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Aline Mânica

ATP COMO MODULADOR DO SISTEMA PURINÉRGICO E INFLAMAÇÃO E STATUS REDOX EM PACIENTES COM MELANOMA

Santa Maria, RS
2019

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de concentração em Enzimologia Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

Orientadora: Prof.ª Dr.ª Margarete Dulce Bagatini
Coorientadora: Prof.ª Dr.ª Vera Maria Melchiors Morsch

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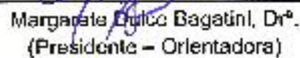

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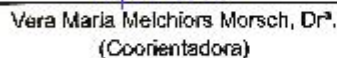

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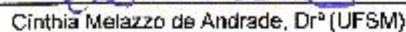

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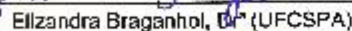

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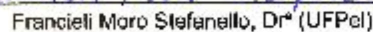

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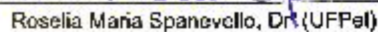

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2019

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RESUMO

ATP COMO MODULADOR DO SISTEMA PURINÉRGICO E INFLAMAÇÃO E STATUS REDOX EM PACIENTES COM MELANOMA

AUTORA: Aline Manica
ORIENTADORA: Margarete Dulce Bagatini
CO-ORIENTADORA: Vera Maria Melchiors Morsch

O melanoma é um tipo de câncer de pele, formado nos melanócitos, células produtoras do pigmento melanina. Os melanócitos da epiderme são os locais mais comuns de origem, porém também podem se desenvolver em melanócitos do olho, das superfícies mucosas, das vias respiratórias, nas superfícies geniturinárias e gastrointestinais. É uma das neoplasias conhecidas mais agressivas, com elevada capacidade de proliferação, estímulo da angiogênese, comprometimento dos sucessivos níveis da derme e geração de metástases, por via linfática ou hematogênica. A frequência de novos casos aumenta a cada ano e a incidência é crescente, especialmente na população jovem da região Sul do Brasil, tornando-se um importante problema de saúde pública. Sendo assim, tendo em vista de que a sinalização purinérgica, resposta inflamatória e o estresse oxidativo são importantes vias que influenciam a iniciação, a progressão e o desenvolvimento de melanomas e considerando que a região geográfica de estudo escolhida tem alta prevalência, este estudo teve por objetivo avaliar os efeitos do ATP no sistema purinérgico, marcadores inflamatórios e status redox *in vivo* e *in vitro* para elucidar as alterações do microambiente tumoral e entender a fisiopatologia do melanoma, ao passo que podem justificar a alta agressividade deste tipo de câncer. Este trabalho consistiu em coleta de sangue venoso de pacientes com melanoma antes e após procedimento cirúrgico de retirada do tumor e um grupo de pacientes controles da pesquisa e prodeceram-se análises *in vivo* e cultivo das (células polimorfonucleares do sangue periférico) PBMC destes pacientes. Foram realizadas análises enzimáticas, quantificação de nucleotídeos, dosagem de parâmetros inflamatórios e oxidativos e cultura PBMC. Foram utilizadas diferentes concentrações de ATP nas analises in vitro: 0,05 a 50 uM de ATP por 24 e 48 horas. Quanto aos aspectos éticos, o presente estudo foi aprovado pelo comitê de ética da Universidade Federal da Fronteira Sul, sob o parecer número 822.782. Os resultados encontrados demonstraram que os pacientes com melanoma após remoção cirúrgica apresentaram uma diminuição da hidrólise dos nucleotídeos. Em contraste, os pacientes pré cirurgia ou sem nenhum tratamento tiveram um aumento das hidrólises de ATP, ADP e AMP. Tais alterações estariam promovendo um aumento de ATP extracelular, o qual foi confirmado pela sua quantificação no soro dos pacientes. Além disso, os pacientes com melanoma após remoção cirurgica do tumor apresentaram um ambiente inflamatório descompensado, evidenciado pelo aumento das interleucinas IL-2, IL-4, IL-6, TNF, IFN-Y, IL-17A, IL-10 e aumento da atividade da adenosina desaminase (ADA). Em relação aos parâmetros de estresse oxidativo, foi observado uma diminuição dos danos oxidativos e um aumento das defesas antioxidantes após o processo cirúrgico, confirmando a efetividade do processo cirúrgico para lesões localizadas. Nossa hipótese principal gera em torno dos efeitos sinalizadores, imunossupressores e oxidante do ATP extracelular, tanto nas análises *in vivo*, quanto *in vitro*. Concluímos através desta tese que o ATP é uma importante mólecula envolvida no processo de melanomagênese e progressão tumoral podendo servir como um marcador progóstico. Dessa forma, através do controle dos seus níveis nas células, pode-se melhorar o prognóstico para pacientes acometidos por esse câncer de alta letalidade e agressividade.

Palavras chave: Melanoma. Sistema purinérgico. Estresse oxidativo. Inflamação.

ABSTRACT

EVALUATION OF THE EFFECTS OF ATP IN VIVO AND IN VITRO ON THE PURINERGIC SYSTEM, INFLAMMATORY PARAMETERS AND OXIDATIVE STRESS IN PATIENTS WITH MELANOMA

AUTHOR: Aline Manica
ADVISOR: Margarete Dulce Bagatini
CO-ADVISOR: Vera Maria Melchiors Morsch

Melanoma is a type of skin cancer, formed in melanocytes, cells that produce the pigment melanin. The melanocytes of the epidermis are the most common places of melanoma's origin; however, it can be also developed in melanocytes of the eye, mucous surfaces, respiratory tract, genitourinary and gastrointestinal tract. It is one of the most aggressive known neoplasms, with high proliferation capacity, stimulation of angiogenesis, involvement of successive levels of the dermis and generation of metastases, by lymphatic or hematogenous route. The frequency of new cases increases every year, especially in the young population of southern Brazil, becoming an important public health problem. Thus, considering that purinergic signaling, inflammatory response and oxidative stress are important pathways that influence the initiation, progression and development of melanomas, and that the geographical region of study chosen has high prevalence of this type of cancer, this study aimed to evaluate the effects of ATP on the purinergic system, inflammatory markers and oxidative stress in patients with melanoma to elucidate the changes in the tumor microenvironment and understand the pathophysiology of melanoma, whereas they may justify the high aggressiveness of this type of cancer. Our work consisted of collecting venous blood from patients with melanoma before and after surgical procedure of removing the tumor and a group of control patients of the study. All patients were selected and monitored by a medical team. Enzymatic analyses, nucleotide quantification, inflammatory and oxidative parameters and culture of polymorphonuclear cells of peripheral blood were performed. Regarding ethical aspects, this study was approved by the ethics committee of the Federal University of the Southern Frontier, under assessment number 822,782. The results found showed that patients with melanoma after surgical removal presented a reduction in the hydrolysis of nucleotides. In contrast, pre-surgery or untreated patients had an increase in ATP, ADP and AMP hydrolysis. Such alterations would be promoting an increase in extracellular ATP, which was confirmed by its quantification in the patient's serum. In addition, patients with melanoma after surgical removal of the tumor presented a decompensated inflammatory environment, evidenced by increased interleukins IL-2, IL-4, IL-6, TNF, IFN-Y, IL- 17A, IL-10 and increased ADA activity. Regarding the parameters of oxidative stress, it was observed a reduction in oxidative damage and an increase in antioxidant defenses after the surgical process, confirming the effectiveness of the surgical process for localized lesions. Our main hypothesis concerns the signaling, immunosuppressive and oxidizing effects of extracellular ATP, both in vivo and in vitro analyses. We conclude through this thesis that ATP is an important molecule involved in the process of melanomagenesis and tumor progression and may serve as a tool in clinical practice. Thus, by controlling their levels in the cells, one can improve the prognosis for patients affected by this cancer of high lethality and aggressiveness.

Keywords: Melanoma. Purinergic system. Oxidative stress. Inflammation.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

FIGURA 1 Estimativa para o ano de 2018/2019 das taxas brutas de incidência (cada 100.000 habitantes) estado e capitais e número de novos casos de câncer de pele melanoma e não melanoma, segundo sexo na região sul do Brasil 20
FIGURA 2 Representação espacial das taxas ajustadas de incidência por 100 mil homens e mulheres, estimadas para o ano de 2018, segundo Unidade da Federação....................21
FIGURA 3 Critérios ABCDE. Achados reconhecidamente suspeitos quando presentes em lesões melanocíticas que podem ser detectados nas fases iniciais de desenvolvimento do tumor....................27
FIGURA 4 Quebra do ATP em Adenosina 32
FIGURA 5 Substâncias antioxidantes versus fatores que favorecem o aumento da produção de EROS 38

QUADRO 1 Fototipos de pele, segundo classificação de Fitzpatrick 25
QUADRO 2 Nomenclatura e preferência por substrato dos membros da família NTPDase....................33

ARTIGO 1

FIGURA 1 E-NTPDase and E-5′-Nucleotidase activities 44
FIGURA 2 Quantitative ATP determination....................44
FIGURA 3 Expression of E-NTPDase1/CD39 and E-5′Nucleotidase/CD73 protein in platelets and lymphocytes of control group (CT) and melanoma group (CM)....................45
FIGURA 4 A, Interleukin-2 (IL-2), (B) Interleukin-4 (IL-4), (C) Interleukin-6 (IL-6), (D) Tumor Necrosis Factor (TNF), (E) Interferon-γ (IFN-γ) (F) Interleukin-17A (IL-17A), protein levels....................45
FIGURA 5 ADA activity and Interleukin-10 (IL-10) protein levels....................46

ARTIGO 2

FIGURA 1 ATP, ADP and AMP hydrolysis in platelets and lymphocytes in controls (CT) and melanoma patients (CM)....................52
FIGURA 2 Quantitative ATP determination in control (CT) and melanoma patients (CM) 52
FIGURA 3 ADA (Adenosine desaminase – hydrolyzing adenosine) in platelets and in lymphocytes of control (CT) and melanoma (CM) groups....53
FIGURA 4 Effects on ATP, ADP and AMP hydrolysis after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for 24 hours, in (PBMCs) of control (CT) and

melanoma patients…………………………………………………..53
FIGURA 5 Effects on ATP, ADP and AMP hydrolysis after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 µM for 48 hours, in PBMCs of control (CT) and melanoma patients..54
FIGURA 6 ATP quantification in control (CT) and melanoma (CM) PBMCs cells after treatment with Adenosine Triphosphate (ATP) with 0,05, 0,5, 5, 10 and 50 µM) for 24 and 48 hours 54
FIGURA 7 Effects on ADA (Adenosine desaminase – hydrolyzing adenosine) activity after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 µM for (A) 24 hours and (B) 48 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients (CM)..55
FIGURA 8 Cell viability determined after 24 hours (A) and 48 hours (B) by MTT assay in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients (CM) 55

MANUSCRITO

FIGURA 1 MDA production by TBARS assay. MDA production in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) 72
FIGURA 2 Myeloperoxidase activity in vivo and in vitro. Myeloperoxidase activity in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) 72
FIGURA 3 Non-protein thiols (NPSH) quantitation in vivo and in vitro. NPSH in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) 73
FIGURA 4 Protein thiols (PSH) quantitation in vivo and in vitro. PSH in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) 73
FIGURA 5 GSH determination. Quantitation of GSH in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) 74
FIGURA 6 Ascorbic acid assay in serum and PBMC cells. Quantitation in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) 74

LISTA DE TABELAS

ARTIGO 1

TABELA 1 Clinical characteristic 42

ARTIGO 2

TABELA 1 Clinical characteristics of studied CT and CM patients.................51

MANUSCRITO

TABELA 1 Clinical characteristic of studies CT and CM patients before and after treatment. 71

LISTA DE ABREVIATURAS E SIGLAS

a.C.	Antes de Cristo
ADA	Adenosina deaminase
ADP	Difosfato de adenosina
AJCC	*American Joint Committee on Cancer*
AMP	Monofostato de adenosina
5’AMPc	5’ Monofosfato de adenina ciclíco
ATP	Trisfosfato de adenosina
BRAF	Proto-oncogene B-Raf
CCL5	Chemokine (C-C motif) ligand 5
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
CXCL13	Chemokine (C-X-C motif) ligand 13
DNA	Ácido desoxirribonucleico
E-NPP	Ectonucleotídeo pirofosfatase/fosfodiesterases
E-NTPDase	Ecto-nucleosídeo trifosfato difosfohidrolase
EROS	Espécies reativas de oxigênio
FAL	Fosfatase alcalina
FDA	*Food and Drug Administration*
GSH	Glutationa reduzida
H_2O_2	Peróxido de hidrogênio
HDL	Lipoproteina de alta densidade
HO^-	Radical hidroxila
IFN-α	Interferon alfa
IFN-γ	Interferon gama
IL	Interleucina
INCA	Instituto Nacional do Câncer
LDL	Lipoproteina de baixa densidade
LS	Linfonodo Sentinela
MES	Melanoma extensivo superficial
MCP-1	Monocyte chemoattractant protein 1
MLA	Melanoma lentiginoso acral
MLM	Melanoma lentigo maligno
MN	Melanoma nodular
mm	Milimetros
mm^3	Milimetros cúbicos
NAD^+	Nicotinamida adenina dinucleotídeo oxidado
O_2^{-1}	Anion superóxido
OMS	Organização Mundial da Saúde
P_i	Fosfato inorgânico
PP_i	Difosfato inorgânico
RUV	Radiação ultravioleta

TERT	Telomerase reverse transcriptase
UDP	Uridina difosfato
UICC	União internacional do controle de cancer
UTP	Uridina-5'-trifosfato
UV	Ultravioleta
UVA	Ultravioleta A
UVB	Ultravioleta B
UVC	Ultravioleta C

LISTA DE APÊNDICES

APENDICE 1 Termo de consentimento livre e esclarecido..........................81

