemiluminescence (CL). Catalase activity, results are expressed as
 11 mean \pm SEM. The statistical significance of the differences was analyzed by one-way analysis of
 12 variance (ANOVA) followed by Tukey's post-hoc test and $p < 0.05$ was considered significant.
 13 Malondialdehyde levels, statistical significance of the differences was analyzed by Kruskal-Wallis
 14 analysis of variance (ANOVA) followed by Dunn's post-hoc test and $p < 0.05$ was considered
 15 significant. Chemiluminescence. PTM (C+): mice with untreated pulmonary metastasis. PTM (MET):
 16 mice with pulmonary metastasis treated with metformin. PTM (DTIC): mice with pulmonary
 17 metastasis treated with dacarbazine. PTM (MET-DTIC): mice with pulmonary metastasis treated
 18 with metformin more dacarbazine.
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Figure 16. Metastatic lung melanoma nodules in animals pretreated with metformin for 5 days prior to inoculation with B16F10 cells. Pulmonary tissue histology. A) PTM (C+): mice with untreated pulmonary metastasis. B) PTM (MET): mice with pulmonary metastasis treated with metformin. C) PTM (DTIC): mice with pulmonary metastasis treated with dacarbazine. D) PTM (MET-DTIC): mice with pulmonary metastasis treated with metformin more dacarbazine. Staining of hematoxylin and eosin. Photos representative in 200x.



1 **Figure 17. Immunohistochemical analysis in the tumor environment and adjacent lung**
 2 **metastatic of melanoma in animals treated with metformin during 5 days before inoculation**
 3 **with B16F10 cells and treatment.** Percentage of nuclear labeling for p53 in tumor A) and
 4 adjacent region B), PCNA for tumor C) and adjacent region D), and Nrf2 for tumor E) and region
 5 adjacent to tumor F). G) Images representative of the nuclear marking p53, PCNA and Nrf2 in
 6 tumor and adjacent tissue. Statistical significance of the differences was analyzed by Kruskal-
 7 Wallis analysis of variance (ANOVA) followed by Dunn's post-hoc test and $p < 0.05$ was considered
 8 significant. PTM (C+): mice with untreated pulmonary metastasis. PTM (MET): mice with
 9 pulmonary metastasis treated with metformin. PTM (DTIC): mice with pulmonary metastasis
 10 treated with dacarbazine. PTM (MET-DTIC): mice with pulmonary metastasis treated with
 11 metformin more dacarbazine.
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	TISSUE ARCHITECTURE	IMPLANTATION POINTS	SIZE OF THE NODULE	MELANIN	INFLAMMATORY INFILTRATE
C-	-	-	-	-	-
C+	+	+++	++++	+	+
MET	+	+++	+++	-	+
DTIC	+	++	++	+	+
MET-DTIC	-	++	+	+	+

15 **Tabela 1. Histological analysis of lung tissue of mice with melanoma metastasis.**

16 Criteria analyzed: pulmonary architecture, implantation numbers of metastatic nodules, size of
 17 metastatic nodules, presence or absence of melanima and inflammatory infiltrate in the tumor
 18 area. C-: mice without pulmonary metastasis. C+: mice with untreated pulmonary metastasis.
 19 MET: mice with pulmonary metastasis treated with metformin. DTIC: mice with pulmonary
 20 metastasis treated with dacarbazine. MET-DTIC: mice with pulmonary metastasis treated with
 21 metformin more dacarbazine.
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	TISSUE ARCHITECTURE	IMPLANTATION POINTS	SIZE OF THE NODULE	MELANIN	INFLAMMATORY INFILTRATE
PTM (C+)	+	++	++	+	+
PTM (MET)	+	+	++	-	+
PTM (DTIC)	+	+	++	+	+
PTM (MET-DTIC)	-	+	+	+	+

23 **Tabela 2. Histological analysis of lung tissue of metformin pre-treated melanoma**
 24 **metastases and inoculated with B16F10 cells.** Criteria analyzed: pulmonary architecture,
 25 implantation numbers of metastatic nodules, size of metastatic nodules, presence or absence of
 26 melanima and inflammatory infiltrate in the tumor area. PTM (C+): mice with untreated pulmonary
 27 metastasis. PTM (MET): mice with pulmonary metastasis treated with metformin. PTM (DTIC):

1 mice with pulmonary metastasis treated with dacarbazine. PTM (MET-DTIC): mice with
2 pulmonary metastasis treated with metformin more dacarbazine.
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APÊNDICE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 128/2017

Londrina, 26 de Outubro de 2017.

Prezado (a) professor (a)

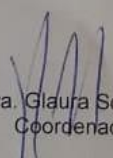
Certificamos que o projeto intitulado: "Terapia combinada de metformina e dacarbazina ou IL-2 no tratamento de melanoma metastático in vivo e in vitro." protocolo CEUA nº13281.2017.07 sob a responsabilidade de **Alessandra Lourenço Cecchini Armani**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **10/10/2017**

O objetivo do projeto é avaliar a atividade da Metformina associada a dacarbazina ou a IL-2 no tratamento de melanoma metastático melanogênico e amelanogênico na resposta celular ao estresse oxidativo in vivo e in vitro. Grau de invasividade=2

Vigência do Projeto	01/08/2017 a 31/07/2020
Espécie/linhagem	Camundongo heterogênico / C57BL6
Nº de animais	50
Peso/Idade	25 - 30g / 8 semanas
Sexo	Machos.
Origem	Universidade de São Paulo - USP
Amostras a serem coletadas	Sangue e pulmão.

Cumprir orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente.



Prof. Dra. Glaucia Scantamburlo Alves Fernandes
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)

Prof. (a) Dr (a). Alessandra Lourenço Cecchini ArmaniResponsável pelo projeto
Departamento de Patologia GeralC/C para a Chefia do Depto. de Patologia Geral
C/C para a Direção do Centro de Ciências Patológicas