APENDICE 2 Fichas de coleta para pacientes com melanoma e controles..83

LISTA DE ANEXOS

ANEXO Parecer consubstanciado do CEP...84

SUMÁRIO

1. INTRODUÇÃO **18**
2 OBJETIVOS **20**
2.1 OBJETIVO GERAL 20
2.2 OBJETIVOS ESPECÍFICOS 20
3 REVISÃO BIBLIOGRÁFICA **21**
3.1 EPIDEMIOLOGIA DO MELANOMA 21
3.2 HISTÓRICO DO MELANOMA 23
3.3 FISIOPATOLOGIA DO MELANOMA 24
3.4 CLASSIFICAÇÃO CLÍNICA 28
3.5 SISTEMA PURINÉRGICO 31
3.6 ECTOENZIMAS 33
3.7 RECEPTORES PURINÉRGICOS 35
4 INFLAMAÇÃO X ESTRESSE OXIDATIVO X MELANOMA **37**
5 ARTIGOS E MANUSCRITO **41**
5.1 ARTIGO 1: 41
HIGH LEVELS OF EXTRACELLULAR ATP LEAD TO CHRONIC INFLAMMATORY RESPONSE IN MELANOMA PATIENTS. 41
5.2 ARTIGO 2: 50
THE SIGNALING EFFECTS OF ATP ON MELANOMA-LIKE SKIN CÂNCER 50
5.3 MANUSCRITO: 59
MELANOMA AND OXIDATIVE STRESS: COULD ATP TO BE A KEY MOLECULE? 59
6 CONCLUSÃO **81**
REFERÊNCIAS **82**
APÊNDICE **91**
APÊNDICE 1: TERMO DE CONSENTIMENTO 91
APÊNDICE 2: FICHAS DE COLETA PARA PACIENTES COM MELANOMA E CONTROLES 93
ANEXO: APROVAÇÃO COMITE DE ÉTICA **94**

APRESENTAÇÃO

Esta tese está organizada da seguinte forma: primeiramente são apresentados a introdução, os objetivos e a revisão bibliográfica. A seguir, os resultados e materiais e métodos são apresentados na forma de dois artigos publicados e um manuscrito os quais foram apresentados de acordo com as normas das revistas os quais estão publicados/submetido.

Os itens discussão e conclusão encontrados ao final desta tese, contém interpretação e comentários gerais referente aos artigos e ao manuscrito. As referências bibliográficas apresentadas ao final referem-se aos itens introdução, revisão bibliográfica e discussão.

1. INTRODUÇÃO

Devido à alta incidência e mortalidade, o melanoma é considerado o câncer de pele de maior importância médica (FERREIRA; NASCIMENTO, 2016). Embora as taxas globais de sobrevivência tenham melhorado, as estimativas não são promissoras para aqueles que são diagnosticados nos estágios mais avançados da doença. A taxa de sobrevivência de 5 anos para indivíduos com melanoma detectado nos estágios iniciais é de cerca de 98%, diminuindo para 63,8 e 15% para um tumor diagnosticado em estádios regionais e distantes. Sendo assim, quase um sexto dos indivíduos diagnosticados com melanoma têm uma baixa possibilidade de sobrevivência (PADDOCK et al., 2016).

A frequência de novos casos aumenta a cada ano e a incidência é crescente, especialmente na população da região Sul do Brasil, tornando-se um importante problema de saúde pública (CALLAHAN; FLAHERTY; POSTOW, 2016; OLIVEIRA et al., 2016). Fatores de risco constitucionais e ambientais estão associados ao aparecimento dos melanomas, tais como: cor da pele, olhos e cabelos claros ou muito claros, sensibilidade ao sol, exposição a radiação solar desde a infância, dentre outros. Entretanto, pesquisas recentes apontam que elementos ainda não estudados possam estar contribuindo para o aumento do número de casos e entre esses fatores, aponta-se o envolvimento do sistema purinérgico (CALLAHAN; FLAHERTY; POSTOW, 2016; DI VIRGILIO; ADINOLFI, 2017).

Estudos usando trifosfato de adenosina (ATP) e melanomas ainda são restritos e pouco explorados, mesmo sabendo que a sinalização purinérgica é uma das vias que influenciam a iniciação, a progressão e o desenvolvimento de melanomas. Mas, além desse sistema, existem outros fatores que podem estar implicados no desenvolvimento e na progressão do melanoma, tais como a intensa resposta inflamatória e o estresse oxidativo (CAZES; RONAI, 2016).

Sabe-se que o processo inflamatório desempenha um papel crucial na melanomagênese, pois muitas das causas e fatores de risco para o câncer estão associadas com algum tipo de inflamação crônica (GRIVENNIKOV; GRETEN; KARIN, 2011). A inflamação é um dos mais importantes sistemas de adaptação do nosso organismo aos desafios contínuos que provêm do ambiente externo e interno (RITEAU et al., 2012). Vários tipos de inflamação -

diferindo por causa, mecanismo e intensidade - podem promover o desenvolvimento e a progressão de câncer. Pode-se citar a autoimunidade e a desregulação do sistema imune como um tipo de inflamação que leva ao desenvolvimento de melanoma (GRIVENNIKOV; GRETEN; KARIN, 2011).

Além disso, as citocinas inflamatórias favorecem o crescimento e a progressão de melanomas. Um aumento na interleucina (IL) - 2 e IL-6 podem indicar um prognóstico desfavorável em pacientes com estágio IV, bem como níveis elevados de IL-4, fator de necrose tumoral alfa (TNF-α) e interferon gama (IFN-ɣ), que estimulam a proliferação, migração e angiogênese das células endoteliais. Sabe-se ainda que no microambiente de tumores primários de melanoma são encontradas altas concentrações de IL-1α, IL-1β, IL-6, IL-8, TNF-α, fator de necrose tumoral alfa beta (TGF-β) (BARRIERS, 2016; GRIVENNIKOV; GRETEN; KARIN, 2011).

Já o estresse oxidativo tem ação em todas as etapas do desenvolvimento do melanoma, bem como na modulação das vias intracelulares relacionadas à proliferação celular e a morte (SANCHES et al., 2017). Os altos níveis intracelulares de espécies reativas de oxigênio (EROs) podem promover dano oxidativo associado à morte celular através da oxidação de proteínas, DNA e lipídios (PEIRIS-PAGÈS et al., 2015). O estresse oxidativo pode afetar o equilíbrio homeostático de melanócitos, devido ao estado pró-oxidante gerado durante a síntese de melanina, ameaçando sua sobrevivência ou induzindo transformação maligna (DENAT et al., 2014).

Estudos tem demonstrado que mutações em vários genes associados ao melanoma resultam ou pioram com o desequilíbrio do estresse oxidativo (JENKINS et al., 2011; LANDI, 2006), além de que, um nível elevado de estresse oxidativo em melanomas aumenta sua agressividade (HAMBRIGHT et al., 2015). Portanto, considerando que a região geográfica de estudo escolhida tem alta prevalência e que quando diagnosticado em estágio inicial, o melanoma tem bom prognóstico com altas possibilidades de cura, o estudo do sistema purinérgico, marcadores inflamatórios e estresse oxidativo em pacientes com melanoma é de fundamental relevância para melhor compreensão dessa doença e assim buscarmos por terapias que reduzam a morbimortalidade.

2 OBJETIVOS

➢ 2.1 Objetivo geral

Avaliar os efeitos do ATP no sistema purinérgico, marcadores inflamatórios e estresse oxidativo em pacientes com melanoma.

➢ 2.2 Objetivos específicos

In vivo

- Analisar a atividade e a expressão de enzimas do sistema purinérgico em linfócitos e plaquetas de indivíduos com melanoma (antes e após tratamento cirúrgico) e controles;
- Avaliar citocinas pró e anti-inflamatórias em sangue total de indivíduos diagnosticados com melanoma cutâneo e controles;
- Verificar parâmetros de estresse oxidativo e atividade de enzimas antioxidantes em soro e plasma de indivíduos diagnosticados com melanoma cutâneo e controles;

In vitro

- Verificar a viabilidade e morte celular da cultura de PBMC dos indivíduos com melanoma através de microscopia e teste MTT;
- Avaliar o efeito no sistema purinérgico da adição de ATP em diferentes concentrações na cultura de leucócitos dos indivíduos com melanoma e controles;
- Determinar o estresse oxidativo na cultura dos leucócitos após adição de diferentes concentrações de ATP.

3 REVISÃO BIBLIOGRÁFICA

3.1 EPIDEMIOLOGIA DO MELANOMA

Para o Brasil, estimam-se 85.170 casos novos de câncer de pele entre homens e 80.410 nas mulheres para cada ano do biênio 2018-2019. Esses valores correspondem a um risco estimado de 82,53 casos novos a cada 100 mil homens e 75,84 para cada 100 mil mulheres. O câncer de pele é o tipo de câncer mais incidente em ambos os sexos na população brasileira (INCA, 2018).

Considerando-se esse alto número de casos de câncer de pele na região Sul do Brasil (Figura 1), torna-se de fundamental importância o seu estudo, principalmente o melanoma, por ser a forma mais agressiva e letal dentre esses tipos de canceres.

Figura 1: Estimativa para o ano de 2018/2019 das taxas brutas de incidência (cada 100.000 habitantes) estado e capitais e número de novos casos de câncer de pele melanoma e não melanoma, segundo sexo na região sul do Brasil.

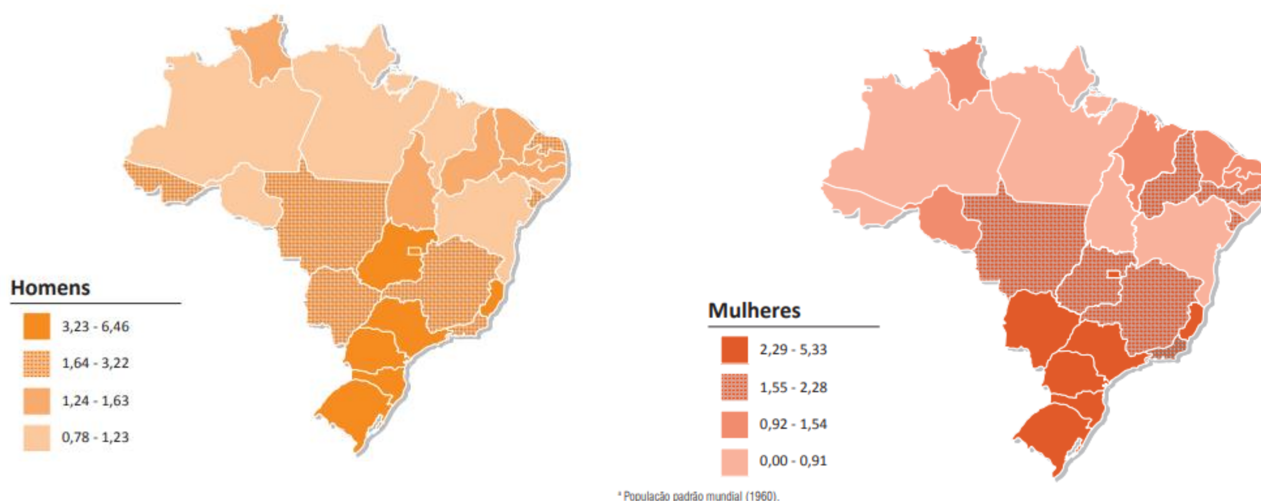
Localização Primária da Neoplasia Maligna	Estimativa dos Casos Novos											
	Homens						Mulheres					
	Estados			Capitais			Estados			Capitais		
	Casos	Taxa Bruta	Taxa Ajust.	Casos	Taxa Bruta	Taxa Ajust.	Casos	Taxa Bruta	Taxa Ajust.	Casos	Taxa Bruta	Taxa Ajust.
Próstata	14.290	96,85	82,05	1.840	100,60	79,97	-	-	-	-	-	-
Mama Feminina	-	-	-	-	-	-	11.030	73,07	59,13	1.940	94,77	66,69
Colo do Útero	-	-	-	-	-	-	2.130	14,07	11,42	300	14,76	10,38
Traqueia, Brônquio e Pulmão	5.350	36,27	31,78	640	34,92	38,93	3.110	20,59	14,91	530	25,93	16,86
Cólon e Reto	3.270	22,17	20,03	590	32,35	25,16	3.460	22,92	18,78	700	33,86	20,84
Estômago	2.520	17,12	14,98	310	16,81	10,95	1.350	8,95	5,96	210	10,28	5,07
Cavidade Oral	2.280	15,40	12,92	290	15,88	14,42	540	3,59	2,51	100	4,48	2,79
Laringe	1.570	10,57	9,75	160	9,11	8,94	190	1,30	0,96	30	1,06	0,78
Bexiga	1.370	9,23	8,43	210	11,52	10,13	670	4,50	3,52	130	5,93	4,45
Esôfago	2.520	17,10	13,99	210	11,68	7,69	740	4,94	3,31	80	3,53	1,02
Ovário	-	-	-	-	-	-	1.080	7,12	5,62	200	9,53	6,54
Linfoma de Hodgkin	360	2,47	2,13	50	2,78	2,54	200	1,29	1,16	40	1,65	1,30
Linfoma não Hodgkin	1.080	7,36	6,31	170	9,32	8,72	890	5,96	4,19	170	8,68	5,66
Glândula Tireoide	380	2,55	2,15	70	3,57	2,47	740	4,91	4,31	130	6,30	5,17
Sistema Nervoso Central	1.500	10,17	8,85	170	9,79	8,97	1.290	8,52	6,73	150	7,78	5,99
Leucemias	1.280	8,67	8,30	160	9,12	9,68	980	6,50	5,53	130	6,22	5,00
Corpo do Útero	-	-	-	-	-	-	1.070	7,17	5,55	240	11,84	8,07
Pele Melanoma	1.040	7,02	5,71	160	8,44	7,65	960	6,35	4,74	140	6,74	4,98
Outras Localizações	10.140	68,70	63,27	1.300	70,45	53,93	8.030	53,21	40,28	1.130	54,95	35,09
Todas as Neoplasias, exceto Pele não Melanoma	48.950	331,88	373,62	6.330	344,78	361,69	38.460	254,80	270,91	6.350	309,71	261,91
Pele não Melanoma	23.610	160,08	-	1.960	106,60	-	14.710	97,46	-	1.560	76,37	-
Todas as Neoplasias	**72.560**	**491,95**	**-**	**8.290**	**451,54**	**-**	**53.170**	**352,25**	**-**	**7.910**	**385,79**	

Fonte: Adaptado de (INCA, 2018).

Embora a incidência de melanoma represente menos de 10% dos cânceres de pele, ele é considerado o responsável pela maioria das mortes relacionadas com câncer de pele (PASTUSHENKO et al., 2014). Sua incidência mundial aumentou significativamente nos últimos 30 anos (AMERICAN CANCER SOCIETY, 2019). Nos Estados Unidos, por exemplo, o melanoma é a causa de 65% de todas as mortes atribuíveis a malignidades cutâneas (PASTUSHENKO et al., 2014; RASTRELLI et al., 2014; WICK, 2016).

No Brasil os maiores índices de melanoma se apresentam na região Sul, como demonstrado na Figura 2 (INCA, 2018; MORENO et al., 2012).

Figura 2: Representação espacial das taxas ajustadas de incidência por 100 mil homens e mulheres, estimadas para o ano de 2018, segundo Unidade da Federação.



Fonte: Adaptado de (INCA, 2018).

Como o processo de colonização não foi uniforme, a distribuição dos diversos grupos étnicos variou muito pelo país (IBGE, 2018). Na região Sul acabaram concentrando-se uma maioria de caucasianos, expostos a radiação solar desde a infância (MORENO; CIOTTA, 2017). Os dados de incidência e prevalência de melanoma nesta região do país se assemelha muito com o que é registrado na Austrália, país onde são registrados os maiores índices de prevalência e incidência de melanoma do planeta (MORENO et al., 2012, 2018).

Além da composição da população, pode-se justificar tais índices pela exposição à radiação ultravioleta (RUV). Por sua grande extensão territorial, o Brasil possui diferentes faixas de clima zonal que pode estar associada a exposições distintas do ponto de vista ocupacional, recreativa e relacionado ao índice ultravioleta (WORLD HEALTH STATISTICS, 2014).

A faixa territorial brasileira que compreende os estados de Santa Catarina e do Rio Grande do Sul está entre os paralelos 26º e 31º (MENDES, 2014). Entre esses mesmos paralelos, encontra-se a Austrália, país em que o melanoma é o terceiro câncer mais comum em homens e mulheres, causando 75% das mortes por câncer de pele (EGGERMONT; SPATZ; ROBERT, 2014; MELANOMA INSTITUTE AUSTRALIA, 2019; MORENO et al., 2012).

Conforme dados apresentados na Figura 2, o INCA estima para 2018/2019, que sejam diagnosticados 6.260 novos casos de câncer de pele melanoma (2.920 casos novos em homens e 3.340 casos novos em mulheres) no Brasil. Como a notificação compulsória dos casos de câncer ainda não é uma realidade, ocorrendo em apenas 30% do país, a existência de um número considerável de sub registros é possível e o número de casos pode ser ainda maior (INCA, 2018).

3.2 HISTÓRICO DO MELANOMA

Nas escrituras de Hipócrates encontra--se a primeira descrição que faz referência ao melanoma, durante o período 460 a 375 a.C. Tendo em vista sua raridade e a elevada incidência de outras doenças, principalmente as infecto-parasitárias, o melanoma só foi reconhecido após o início do século XIX, quando alguns pesquisadores como Eiselt começaram a estudar o que designavam na época como “tumores negros fatais com metástases e fluido negro no corpo” (1861). Robert Carswell o caracterizou e o denominou como “lesões malignas pigmentadas da pele” (WAINSTEIN; BELFORT, 2004).

Nos Estados Unidos, a primeira descrição de um caso de melanoma ocorreu em 1837, por Isaac Parrish. As primeiras cirurgias para a ressecção do melanoma foram realizadas na França em casos de melanoma na mão, pés e genitália, entre 1829 e 1842, e na Inglaterra por Pemberton em 1858 (WAINSTEIN; BELFORT, 2004; WICK, 2016).

Em 1840, Samuel Cooper definiu que a melanose - melanoma disseminado, deveria ser encarada como uma doença intratável. Já no final do século XIX, Herbert Snow (NEUHAUS; CLARK; THOMAS, 2004) iniciou os esvaziamentos linfonodais. A partir da metade do século XX, importantes estudos que correlacionaram as dimensões do tumor primário, com o curso clínico foram realizados e alguns são utilizados, pela sua importância e significância, até hoje: Lehman (LEHMAN; CROSS; RICHEY, 1966), Clark (CLARK et al., 1969) e Breslow (BRESLOW, 1970).

Breslow em 1970 determinou os padrões de espessura do tumor e logo foi utilizado como um recurso de prognóstico para o melanoma (MERVIC, 2012). Em seu estudo, ele definiu que: pacientes com tumores mais finos do que 0,76 milímetros (mm) seriam designados de pacientes de baixo risco, pois raramente vão gerar metástase; pacientes de risco intermediário seriam aqueles nos quais o tumor tivesse a espessura de 0,76 até 1,5 mm; pacientes de alto risco deveriam apresentar uma espessura de 1,5 até 4,0 mm. Para pacientes com tumores mais espessos que 4,0 mm o risco de gerar metástase seria altíssimo (MERVIC, 2012).

Já o estudo de Clark (CLARK et al., 1969), descreve as alterações histológicas que ocorrem na progressão do melanócito normal ao melanoma:

- Inicia-se pelo desenvolvimento de um nevo benigno (composto por melanócitos névicos);
- Segue com o desenvolvimento de atipia citológica, que pode surgir a partir de um nevo pré-existente ou de um novo;
- Já com a transformação maligna presente, observa-se a fase de crescimento radial, quando a lesão passa a proliferar na epiderme e crescimento vertical, quando avança pela derme (LEONARDI et al., 2018).

3.3 FISIOPATOLOGIA DO MELANOMA

A pele é o maior órgão do corpo, considerada uma barreira contra lesões e RUV, protege contra o atrito, a perda de água e evita entrada de agentes tóxicos, dentre outras funções. É composta pela epiderme (epitélio estratificado pavimentoso queratinizado) e pela derme (tecido conjuntivo). Subjacente,

unindo-a aos órgãos, há a hipoderme (ou fáscia subcutânea) uma mistura de tecido conjuntivo frouxo e adiposo (JUNQUEIRA; CARNEIRO; ABRAHAMSOHN, 2017).

A epiderme contém dois tipos de células, as células de Langerhans e os melanócitos. As células de Langerhans são células dendríticas processadoras de antígenos e os melanócitos são as células produtoras do pigmento melanina. O pigmento melanina é sintetizado em organelas especiais dos melanócitos, chamadas de melanossomas, em uma cascata enzimática envolvendo principalmente a tiroquinase e suas proteínas (BANDARCHI et al., 2010; JUNQUEIRA; CARNEIRO; ABRAHAMSOHN, 2017).

Os melanócitos são embriologicamente derivados a partir de uma população germinativa de melanoblastos originários de células da crista neural, pouco tempo após o fechamento do tubo neural. Na maioria das espécies, os melanoblastos começam o processo de melanização imediatamente antes ou logo depois de alcançar seu destino (JUNQUEIRA; CARNEIRO; ABRAHAMSOHN, 2017). Dois tipos de pigmentos são produzidos, o marrom/preto que exibe características fotoprotetoras, e o laranja/amarelo que tem fraca propriedade fotoprotetora. A melanina fornece uma eficiente proteção contra os efeitos prejudiciais da radiação ultravioleta, por reduzir o dano causado ao DNA e a instabilidade genômica causados pela radiação (BANDARCHI et al., 2010; CALLAHAN; FLAHERTY; POSTOW, 2016).

O melanoma forma-se então a partir da transformação maligna dos melanócitos, envolvendo fatores ambientais e genéticos. Os melanócitos da epiderme são os locais mais comuns de origem, porém tumores primários também podem ser encontrados revestindo a camada coroidal do olho ou as superfícies mucosas das vias respiratórias, superfícies geniturinárias e gastrointestinais (VALKO-ROKYTOVSKÁ et al., 2018). O melanoma é uma das neoplasias cutâneas mais agressivas, com elevada capacidade de proliferação, estímulo da angiogênese, comprometimento dos sucessivos níveis da derme e geração de metástases, por via linfática ou hematogênica (WICK, 2016).

As transformações genéticas malignas do melanoma podem ser advindas de mutações chamadas de condutoras ou *driver mutations*: mutação V600E no gene v-raf murine sarcoma viral oncogene homolog B1 (BRAF) e mutação na região promotora da enzima telomerase (TERT), responsável pela

evolução de lesão intermediária benigna para melanoma *in situ*. Mutações no gene neuroblastoma RAS viral oncogene homolog (NRAS), Cyclin dependent kinase inhibitor 2A (CDKN2A), Cyclin-dependent kinase 4 (CDK4), BRAF e Receptor de melanocortina 1 (MC1R) (CALLAHAN; FLAHERTY; POSTOW, 2016; SHAIN et al., 2015) também podem desencadear a evolução de lesões benignas para malignas (CHENG et al., 2018).

Além das alterações genéticas, existem alguns fatores de risco associados ao desenvolvimento do melanoma: até 65% dos melanomas malignos estão relacionadas à exposição intermitente a RUV; número elevado de nevos melanocíticos na pele (>100) – nevos são pequenos tumores cutâneos, geralmente hiperpigmentados, cor da pele/olhos/cabelo, de acordo com classificação de Fitzpatrick (Quadro 1) e histórico familiar de câncer e de queimaduras solares, principalmente na infância (BEHRENS et al., 2018).

Essas características de cor de pele, olhos e cabelos, estão diretamente relacionadas com a capacidade de proteger contra os raios UV, muito conhecidos por provocar alterações no DNA e aumentar o risco de câncer (BANDARCHI et al., 2010; BERTOLOTTO, 2013; MARTINS-COSTA et al., 2013). Fototipos I e II de Fitzpatrick, ou seja, indivíduos que apresentam pele, cabelos e olhos claros e se queimam facilmente ao invés de se bronzear, tem alta probabilidade de desenvolver melanoma (Quadro 1). O uso de camas de bronzeamento, também é um fator de risco importante para o seu desenvolvimento (CALLAHAN; FLAHERTY; POSTOW, 2016; SHAIN et al., 2015).

Quadro 1: Fototipos de pele, segundo classificação de Fitzpatrick

	Fototipo I	**Fototipo II**	**Fototipo III**	**Fototipo IV**	**Fototipo V**	**Fototipo VI**
Fototipo (pele)	Branca	Branca	Morena Clara	Morena Moderada	Morena escura	Negra
Sensibilidade ao sol	Muito sensível	Sensível	Normal	Normal	Pouco sensível	Insensível
Características	Queima com facilidade e nunca bronzeia	Queima com facilidade e bronzeia pouco	Queima e bronzeia moderada-mente	Queima pouco e bronzeia com facilidade	Queima raramente e bronzeia bastante	Nunca queima. Pele totalmente pigmentada

Fonte: Adaptado de FITZPATRICK, MOSHER (2000).

Como citado anteriormente, a RUV é considerada um dos principiais fatores de risco para o desenvolvimento de melanoma. O sol emite 3 tipos de radiações, a ultravioleta A (UVA), ultravioleta B (UVB) e ultravioleta C (UVC). Todos os tipos estão relacionados a lesão do DNA, carcinogênese, inflamação e propriedades imunossupressoras, e contribuem para o desenvolvimento do melanoma, porém com maior importância para a UVB (VOLKOVOVA et al., 2012). É possível que EROS geradas pela UVA também contribuam para a transformação do melanócito, pois a UVA gera altos níveis de EROS quando ativa fotossensibilizadores endógenos (porfirinas, quinonas, riboflavina), que promovem a oxidação de guaninas (PFEIFER; BESARATINIA, 2012).

A exposição à RUV não se faz somente pelas fontes naturais, também ocorre pelas camas de bronzeamento artificial. Essa exposição é considerada carcinogênica do Grupo 1 pela Agência Internacional de Pesquisa em Câncer e sua utilização foi banida no Brasil em 2008 (ANVISA, 2012). Estudos demonstram aumento de duas vezes no risco de desenvolver melanoma de início precoce (abaixo de 40 anos) em indivíduos com elevada exposição (mais de 10 sessões) (CUST et al., 2011; NIELSEN et al., 2012).

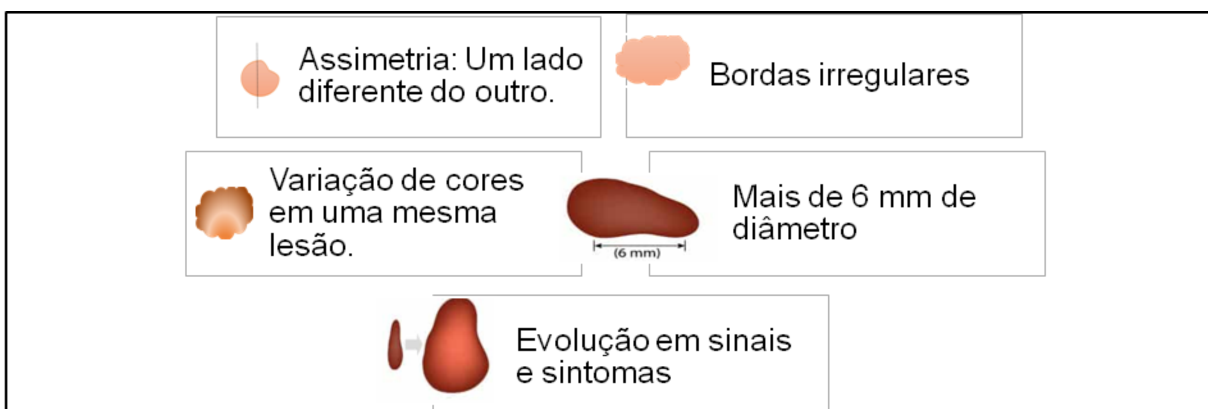
O achado clínico mais importante no diagnóstico do melanoma é o de uma lesão pigmentada que muda visivelmente durante um período de meses a anos (D'ISCHIA et al., 2015; SAÚDE, 2013). Como regra geral, qualquer lesão que mude de cor, forma, dimensão ou que apresente elevação deve ser investigada. Melanomas típicos geralmente se apresentam assimétricos, com irregularidades da borda, variação de cor e diâmetro superior a 6 mm^3. As lesões mais avançadas podem apresentar sangramento, prurido, dor e ulceração (CALLAHAN; FLAHERTY; POSTOW, 2016).

Apesar de apresentarem características comuns, muitas exceções e variações podem ocorrer e alguns critérios podem ser utilizados para determinar maior probabilidade da lesão cutânea de fato tratar-se de melanoma. Sendo assim, foram criados os critérios ABCDE, desenvolvidos a fim de identificar e tratar lesões iniciais e potencialmente curáveis (Figura 3) (VALKO-ROKYTOVSKÁ et al., 2018):

- A – Assimetria;
- B – Bordas (irregulares);

- C – Cor (variação ou vários tons na mesma lesão);
- D – Diâmetro (mais de 6 mm de diâmetro);
- E – Evolução.

Figura 3: Critérios ABCDE. Achados reconhecidamente suspeitos quando presentes em lesões melanocíticas que podem ser detectados nas fases iniciais de desenvolvimento do tumor



Fonte: (Autor, 2019).

3.4 CLASSIFICAÇÃO CLÍNICA

O melanoma pode ser subdividido, de acordo com suas características clínicas, padrões microscópicos de crescimento, localização anatômica do tumor primário e idade do paciente (RASTRELLI et al., 2014).

Melanoma Extensivo Superficial (MES): é o subtipo mais frequente caracterizado por crescimento radial. Fatores importantes da sua etiologia variam de exposição solar aguda na infância e exposição intermitente na vida adulta. Comumente observado nas pernas em mulheres e no tronco em homens (BATTISTI et al., 2009; EGGERMONT; SPATZ; ROBERT, 2014; SAÚDE, 2013).

Melanoma Lentigo Maligno (MLM): é uma forma de melanoma que ocorre na pele de idosos em regiões cronicamente expostas ao sol, principalmente na face, cuja lesão está associada à atrofia epidérmica e elastose solar (CALLAHAN; FLAHERTY; POSTOW, 2016; SAÚDE, 2013).

Melanoma Lentiginoso Acral (MLA): é um subtipo de melanoma cutâneo com características histopatológicas bem distintas e acomete as palmas das mãos, plantas dos pés e regiões subungueais. Sua incidência é de até 80% na

população negra, e 77% na população asiática (CALLAHAN; FLAHERTY; POSTOW, 2016; SAÚDE, 2013).

Melanoma nodular (MN): é o segundo mais comum (15 a 30% dos casos), ocorre mais frequentemente na quinta e sexta décadas de vida e no sexo masculino. Apresenta-se como lesão papulosa ou nodular, elevada, de cor castanha, negra ou azulada. São frequentes a ulceração e o sangramento, porém existe a variante amelanótica, com superfície eritematosa. Apresenta crescimento vertical com metástases precoces (GARBE; LEITER, 2009; SAÚDE, 2013).

Além dessas formas clássicas de melanoma, existem também formas mais raras, como o melanoma de mucosas, ocular, desmoplásicos, associados a nevos congênitos, infantil, nevoide, persistente e associado a nevo azul (EGGERMONT; SPATZ; ROBERT, 2014; O'SULLIVAN; O'CONNOR, 2018). Diferenciar os vários estágios do melanoma, bem como seus tipos tem um papel fundamental em termos de prognóstico (MERVIC, 2012).

Para auxiliar no diagnóstico clínico, utiliza-se a dermatoscopia, um método não invasivo baseado em amplificação da imagem cutânea, com a utilização do dermatoscópio, que torna a epiderme translúcida e permite a observação de estruturas da epiderme e da derme superficial (SAÚDE, 2013; VESTERGAARD et al., 2008).

Já o diagnóstico histopatológico, baseia-se em critérios arquiteturais na epiderme (extensão da lesão, simetria das alterações, padrão de distribuição dos melanócitos, circunscrição, predomínio de células isoladas versus ninhos de células, configuração da epiderme), e aspectos citológicos dos melanócitos na epiderme e na derme, além de maturação celular e atividade mitótica (MIHM; MULE, 2015; SAÚDE, 2013).

Para a determinação do prognóstico, avalia-se o estadiamento do melanoma, ou seja, a determinação da extensão daquele tumor no organismo, levando em conta aspectos do tumor primário (T), do eventual comprometimento linfático regional (N) ou a presença de metástases (M). Tais critérios são os definidos pela *Union International for Cancer Control* (UICC) e pelo *American Joint Committee on Cancer* (AJCC) (AMERICAN JOINT COMMITTEE ON CANCER, 2017).

Uma das suas características mais marcantes é a elevada capacidade de gerar metástases, tanto regionais quanto sistêmicas. Para avaliação das metástases regionais é realizada a técnica chamada biópsia do linfonodo sentinela (LS). Esta técnica foi desenvolvida durante as décadas de 1980 e 1990, com elevada sensibilidade e especificidade, tornando-se o procedimento padrão (WONG et al., 2018).

O LS corresponde ao primeiro linfonodo da base linfática que recebe a drenagem do local de implantação tumoral e permite predizer o estado de toda essa cadeia linfática (SAPIENZA, 2018; WONG et al., 2018). Além de ser uma técnica melhor do que a ressecção de toda cadeia, a melhoria no estadiamento e informações prognósticas obtidas a partir da detecção do LS explicam a rápida aceitação e disseminação dessa técnica (DELGADO; ZOMMORODI, 2019; MASOUD et al., 2018).

Após o diagnóstico de um melanoma, aproximadamente 3% dos pacientes irão desenvolver um segundo tumor em um período de até três anos. O risco pode ser maior em pacientes com história familiar, chegando a 33% de incidência de um segundo melanoma num período de até cinco anos (D'ISCHIA et al., 2015; MANICA et al., 2018). O prognóstico piora quanto mais profunda a lesão se estender, devido à maior propensão à metástase (BERTOLOTTO, 2013).

Nos últimos anos, vários tratamentos foram aprovados pela Food and Drug Administration (FDA) para melanoma, os quais variam em virtude das características do tumor (localização, estágio e perfil genético). Dentre as opções terapêuticas utilizadas podem ser citadas: ressecção cirúrgica (diferem de acordo com as características clínico-patológicas), quimioterapia, radioterapia, terapia fotodinâmica, imunoterapia (aumento da resposta imune, estimulando a atuação dos linfócitos) ou terapia direcionada. Apesar de raramente ser indicado para tratamento de tumores primários, a radioterapia pode ser útil para o tratamento de metástases cutâneas, ósseas e cerebrais (DOMINGUES et al., 2018).

A terapia para melanoma possui limitações relevantes: incidência de graves efeitos adversos, reações imunes e baixa eficiência em virtude da falta de especificidade para as células tumorais (DOMINGUES et al., 2018). Dessa forma, a identificação de fatores moleculares envolvidos na patogênese da

transformação maligna das células melanocíticas, entender o envolvimento de moléculas sinalizadoras e/ou inflamatórias, bem como seus efeitos no melanoma é de suma importância, para um diagnóstico mais precoce e preciso e consequentemente um prognóstico mais favorável.

3.5 SISTEMA PURINÉRGICO

O termo sinalização purinérgica foi designado para descrever um sistema mensageiro (SHABBIR; BURNSTOCK, 2009) extracelular capaz de sinalizar uma série de efeitos biológicos (BURNSTOCK, 2007, 2018). Dentre esses efeitos biológicos, é importante destacar o controle da dor e inflamação, contração do músculo liso, neurotransmissão, secreção endócrina e exócrina, resposta imune, agregação plaquetária e modulação da função cardíaca (BURNSTOCK, 2016a).

Esses mensageiros extracelulares – ATP, difosfato de adenosina (ADP), monofosfato de adenosina (AMP) e adenosina (Ade), tiveram as primeiras descrições de seus efeitos em 1929 por Drury e Szent-Györgyi, que descreveram suas potentes ações no coração dos mamíferos (BURNSTOCK, 2007). Em 1959, Holton sugeriu que o ATP poderia atuar como um mensageiro neuronal durante uma estimulação que induziria a alterações no tônus vascular. Porém, somente alguns anos mais tarde, Burnstock introduziu o conceito de transmissão purinérgica (BURNSTOCK, 2016b).

Dentre as purinas extracelulares, o ATP é a molécula chave que participa de vários processos fisiológicos, incluindo resposta imune, neurotransmissão, tônus vascular, sensação de dor, proliferação, diferenciação, desenvolvimento e morte celular (BURNSTOCK, 2016; DI VIRGILIO; ADINOLFI, 2017).

A atividade anticancerígena do ATP foi primeiramente descrita por Rapaport em 1983, que descobriu que a injeção intraperitoneal dessa molécula em ratos portadores de um tumor resultou em significativa atividade contra o crescimento de carcinomas mais agressivos (QIAN et al., 2016; ZIMMERMANN, 2016). A adição de ATP exógeno inibiu o crescimento de células de câncer de cólon e adenocarcinoma pancreático, por inibir o ciclo celular na fase S (SPYCHALA, 2000). Para executar tais funções, o ATP pode

se ligar em dois tipos de receptores: P2X e P2Y (BURNSTOCK, 2016; DI VIRGILIO; ADINOLFI, 2017).

Sabe-se que as células de melanoma expressam tais receptores e seus agonistas, tais como ATP produzem uma redução no número de células de melanoma em cultura (WHITE et al., 2009). Esse fato tem fundamental importância pois essa doença maligna tem alto risco de recorrência após o tratamento do tumor primário e quando está em estágios avançados tem poucas chances de sobrevivência. Os tratamentos com quimioterapia e imunoterapia podem beneficiar alguns pacientes mas nenhum destes tratamentos são totalmente efetivos (CALLAHAN; FLAHERTY; POSTOW, 2016).

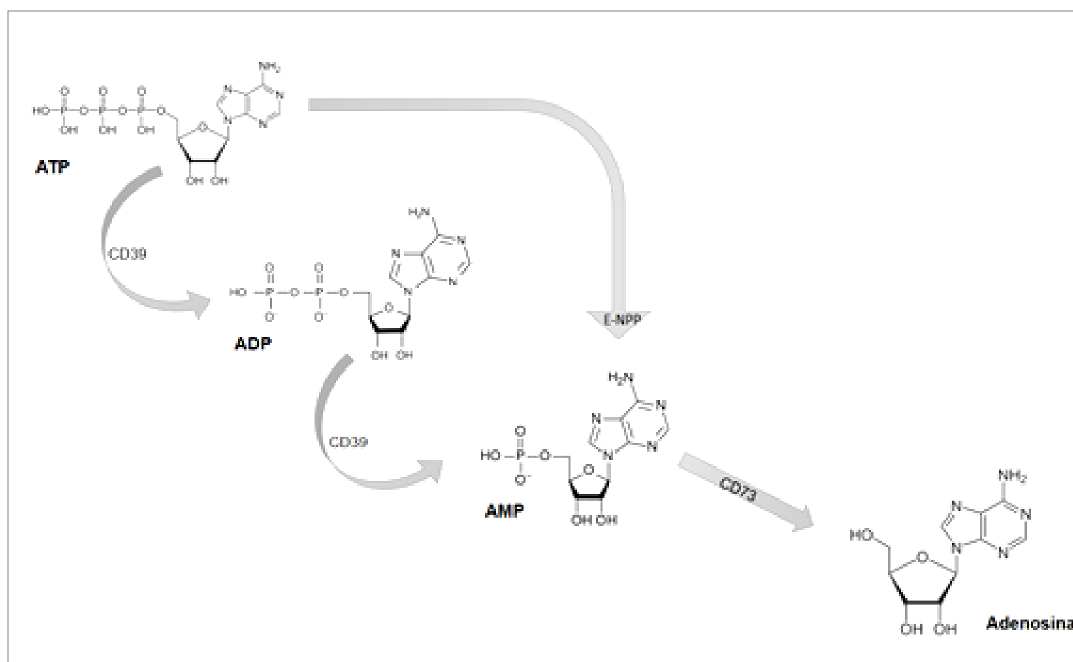
A presença do ATP no espaço extracelular interfere com o metabolismo celular do câncer e na imunidade antitumoral. Seu efeito dependerá da sua concentração, taxa de degradação e expressão dos receptores pelas células cancerígenas e pelas células inflamatórias infiltrantes, pois o ATP tem uma função imunomoduladora no microambiente tumoral (DI VIRGILIO; ADINOLFI, 2017).

O ATP liberado em um meio com células tumorais se acumula em grandes concentrações extracelularmente e, além de atuar como sinal de perigo também pode matar as células tumorais adjacentes via ligação aos receptores P2X7 (DI VIRGILIO; ADINOLFI, 2017). A liberação do ATP extracelular pelas células tumorais de uma linhagem de melanoma (B16) contribuiu para o recrutamento e a estimulação de células T reguladoras, resultando em um ambiente imunossupressor (RING; ENK; MAHNKE, 2011).

Os demais nucleotídeos e o nucleosídeo adenosina possuem efeitos no câncer. Maldonado e colaboradores (MALDONADO et al., 2012) ao estudarem o papel do sistema purinérgico em pacientes com câncer de útero e neoplasia cervical verificaram sua influência nessas neoplasias e em seus diferentes estágios de desenvolvimento, observando que a hidrólise do ADP e do AMP foram variáveis frente aos diferentes estágios do câncer enquanto que o ATP que teve seus níveis diminuídos em todas as etapas avaliadas nos pacientes quando comparado com grupo controle. O sistema purinérgico, além dos tumores já citados, foi encontrado alterado em outros tipos de tumores, tais como o câncer de pulmão, mama, colo uterino dentre outros (BATTISTI et al.,

2013; MALDONADO et al., 2012; MANDAPATHIL, 2016; SHABBIR; BURNSTOCK, 2009; ZANINI et al., 2012). A Figura 4 ilustra o processo de hidrólise dos nucleotídeos até a formação de adenosina e suas respectivas enzimas.

Fig. 4: Quebra do ATP em Adenosina (ATP: trisfosfato de adenosina; ADP: difosfato de adenosina; AMP: monofosfato de adenosina; CD39: Ecto-Nucleosídeo Trifosfato Difosfoidrolase/E-NTPDases; CD73: E-5'-Nucleotidase; E-NPP: Ecto-Nucleotídeo Pirofosfato/Fosfodiesterase; FAL: Fosfatase alcalina).



Fonte: Adaptado (BURNSTOCK, 2018).

A liberação e a concentração extracelular dos nucleotídeos são influenciadas por vários fatores, tais como: secreção ou lise celular, permeabilidade seletiva da membrana plasmática, exocitose de vesículas secretoras (corpos densos plaquetários) e ação catalítica de enzimas (ROBSON; SÉVIGNY; ZIMMERMANN, 2006; ZIMMERMANN, 2016).

3.6 ECTOENZIMAS

A concentração dos nucleotídeos extracelulares pode ser modulada por um conjunto de enzimas denominadas de ectonucleotidases (ALLARD et al., 2017; ANTONIOLI et al., 2013; BONNEFOY et al., 2015). Nesse processo, diferentes famílias de ectonucleotidases trabalham de forma organizada, dentre

as quais estão incluídas: Ecto-Nucleotídeo Pirofosfato/Fosfodiesterase (E-NPP), Ecto-Nucleosídeo Trifosfato Difosfoidrolase (E-NTPDases/CD39), E-5'-Nucleotidase (CD73), e a Fosfatase alcalina (FAL) (ZIMMERMANN, 2001).

As E-NPPs são glicoproteínas transmembrana tipo II, capazes de hidrolisar 3'-5'-monofosfato de adenosina cíclico (5'-AMPc) a AMP; ATP a AMP e difosfato inorgânico (PPi); ADP a AMP e fosfato inorgânico (Pi); nicotinamida adenina dinucleotídeo oxidado (NAD^+) a AMP e nicotinamida mononucleotídeo. Embora essa família de enzimas seja composta por 7 membros, apenas a E-NPP 1, 2 e 3 são capazes de hidrolisar nucleotídeos (BATTISTI et al., 2013; ZIMMERMANN, 2001).

As E-NTPDases são enzimas muito eficientes e controlam a biodisponibilidade de ATP (CARDOSO et al., 2015; ROBSON; SÉVIGNY; ZIMMERMANN, 2006). Essa família de enzimas é constituída por oito membros – E-NTPDases 1 – 8 que diferem entre si quanto à especificidade de substrato, distribuição tecidual e localização tecidual. Os membros 1, 2, 3 e 8 são as principais enzimas responsáveis pela hidrólise de nucleotídeos tri e difosfatos na superfície da célula sob condições fisiológicas. As E-NTPDases 4, 5, 6 e 7 estão principalmente associadas com organelas intracelulares (Quadro 2).

Quadro 2: Nomenclatura e preferência por substrato dos membros da família NTPDase.

Nome da proteína	**Nomes adicionais**	**Preferência por substrato**
NTPDase1	CD39, ATPDase, ecto-apirase	ATP → ADP (1:1)
NTPDase2	CD39L1, ecto-ATPase	ATP→ → ADP (30:1)
NTPDase3	CD39L3, HB6	ATP → ADP (3:1)
NTPDase4	UDPase, LALP70	UDP → GDP
NTPDase5	CD39L4, ER-UDPase, PCPH	UDP→GDP/ UDP→ADP
NTPDase6	CD39L2	GDP → IDP → UDP
NTPDase7	LALP1	GDP → IDP → UDP
NTPDase8	Ecto-ATPase, hATPDase	ATP→ADP ADP → AMP

Fonte: Adaptado (ROBSON; SÉVIGNY; ZIMMERMANN, 2006).

Essa classe de ectoenzimas apresenta uma distribuição tecidual diferenciada, podendo ser expressa simultaneamente por um mesmo tipo celular. Se ancoram à membrana plasmática via domínios hidrofóbicos com um duplo sítio ativo voltado para o meio extracelular (ROBSON; SÉVIGNY; ZIMMERMANN, 2006; YEGUTKIN, 2008).

A família das E-5'-Nucleotidase tem sete membros, seis citosólicos e uma ectoenzima, a 5'-Nucleotidase, que também está ancorada à membrana plasmática. Na maioria dos tecidos, sua atividade é o passo limitante da formação de adenosina a partir do AMP (ANTONIOLI et al., 2013; MONTEIRO et al., 2018).

As FALs em condições alcalinas removem o Pi de uma ampla gama de substratos, incluindo os nucleotídeos (ATP, ADP e AMP). Uma única enzima pode assim catalisar toda a cadeia de hidrólise a partir de um ATP para o respectivo nucleosídeo - adenosina. A FAL é ancorada à membrana similar às outras ectoenzimas (ZIMMERMANN, 2001)

A ADA catalisa a desaminação de adenosina e desoxiadenosina em inosina e desoxi inosina. Apesar de grande parte desta enzima estar presente no meio intracelular, ela também está localizada na superfície celular. É encontrada alterada em diversas situações patológicas, inclusive na presença de certos tumores (SPYCHALA, 2000). Essa importante enzima se divide em duas isoformas: ADA1 e ADA2. A primeira é encontrada predominantemente nos tecidos, enquanto a ADA2 é o principal componente do soro. Ambas apresentam diferenças estruturais e cinéticas (ANTONIOLI et al., 2012).

Em conjunto, as enzimas descritas acima são capazes de regular a concentração extracelular do ATP e seus metabólitos, os quais apresentam importante relação com o desenvolvimento e a alta agressividade do melanoma.

3.7 RECEPTORES PURINÉRGICOS

Os nucleotídeos e o nucleosideo de adenina interagem com receptores purinérgicos específicos mediando eventos de resposta imune, inflamação, agregação plaquetária dentre outros (DI VIRGILIO; ADINOLFI, 2017). Os receptores do sistema purinérgico podem ser divididos em dois grupos principais: os receptores de adenosina, também chamados receptores P1 e os receptores P2, que reconhecem ATP, ADP, Uridina-5'-trifosfato (UTP), e Uridina difosfato (UDP) (BURNSTOCK, 2014).

Os receptores P1 apresentam quatro subtipos de acordo com suas características: A_1, A_{2A}, A_{2B} e A_3. Baseado em diferenças de estrutura molecular e mecanismos de transdução de sinal, os receptores P2 também foram subdivididos em duas classes: canais iônicos e acoplados à proteína G. Os receptores P2 foram então denominados de receptores P2X e P2Y (sete receptores P2X – ligados a canais iônicos: $P2X_{1-7}$, e oito receptores P2Y – à qual pertencem purinoreceptores metabotrópicos acoplados à proteína G: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) (KRÜGEL, 2016; WOODS et al., 2016).

O ATP exerce seus efeitos através da ligação e ativação aos receptores P2. Sendo assim, alguns subtipos de receptores de P2 ($P2Y_1$, $P2Y_2$, $P2Y_{11}$, $P2X_5$ e o $P2X_7$) têm sido implicados com envolvimento com o câncer: modulação da proliferação de células (receptores $P2Y_1$ e $P2Y_2$) estimulação da diferenciação com a subsequente inibição da proliferação - receptores $P2X_5$ e $P2Y_{11}$ - ou indução da morte celular (apoptose) - receptores $P2X_7$ (BURNSTOCK, 2007).

A função dos receptores P2Y em melanomas é regular a proliferação celular. Receptores $P2Y_1$ ativados causam uma diminuição no número de células, enquanto os receptores $P2Y_2$ ativados causam um aumento no número de células. Receptores $P2Y_6$ também estão presentes no melanoma, mas foi demonstrado não ter um efeito sobre o número de células, sugerindo-se então que eles podem estar desempenhando outro papel na regulação celular (WHITE et al., 2009; ZIMMERMANN, 2016). Através da ligação com seus receptores, o ATP pode mediar eventos que levam a uma imunossupressão e quadros inflamatórios, favorecendo assim a progressão tumoral.

4 INFLAMAÇÃO X ESTRESSE OXIDATIVO X MELANOMA

A sobrevivência de todos os organismos requer a eliminação de invasores estranhos, como agentes infecciosos e tecidos lesados. Essas funções são mediadas por uma resposta complexa do hospedeiro chamada inflamação. A inflamação é considerada uma resposta protetora que envolve células, mediadores, vasos sanguíneos, dentre outros, destinada a eliminar a causa inicial da lesão celular e iniciar o processo de reparo (KUMAR; ABBAS, ASTER, 2013).

Lesões cutâneas iniciam processo inflamatório que ocorre em três estágios: dilatação dos capilares para aumentar o fluxo sanguíneo local, alterações estruturais microvasculares a fim de extravasar proteínas da circulação sanguínea para o local da inflamação e transmigração leucocitária através do endotélio vascular, acumulando-se no local do dano. Além disso as células inflamatórias atuam como fonte importante na liberação de citocinas e fatores de crescimento, que são necessários para o recrutamento celular, ativação e proliferação (SHALAPOUR; KARIN, 2015).

Esse processo inflamatório no tecido cutâneo associado ao dano tecidual tem forte ligação com o desenvolvimento de câncer. A associação entre o desenvolvimento tumoral e inflamação já vem sendo estudada desde 1863, quando Rudolf Virchow notou a presença de leucócitos em tecidos neoplásicos, sugerindo a origem de células tumorais em locais de inflamação crônica (SHALAPOUR; KARIN, 2015). Estudos recentes demonstram que a inflamação é um componente crítico na progressão e desenvolvimento tumoral (GRIVENNIKOV; GRETEN; KARIN, 2011; LANDSKRON et al., 2014; MANICA et al., 2018).

No melanoma, a correlação entre a inflamação, mediadores, células imunossupressoras e desfecho clínico do paciente ainda é uma questão de intensa pesquisa. No entanto, as evidências sugerem cada vez mais que a inflamação também pode estar associada a um prognóstico clínico desfavorável nesses pacientes (DUNN; ELLIS; FUJITA, 2012; HÖLZEL; TÜTING, 2016; RIBATTI et al., 2001).

Estudos sobre polimorfismos genéticos no melanoma, especialmente polimorfismos de nucleotídeo único, têm demonstrado que genótipos de

citocinas como IL-6, TNF-a, IFN-γ, IL-10 e fator de transformação do crescimento beta 1 (TGF-β1) podem desempenhar um papel na progressão do melanoma por facilitar o escape da vigilância imunológica (SHALAPOUR; KARIN, 2015; NEAGU et al., 2015).

Na pele as citocinas são produzidas pelos queratinócitos, células de Langerhans, melanócitos, mastócitos, macrófagos, mas também por células inflamatórias recrutadas: neutrófilos, eosinófilos e linfócitos. Quando há um estímulo inflamatório prolongado, a produção de citocinas é excessiva e, além de ter um efeito deletério no tecido inflamado, pode afetar células distantes do local da inflamação inicial (MANICA et al., 2018; NEAGU et al., 2015).

As próprias células de melanoma produzem citocinas que são conhecidas por estarem associadas com invasividade e agressividade, as quais incluem IL-6, IL-8, quimiocina CXC L-13 (CXCL 13), quimiocina CCL-5 (CCL5) e proteína quimiotática de monócitos-1 (MCP-1, também conhecida como CCL2) (HOWELL et al., 2003). O ambiente inflamatório criado tanto pelo melanoma, quanto pelo microambiente o qual está instalado, favorece a plasticidade do melanoma. Essa interação entre a inflamação e a plasticidade das células do melanoma surgiu como um determinante crítico da progressão metastática (HÖLZEL; TÜTING, 2016).

Importante ressaltar que o tipo de inflamação e subtipos de células imunes presentes são importantes, uma vez que os melanomas primários com infiltrados de células T têm menor risco de desenvolver metástases à distância (BALD et al., 2014). Tecidos que apresentam ambientes inflamatórios fornecem um microambiente rico em EROs, resultando em estresse oxidativo.

O estresse causado por agentes como RUV e inflamação é um importante fator de risco para o desenvolvimento de tumores de pele, incluindo o melanoma (BISEVAC et al., 2018). No entanto poucos estudos foram feitos até o momento para entender essa conexão.

A instalação do processo de estresse oxidativo decorre da existência de um desequilíbrio entre compostos oxidantes e antioxidantes, em favor da geração excessiva de espécies reativas ou pela deficiência na sua remoção. Esse estado conduz à oxidação de biomoléculas com consequente perda de suas funções biológicas com dano contra células e tecidos (BARBOSA et al., 2010).

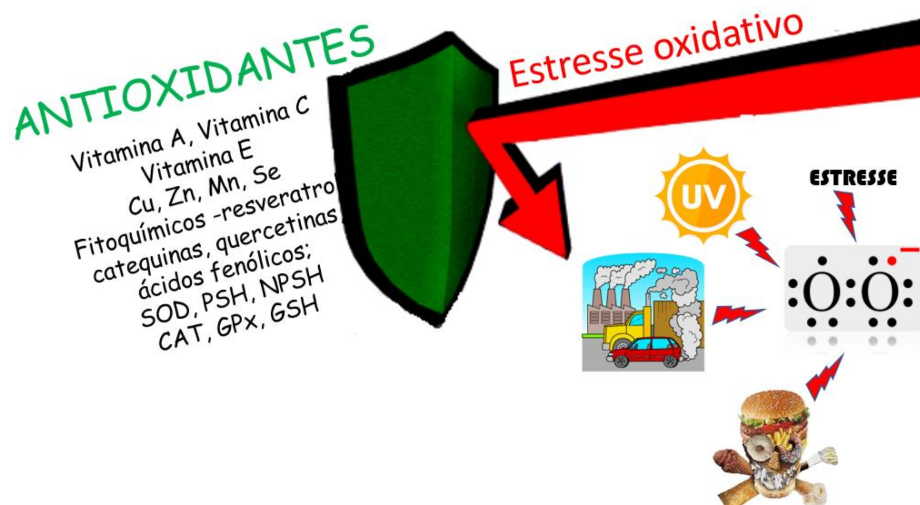
Sabe-se que o estresse oxidativo está envolvido no processo de carcinogênese através de vários mecanismos: metabolismo energético alterado, que pode ser atribuído a sintomas como caquexia, náusea e vômito, os quais impedem uma nutrição normal e, portanto, um suprimento normal de nutrientes, como vitaminas antioxidantes, que levam a um desequilíbrio entre antioxidantes e oxidantes; ativação crônica inespecífica do sistema imune com uma produção excessiva de citocinas pró-inflamatórias, o que, por sua vez, podem aumentar a produção de EROs ou pelo efeito de medicamentos usados no tratamento do câncer (CANNAVÒ et al., 2019).

Vários parâmetros bioquímicos (peroxidação lipídica, níveis de glutationa, atividade da superóxido dismutase e da catalase) e perfil lipídico plasmático (lipídios totais, colesterol total, triglicerídeos totais, lipoproteínas de alta densidade (HDL) e lipoproteínas de baixa densidade (LDL)) podem ser avaliados para estabelecer níveis de estresse oxidativo ou atividade antioxidante (PISOSCHI; POP, 2015). Além desses, uma característica bioquímica única do melanócito é que a geração de melanina produz peróxido de hidrogênio e consumo de glutationa reduzida (GSH), uma importante defesa antioxidante (OBRADOR et al., 2019).

O sistema de defesa antioxidante tem a função de inibir e/ou reduzir os danos causados pela ação deletéria dos radicais livres ou das espécies reativas de oxigênio (EROs). Usualmente, esse sistema é dividido em enzimático e não-enzimático. Os antioxidantes são definidos como qualquer substância que, presente em menores concentrações que as do substrato oxidável, seja capaz de atrasar ou inibir a oxidação deste de maneira eficaz (VISSERS; DAS, 2018).

Na figura 5 é possível observar alguns fatores que desencadeiam a produção de EROs/estresse oxidativo e alguns tipos de defesas antioxidantes disponíveis. Uma falha no sistema antioxidante, com acúmulo de EROs, pode contribuir para a capacidade de células de melanoma ficarem mais agressivas, autorenovar-se, inibir antiproteases, invadir tecidos locais e, portanto, promover metástases (OBRADOR et al., 2019).

Fig. 5. Substâncias antioxidantes *versus* fatores que favorecem o aumento da produção de Espécies Reativas de Oxigênio (EROs).



Cu: cobre; Zn: zinco; Mn: manganês; Se: selênio; SOD: superóxido dismutase; PSH: tióis proteicos; NPSH: tióios não proteicos; CAT: catalase; GPx: glutationa peroxidase, GSH: glutationa dismutase. Fonte: (PISOSCHI; POP, 2015).

Sabendo que o estresse oxidativo está elevado em pacientes com melanoma, e pode estar associado aos diferentes estágios de melanoma, além disso, como a inflamação geralmente está associada ao estresse oxidativo e que o sistema purinérgico está intimamente relacionado com quadros de inflamação, essa tese demonstra resultados inéditos da associação desses três fatores no câncer de pele do tipo melanoma.

5 ARTIGOS E MANUSCRITO

5.1 ARTIGO 1:

High levels of extracellular ATP lead to chronic inflammatory response in melanoma patients.

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RESEARCH ARTICLE

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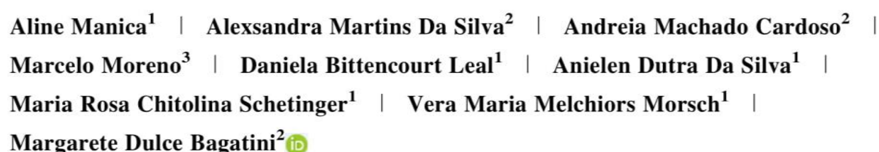
High levels of extracellular ATP lead to chronic inflammatory response in melanoma patients



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Abstract

Skin cancer represents a serious public health problem and melanoma is considered the most significant due to its high metastasis capacity. Evasion mechanisms are the main characteristic of these tumor cells to escape of immune response. Extracellular nucleotides and nucleosides play an important role in inflammatory and immune responses. In this study, we analyzed the expression and activity of purinergic system enzymes in platelets and lymphocytes, ATP levels quantification, as well the level of pro and anti-inflammatory interleukins in the serum of 23 patients with surgical melanoma removal (CM group) and 23 control subjects (CT group). Results showed a decrease in ATP, ADP, and AMP hydrolysis and an increase in ATP levels quantification in CM group. The pro-inflammatory cytokines were elevated in CM group when compared to CT group. These results suggest an inflammatory process, even after surgical removal, due to elevated extracellular ATP levels. Besides, CM group displayed an increase in IL-10 levels and an increased in ADA activity in platelets and lymphocytes. Once adenosine and IL-10 are anti-inflammatory molecules, these results indicate a down-regulation of immune system front to malignant process. The alteration in nucleotide and nucleoside hydrolysis reinforces the purinergic systems role in this cancer. Therefore, even after surgical removal, the purinergic system can develop a chronic inflammatory micro-environment that can influence directly on relapse or metastasis.

KEYWORDS
cytokines, inflammation, purinergic system, skin cancer

1 | INTRODUCTION

The incidence of cancer, mainly skin cancer, has been globally alarming, representing a serious public health problem, and it is expected that incidence of this cancer increases in the coming decades and reaches the general population. Cutaneous Melanoma (CM), a type of skin cancer, accounts for only 4% of malignant skin cancers, but is considered the most serious due to its high metastasis capacity.[1]

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CM is a highly aggressive solid tumor, and its aggressive behavior is characterized by uncontrolled cell proliferation through suppression signals, escape of the immune response, induction of inflammation, angiogenesis, genomic instability, mutations, and resistance to cell death, invasion, and metastasis. However, suppression of anti-tumor immunity and the escape of the immune response is a pivotal step in tumor progression and recurrences.[2]

Intracellular purines are being important participants in metabolic processes such inflammatory and immune responses by the extracellular release of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine. In the extracellular space, ATP is converted to ADP and AMP by enzymes called ectonucleotidases: alkaline phosphatases, ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, CD39, NTPDase 1), and ectonucleotide pyrophosphatases/phosphodiesterases (E-NPPs). Extracellular AMP is primarily converted to adenosine by E-5′Nucleotidase (CD73). Adenosine hydrolysis is terminated by the activity of adenosine deaminase (ADA), which converts adenosine to inosine. These ectoenzymes, nucleotides, and nucleosides are crucial for the functions exerted by the nucleotides in all irrigated tissues, including the skin, where melanocytes are present.[3–5]

ATP is mainly associated to proinflammatory response but adenosine has opposite effects limiting the inflammation by suppressing the actions of immune cells,[6–8] reinforcing the important participation of ectonucleotidases in inflammatory processes.[9–11] Our research group has focused investigations on the interrelation between the purinergic system and homeostasis alterations, and their potential involvement in cancer: breast cancer,[12] lung cancer[13] cervical intraepithelial neoplasia and uterine cancer,[14] and bladder tumor.[15] Cited works clearly demonstrate the role of the purinergic system in cancer development and progression.

Considering, that the enzymatic chain responsible for ATP hydrolysis and adenosine production, present in all immune and vascular cells, has an important role in the control or promotion of inflammation in tumors, the aim of this work is to analyze and clarify the role of adenine nucleotides and adenosine as well as the role of interleukins in the pathophysiology of CM and a possible mechanism of recurrence and metastasis.

2 | MATERIALS AND METHODS

2.1 | Chemical and equipment

The substrates adenosine 5′-triphosphate disodium salt, adenosine 5′diphosphate sodium salt, 5′-monophosphate sodium salt, adenosine, bovine serum albumin, Trizma base, HEPES, Ficoll-Histopaque (Lymphoprep), and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO). The antibodies used for flow cytometry analyses, Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit and BD Citofix®/Citoperm® was obtained from BD Biosciences (Becton, Dickinson and Company, Franklin Lakes, NJ) and the equipment used was BD Accuri C6. The centrifuge used was Sigma 3k-16® refrigerated and the rotors was changed depending the samples. All other reagents used in the experiment were of analytical grade and high purity.

2.2 | Patients and samples

The samples consisted of 23 patients with CM and 23 healthy subjects as a CT group selected according to the characteristics, age, and gender of the CM group. The CM group had previous surgical excision of the melanoma, but now they did not have in pharmacology treatment. Ten milliliters of blood was obtained from each patient and used for platelet and lymphocytes separation, flow cytometry and biochemical analyzes. The same procedure was carried out for the control group.

2.3 | Experimental design

Patients with CM were selected after diagnosis according to the International Classification of Diseases (ICD) and surgical removal of the tumor by an oncologist. Only patients with Breslow thickness T3/T4 ($\geq$2 mm)[16] were selected for analysis. Control patients in the study were those who had no acute or chronic pathology or CM history, in addition to having normal blood pressure and not undergoing any drug therapy. Patients' characteristics are shown in Table 1. All subjects gave written informed consent to participate in the study. The Human Ethics Committee of Federal University Frontier South, Brazil, approved the protocol under number 822.782. All analysis was developed in triplicates to ensure reliability of results.

2.4 | Platelet and lymphocytes separation

Platelet-rich plasma was prepared by the method of Pilla et al[17] modified by Lunkes et al.[18] Total blood was collected with sodium citrate as anticoagulant and centrifuged at 1500 rpm for 10 min. After, the platelet-rich plasma was centrifuged at 5000 rpm for 30 min and washed with 3.5 mM HEPES buffer, pH 7.0 at least twice. The platelet pellets were suspended in HEPES buffer and protein was adjusted to 0.4-0.6 mg/mL.

The mononuclear leukocytes were isolated from human blood collected with EDTA and separated on Ficoll-Histopaque density gradients as described by Böyum.[19] Due to the fact that the methodology described above is employed for separating mononuclear cells, the study

TABLE 1 Clinical characteristic

	CT (23)	CM (23)	*P*-value
Age	47 ± 13	48 ± 12.5	0.8695
Tumor location			
Lower extremities	-	9	
Upper extremities	-	6	
Trunk	-	5	
Head/neak	-	3	
Subtypes			
Superficial spreading melanoma	-	16	
Nodular melanoma	-	5	
Lentigo maligno melanoma	-	2	
Acral lentiginous melanoma	-	-	
Time after surgery			
<1 year	-	3	
1-2 years	-	20	
Sun exposure (%)	24	30	
Fitzpatrick skin classification			
I/II (%)	94	96	
III/IV/V (%)	6	4	
History of sunburn (%)	70	89	

The data were obtained through a documented interview with each participant. CT, control; CM, cutaneous melanoma.

performed by Jaques et al[20] demonstrated that there is a high incidence of lymphocytes (95%) in these samples and the amount of monocytes is practically insignificant. For this reason, we treat the samples as containing only lymphocytes.

2.5 | Protein determination

Protein was measured by the method of Bradford[21] using bovine serum albumin as standard. This assay is based on the binding of the dye Coomassie Blue G-250 to protein, and this binding is accompanied by measuring the absorbance maximum of the solution at 595 nm.

2.6 | E-NTPDase and E-5′-nucleotidase assays

A total of 20 µL of the platelet-rich plasma preparation (0.4-0.6 mg/mL protein) was added to the reaction mixture of E-NTPDase or E-5′nucleotidase and preincubated for 10 min at 37°C, to a final volume of 200 µL. E-NTPDase activity was determined by the method of Lunkes et al.[18] The reaction was started by the addition of ATP or ADP as substrate at a final concentration of 1.0 mM. E-5′-nucleotidase was determined by the method of Heyman et al.[22] Phosphate released by ATP, ADP, and AMP hydrolysis was measured using KH_2PO_4 as standard. Controls were prepared to correct for nonenzymatic hydrolysis and all samples were analyzed in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein.

2.7 | Flow cytometry analysis for CD39 and CD73

The enzymes E-NTPDase (*CD39)* and E-5′-nucleotidase (*CD73)* expression analysis were performed from human blood collected with EDTA by flow cytometry using monoclonal antibodies anti-CD61 (FITC), anti-CD45 (monoclonal antibody), anti-CD39 (FITC), and anti-CD73 (PE) for marking platelet, lymphocytes, E-NTPDase, and E-5′ nucleotidase, respectively. A total of 200 µL of whole blood were incubated with 2 mL of lysis solution for 15 min in the dark and then centrifuged 1000 rpm for 5 min.

The lysis solution is an ammonium chloride-based lysing reagent that lysis red blood cells following monoclonal antibody staining. The lysing solution results in good light scatter separation of lymphocytes and red blood cell debris when analyzed by flow cytometry and does not contain a fixative agent, so leukocytes remain viable after red blood cell lysis. The procedure is only applied to human whole blood red blood cell lysis.

The cells were incubated with specific antibodies for 25 min. After, twice centrifugation 1000 rpm for 5 min and discarding the supernatant by adding 1 mL of PBS. For fixing, 250 µL of BD Citofix® was added until analysis.

2.8 | Quantitative ATP determination

The quantitative ATP determination was developed using commercial kit by bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin in serum of CM and CT group. The assay is based on luciferase's requirement for ATP in producing light—emission maximum ∼560 nm at pH 7.8.[23] This assay is extremely sensitive.

We combined the components of the reaction as follows to make a standard reaction solution and adjust the volumes according to particular requirements. Each reaction contained 1.25 µg/mL of firefly luciferase, 50 µM D-luciferin, and 1 mM DTT in 1X Reaction Buffer. After a 15 min incubation, luminescence was measured.

2.9 | Flow cytometry analysis for cytokines

The BD™ CBA Human Th1/Th2 Cytokine Kit II (Catalog No. 551809) was used to quantitatively measure Interleukin-2

(IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), Interleukin-17A (IL-17A), and Interferon-γ (IFN-γ) protein levels in a single sample. BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry.

Each capture bead was conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)-conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes was measured using flow cytometry for identify particles with fluorescence characteristics of both the bead and the detector.

2.10 | Adenosine deaminase (ADA) determination

ADA activity from platelets and lymphocytes was determined according to Giusti and Galanti[24] based on the direct measurement of ammonia produced when adenosine deaminase acts in excess of adenosine. Briefly, 50 μL of cells reacted with 21 mmol/L of adenosine, pH 6.5 was incubated at 37°C for 60 min. Afterward the reaction was stopped by adding a solution of 106.2 mM phenol and 167.8 mM sodium nitroprussiate and a hypochlorite solution. The amount of ammonia produced was measure at 620 nm and the results were express in units per liter (U/L).

2.11 | Statistical analysis

Statistical analyses were performed with GraphPad Prism 7. Normality was tested by the Shapiro-Wilk test. The differences between the groups, in relation to the variables of the study, were evaluated through analysis unpaired *T* test. The results were presented as mean and standard deviation. The differences in the probability of rejection of the null hypothesis as being less than 5% ($P < 0.05$) were considered statistically significant.

3 | RESULTS

3.1 | Clinical characteristics

The data were obtained through a documented interview with each participant. The characteristics of our study groups are summarized in Table 1.

The average age was similar between control and CM group (47 and 48 years old). The most affected body areas were: lower extremities (39.1%), upper extremities (26%), trunk (21.7%), and head/neck (13%). Differences in the forms of sun exposure, awareness, prevention measures, and medical care were considered as probable reasons for the higher incidence of injuries to the trunk, head, and neck.

The Superficial Spreading Melanoma had greater incidence in the study group (69.5%). Nodular Melanoma and Lentigo Malignant Melanoma had low incidence in this group (21.7% and 8.6%), while the Acral Lentiginous Melanoma subtype was not present. The majority of patients in CM group had the surgical removal between 1 and 2 years following surgery (86.9%) and only 13% less than 1 year following surgery.

Regarding risk factors for CM development, sun exposure was higher in CM group (30%) than in control group (24%), and both showed elevated percentage for Fitzpatrick Phototype I/II (96% and 94%). The main risk factors for the development of CM result from the combination of constitutional/genetic and environmental factors: skin types I/II Fitzpatrick classification, presence of multiple melanocytic nevi, presence of atypical or dysplastic nevi, history of melanoma or other skin cancer, and mutations in genes. One of main risk factor for melanoma development is the history of sunburn. In both groups this factor was elevated (CT and CM group—70% and 89%, respectively) during the life.

3.2 | Purinergic enzymes activity and expression by flow cytometry

To elucidate the importance of purinergic enzymes in melanoma, we first analyzed the hydrolysis of nucleotides ATP, ADP in lymphocytes and platelets, and AMP hydrolysis in platelets of CT and CM group (Figure 1). The E-5′-Nucleotidase activity in lymphocytes is very small; then our technique was not sensitive for its analysis.

As can be observed in Figure 1, nucleotides hydrolysis (ATP, ADP, and AMP) was decreased in CM patients when compared to CT group. These results suggest that CM patients have lower E-NTPDase and E-5′-Nucleotidase activities.

To confirm the enzymatic activity inhibition and ATP accumulation in extracellular medium, extracellular ATP concentration was measured using luciferase/luciferin reagent. The concentration of extracellular ATP increased in CM group when compare to control group (Figure 2).

In order, to confirm the decrease on purinergic enzymes activity, was performed the expression of E-NTPDase (CD39-converted ATP to ADP and AMP) and E-5′-Nucleotidase (CD73-converted AMP to adenosine) by flow cytometry. Differently of activity, the expression of CD39 and CD73 positive cells in platelets and lymphocytes was not statistically different between the groups indicating that these enzymes are being expressed normally in both groups (Figure 3).

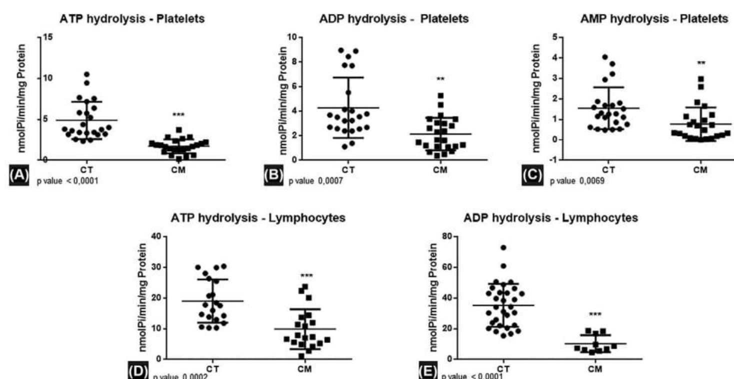

FIGURE 1 E-NTPDase and E-5′-Nucleotidase activities. A, E-NTPDase (hydrolyzing ATP); (B) E-NTPDase (hydrolyzing ADP), and (C) E-5′-Nucleotidase (hydrolyzing AMP) activity in platelets. D, E-NTPDase (hydrolyzing ATP) and (E) E-NTPDase (hydrolyzing ADP) activity in lymphocytes of control (CT) and melanoma (CM) groups. The E-NTPDase and E-5′-nucleotidase assays was followed as described in materials. Data are presented as means ± SEM. ***Indicates a significant difference from the control group (Student's *t* test, $P < 0.0001$, $n = 23$); **Indicates a significant difference from the control group (Student's *t* test, $P < 0.001$, $n = 23$)

3.3 | Inflammatory alterations

To demonstrate the inflammatory profile and to determine the effect of the ATP extracellular release, we examined the quantification of proinflammatory cytokines (IL-2, IL-4, IL-6, TNF, IFN-Y, and IL-17A) (Figure 4) and an anti-inflammatory interleukin (IL-10) (Figure 4C). ADA activity was also performed (Figures 4A and 4B).

As can be observed in Figure 4, cytokines IL-2, IL-4, IL-6, and TNF are significantly increased in CM when compared to CT group. On the other hand, no difference was observed in the groups for IFN-Y and IL-17A (Figures 4E and 4F). These results show that these cytokines remained increased after the surgery.

Based on the pro-inflammatory profile found, we evaluated some anti-inflammatory parameters, such as IL 10 and adenosine hydrolysis as shown in Figure 5. IL 10, an anti-inflammatory interleukin, showed a significant increase in CM group when compared to control (Figure 5C). In relation to ADA activity, an increase was observed in this enzyme in CM patients when compared to control group in platelets and lymphocytes.

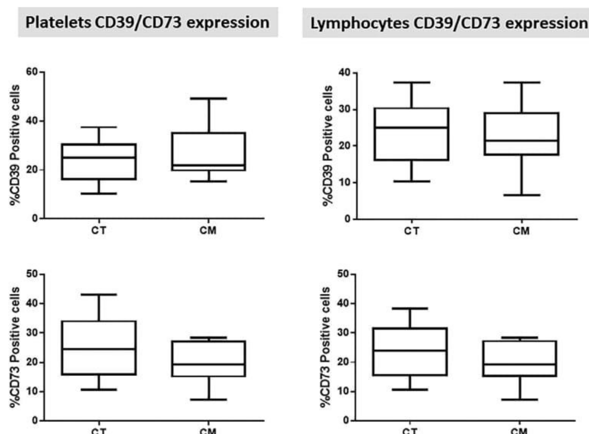

FIGURE 2 Quantitative ATP determination. Extracellular ATP was determined as production of bioluminescence using a luciferin-luciferase reaction system (emission maximum ~560 nm at pH 7.8) through a commercial kit (Invitrogen®). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (Student's *t* test, $P < 0.05$, $n = 23$)

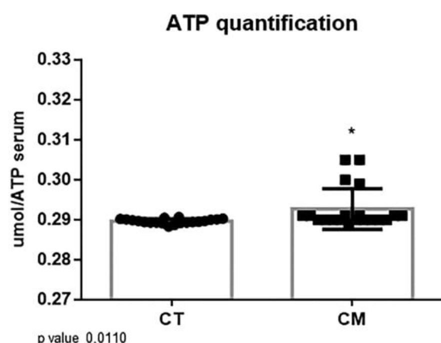

FIGURE 3 Expression of E-NTPDase1/CD39 and E-5′-Nucleotidase/CD73 protein in platelets and lymphocytes of control group (CT) and melanoma group (CM). The analysis were performed from human blood by flow cytometry using monoclonal antibodies anti-CD61 (FITC), anti-CD45 (monoclonal antibody), anti-CD39 (FITC), and anti-CD73 (PE) for marking platelet, lymphocytes, E-NTPDase and E-5′-Nucleotidase, respectively. Data are presented as means ± SEM. No differences was observed between the groups

4 | DISCUSSION

Our results show for the first time, that the inhibition of extracellular ATP hydrolysis, evidenced by significantly decrease on purinergic enzyme activities, lead to accumulation of ATP extracellular. The presence of high ATP levels in the post-surgery CM micro-environment is suggested to be the cause of deleterious changes, which are evidenced by the uncompensated inflammatory profile. Patients with CM are treatment with surgical excision, but recurrences and metastases are common with a relapse rate between 50% and 80%, mainly in Breslow thickness T3/T4 tumor. These recurrences and metastases can be the result of changes in the tumor micro-environment that remain even after its removal.

A deregulation in the metabolism of nucleotides, evidenced by the decrease of ATP and ADP hydrolysis in platelets and lymphocytes (Figures 1A, 1B, 1D, and 1E), and AMP hydrolysis in platelets (Figure 1C), was observed. However, the expression of CD39 and CD73 remained the same in both groups (Figure 2), suggesting that E-NTPDase/CD39 are inhibited and extracellular ATP is accumulated. Although we do not know the reason why this enzyme is inhibited, the explanation for the decrease in ADP and AMP hydrolysis is the reduced activity of CD39, the first enzyme of the cascade, whose consequence is the decrease of ADP as well as AMP production and breakdown.

A study with uterine cervical neoplasia showed that ATP, ADP, and AMP hydrolysis was also decreased in patients treated with conization surgery or radiotherapy, which corroborates with our findings,[14] but in another cancer types the results are varied. In lung cancer, for example, the ATP hydrolysis was not altered while the ADP hydrolysis was decreased and an increase in E-5′-Nucleotidase activity was observed.[13] In addition, other types of cancer were studied with different profiles of activities of the purinergic enzymes[12,14,15] that reinforce the close relationship between the neoplastic diseases and activity of enzymes that hydrolyze adenine nucleotides.

It is interesting to note that even in treated patients, we observed a disruption of ectonucleotidases activity. This is

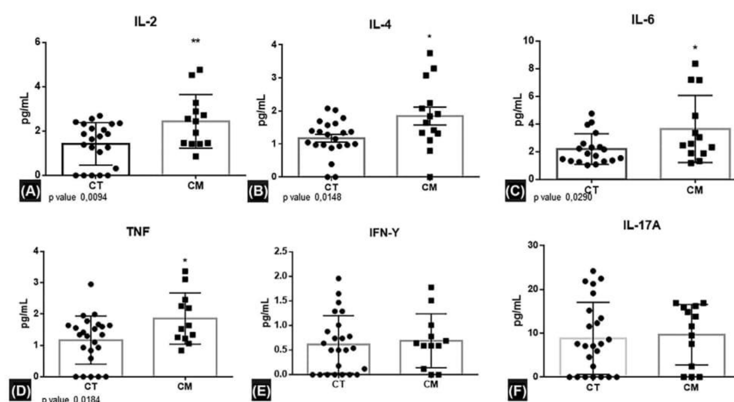

FIGURE 4 A, Interleukin-2 (IL-2), (B) Interleukin-4 (IL-4), (C) Interleukin-6 (IL-6), (D) Tumor Necrosis Factor (TNF), (E) Interferon-γ (IFN-γ) (F) Interleukin-17A (IL-17A), protein levels. They was measured by BD™ CBA Human Th1/Th2 Cytokine Kit II in serum of control (CT) and melanoma (CM) groups. The assay was followed as described in materials. Data are presented as means ± SEM. **Indicates a significant difference from the control group (Student's *t* test, $P < 0.001$, $n = 23$). *Indicates a significant difference from the control group (Student's *t* test, $P < 0.05$, $n = 23$)

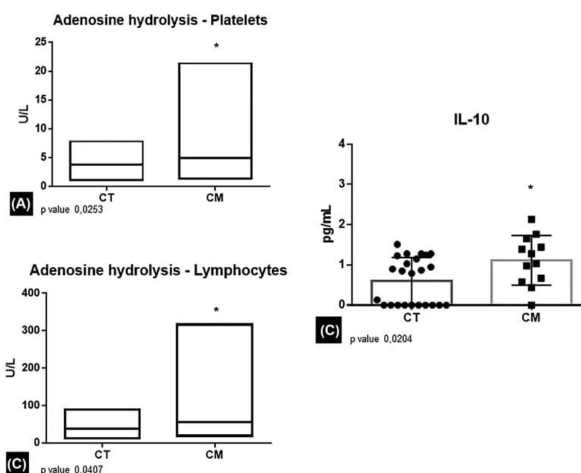

FIGURE 5 ADA activity and Interleukin-10 (IL-10) protein levels. A, ADA (Adenosine desaminase—hydrolyzing adenosine) in platelets and (B) ADA (Adenosine desaminase—hydrolyzing adenosine) in lymphocytes of control (CT) and melanoma (CM) groups. The assay is based on the direct measurement of ammonia produced when adenosine deaminase acts in excess of adenosine. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol the ammonia per minute from adenosine at standard assay conditions. Data are presented as means ± SEM. *Indicates a significant difference from the control group (Student's *t* test, $P < 0.05$, $n = 23$). C, Interleukin-10 (IL-10) protein levels measured by BD™ CBA Human Th1/Th2 Cytokine Kit II in serum of control (CT) and melanoma (CM) groups. *Indicates a significant difference from the control group (Student's *t* test, $P < 0.01$, $n = 23$)

due to chronic phase of purinergic signaling, which lasts days to months (or longer). This process leads to extracellular ATP accumulation for extended periods[4,5] and contributes to tissue damage and inflammation. According our findings, Feng et al[25] showed that extracellular ATP released by tumor cells of some types of cancer, accumulates in high concentrations and may remain elevated for extended periods. However, in CM post-surgery, this is the first work.

High levels of extracellular ATP act as a danger signal molecule initiating an innate immune response through macrophages induction to release a repertory of proinflammatory cytokines by activating transmembrane P2 and P1 receptors.[11] The pro-inflammatory status initiated by elevated ATP levels can be evidenced by the high levels of pro-inflammatory cytokines observed in CM group when compared to the CT group (Figure 4).

It is known that some inflammatory cytokines in the tumor micro-environment may favor melanoma growth and tumor progression.[26] Among these cytokines, we verified high levels of IL-2, IL-4, IL-6, TNF-α, and IFN-γ in CM patients (Figure 4). Increased levels of IL-2 and IL-6 can indicate a poor prognosis in patients with stage IV, and other cytokines (IL-4, TNF-α, and IFN-γ) stimulate endothelial cell proliferation, migration, and angiogenesis, which are important for melanoma growth and metastasis.[27] Corroborating our data,[28] revised by[29] have shown that in the micro-environment of melanoma tumors high concentrations of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and TGF-β are found. The main point showed by our study is: the increased inflammatory process by extracellular ATP lead an immunosuppressive profile even after melanoma withdrawal.

Although what can be first seen is an installed inflammatory process, we observed high levels of IL-10 (Figure 5C) as well as an augment of ADA activity in platelets and lymphocytes (Figures 5A and 5B) in CM group when compared to CT group. The increase in ADA activity suggests that high adenosine levels are being released. Since adenosine and IL-10 are anti-inflammatory molecules, we can infer that these molecules are mediating an immunosuppressive response to protect adjacent tissues of inflammation.[30] This nucleoside has been reported to mediate cell proliferation, angiogenesis, and acts in tumor progression, an immunosuppressive agent.[31]

Interestingly, besides having alternative routes, the local production of adenosine may occur as a product of cooperation between different cell types present at any given area, but more specifically, in areas of inflammation. Furthermore, it has recently been demonstrated, both in mice and in humans, that B-cells express CD39 and CD73 that produce adenosine and inhibit T-cell proliferation.[32] Knowing that immune suppression is closely related to the

development of lymphatic metastases in melanoma patients, the increase in ADA activity can be related to malignant processes even after surgical removal of the tumor.

It is important to emphasize that IL-10 production by melanoma cells increases as melanomas progress, together with the development of metastasis. This alteration in the capacity to generate and secrete IL-10 is associated with the increasing prognostic implications of transition from the radial phase to vertical growth and the developing risk of regional and visceral metastases. The findings of this study corroborate with the hypothesis that IL-10, acting with other cytokines, induces immune down-regulation that may render the development of metastases.[33]

In summary, despite human study limitations, the whole of results suggests that even after surgical CM removal, the purinergic system can develop a chronic inflammatory micro-environment and this can influence directly on relapse or metastasis. This effect involves the deregulation of nucleotide and nucleoside levels in peripheral blood with potential application in CM prevention or treatment.

5 | CONCLUSION

In conclusion, we showed that purinergic system enzymes have altered activity in Breslow thickness T3/T4 tumor even after surgical excision causing an increase in extracellular ATP levels. The modifications observed in this study may reflect the CM micro-environment alterations, whereas after removal of the tumor, they can represent high CM aggressiveness. The regulation of extracellular nucleotides and nucleosides released represent an important aspect of CM. Besides this understanding, the interaction between tumor cells and the immune suppression of anti-tumor immunity will help provide insights that will allow the introduction of future novel therapeutic approaches for the management of patients with melanoma.

CONFLICTS OF INTEREST

None.

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5.2 ARTIGO 2:

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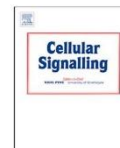

The signaling effects of ATP on melanoma-like skin cancer

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ABSTRACT

Melanoma is a type of skin cancer originated by the malignant transformation of melanocytes. Increasing incidence and mortality require efforts focused on studies and research about this cancer. Its microenvironment is rich in extracellular ATP, but there are no studies evaluating the ectonucleotidases and ATP effects on tumor-derived melanoma cells with known amounts of ATP. This way, the objective of this work was to evaluate the purinergic signaling in the pathophysiology of *in vivo* melanoma and the *in vitro* effects of ATP signaling. We found increased and effective extracellular ATP hydrolysis in platelets and a significant decrease of extracellular ATP levels and adenosine hydrolysis. In addition, we cultured PBMCs of melanoma patients and used ATP salt with specific concentrations to evaluate its signaling effects. The enzymatic activity analysis revealed that even with higher ATP doses cells metabolize adenine nucleotides less efficiently, and present low ATP, ADP and AMP hydrolytic activity in CM compared to CT cells. In summary, we showed for the first time important data about the purinergic signaling in the pathophysiology of melanoma and ATP signaling exercising immunosuppressive effects. Therefore, as already shown for other tumors, the purinergic signaling should be considered a potential target for melanoma management and treatment and could offer novel therapeutic prospects.

1. Introduction

Cutaneous melanoma (CM) or only melanoma is a neoplasm generated through the malignant transformation of epidermal melanocytes, characterized by insidious and fast progression, heterogenic evolution among patients, and significant resistance to diverse therapeutic strategies. Its incidence has been rising worldwide in the last 30 years and although it represents only 4–7% of skin cancers, this type of skin cancer causes approximately 80% of cancer deaths [1].

In recent years, there has been a growing interest in the potential of purinergic signaling for cancer because it plays an important role modulating the inflammatory and immune responses by extracellular biomolecules such as adenine nucleotides (adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) and their derived nucleoside adenosine [2–4]. Their effects depend on nucleotide concentration, expression pattern of purinergic receptors and enzymes, and general dynamics of their synthesis and degradation [5,6].

Levels of extracellular nucleotides and nucleosides are controlled by ecto-enzymes such as ecto-nucleoside triphosphate diphosphohydrolase (*E*-NTPDase; CD39; EC 3.6.1.5) and ecto-adenosine deaminase (E-ADA; EC 3.5.4.4), which are anchored in the cellular surface with their active site facing the extracellular environment [7]. Together, these enzymes constitute a system for the regulation of nucleotide-mediated signaling by controlling the rate, degradation, and formation of nucleosides [8]. In this context, due to this important physiological role, ectonucleotidases have been studied in different pathological and experimental conditions by our research group [9–15].

Among the extracellular purines, ATP is a key extracellular signaling molecule that participates in several physiological processes such as immune response, neurotransmission, vascular tonus, pain sensation, cell proliferation, differentiation, development, and death [10,16–20]. The tumor microenvironment is rich in extracellular ATP [21–23] and its effect depends on both ATP concentration and rate of degradation to adenosine by ecto-nucleotidases. Accumulation of this nucleotide can reflect an active signaling process with relevant

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pathophysiological implications [24].

Previous studies demonstrated that even after surgical excision, melanoma patients showed an increase in extracellular ATP levels [10]. In this case, ATP signaling increases tumor cells and immune cells interaction, causing an immune suppression. ATP effects can justify some of CM aggressiveness mechanisms. While these data indicate that the purinergic system is a strong pivot of melanomagenesis, the understanding of its effects on the pathophysiology of melanoma needs to be better elucidated, mainly in patients not undergoing treatment. Furthermore, increased ATP levels in the tumor microenvironment contribute to cellular and biochemical composition in different ways. Thus, in this work we demonstrated the involvement of signaling effects of ATP in the pathophysiology of melanoma patients (before any treatment intervention) and in cell culture.

2. Materials and methods

2.1. Chemicals and equipment

Chemicals were of the highest available purity and purchased from Sigma (St Louis, MO, USA) or Merck (Darmstadt, Germany) unless otherwise stated. Aqueous solutions were prepared using deionized, filtered water. The centrifuge used was the refrigerated Sigma 3 k-16® and the rotors were changed depending on the samples.

2.2. Patients and samples

Samples consisted of 20 patients with CM and 20 healthy subjects as the control (CT) group. All participants in the CT group were volunteers, free from pathologies that could compromise the research data, with similar gender and age to the CM patients. Patients with decompensated or ischemic heart disease, renal or hepatic impairment, decompensated diabetes, HIV-positive patients, patients with autoimmune diseases, pregnant women and drug users were excluded from the study. Ten milliliters of blood were obtained from each patient at the time of initial diagnosis and used for separation of platelets and lymphocytes, cell culture and biochemical analyses. The same procedure was carried out for the control group.

2.3. Experimental design

Patients with CM were selected according to the International Classification of Diseases (ICD) before surgical removal or any treatment. Control patients in the study were those who had no acute or chronic pathology or CM history in addition to having normal blood pressure and not undergoing any drug therapy. All subjects gave written informed consent to participate in the study. The Human Ethics Committee of Universidade Federal da Fronteira-Sul, Brazil, approved the protocol number 822.782. All analyses were developed in triplicates to ensure reliability of results.

2.4. Platelets and lymphocytes separation

Platelet-rich plasma was prepared by the method of Pilla and col. [25] modified by Lunkes and col. [26]. Total blood was collected with sodium citrate as anticoagulant and centrifuged at 1500 rpm for 10 min. After, the platelet-rich plasma was centrifuged at 5000 rpm for 30 min and washed with 3.5 mM HEPES buffer, pH 7.0 at least twice. The platelet pellets were suspended in HEPES buffer and protein was adjusted to 0.4–0.6 mg/mL.

The mononuclear leukocytes were isolated from human blood collected with EDTA and separated on Ficoll-Histopaque density gradients as described by Bo¨yum [27]. Due to the fact that the methodology described above is employed for separating mononuclear cells, the study performed by Jaques et al. [28] demonstrated a high incidence of lymphocytes (95%) in these samples and a practically insignificant amount of monocytes. For this reason, we treat the samples as containing only lymphocytes.

2.5. Protein determination

Protein was measured by the method of Bradford [29] using bovine serum albumin as standard. This assay is based on the binding of the dye Coomassie Blue G-250 to protein, and this binding is accompanied by measuring the maximum absorbance of the solution at 595 nm.

2.6. E-NTPDase and E-5′-nucleotidase assays

Twenty microliters of platelet-rich plasma preparation (0.4–0.6 mg/mL protein) were added to the reaction mixture of E-NTPDase or E-5'nucleotidase and preincubated for 10 min at 37 °C, to a final volume of 200 μL. E-NTPDase activity was determined by the method of Lunkes and col. [26]. The reaction was started by the addition of ATP or ADP as substrate at a final concentration of 1.0 mM. E-5′-nucleotidase, determined by the method of Heymann and col. [30]. Phosphate released by ATP, ADP and AMP hydrolysis was measured using KH_2PO_4 as standard. Controls were prepared to correct for nonenzymatic hydrolysis, and all samples were analyzed in triplicate. Specific enzyme activities are reported as nmol Pi released/min/mg of protein.

2.7. Quantitative ATP determination

The quantitative ATP determination was developed using a commercial kit for bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin. The assay is based on luciferase's requirement for ATP in producing light – emission maximum ~560 nm at pH 7.8 [31]. This assay is extremely sensitive.

We combined the components of the reaction as follows in order to make a standard reaction solution and adjust the volumes according to particular requirements. Each reaction contained 1.25 μg/mL of firefly luciferase, 50 μM D-luciferin and 1 mM DTT in 1× Reaction Buffer. After a 15-min incubation, luminescence was measured. For determination of ATP levels in PBMCs, ATP concentration was normalized to cell number.

2.8. Adenosine deaminase (ADA) determination

ADA activity from platelets and lymphocytes was determined according to Giusti and Galanti [32] based on the direct measurement of ammonia produced when adenosine deaminase acts in excess of adenosine. Briefly, 50 μL of cells reacted with 21 mmol/L of adenosine, pH 6.5 was incubated at 37 °C for 60 min. Afterwards, the reaction was stopped by adding a solution of 106.2 mM phenol and 167.8 mM sodium nitroprusside as well as a hypochlorite solution. The amount of ammonia produced was measured at 620 nm and the results were expressed in units per liter (U/L).

2.9. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated from fresh blood from healthy participants and melanoma patients within 1–2 h after collection using density medium centrifugation and Ficoll-Paque PLUS (GE Healthcare Bio-Science, Darmstadt, Germany). Blood samples were collected in EDTA medium. Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS), carefully layered onto Ficoll-Paque PLUS, and centrifuged at 400 g for 40 min.

Isolated PBMCs were carefully collected (2–4 mL), resuspended in 15 mL PBS, and centrifuged at 500 g for 15 min. The supernatant was removed, and the pellet was resuspended in 15 mL PBS and centrifuged at 500 g for 10 min [GE Healthcare info]. The supernatant was removed,

and the pellet was resuspended in 1 mL RPMI medium (11.1 mM glucose, supplemented with 3% FBS, 50 units/ml penicillin, 50 g/mL streptomycin) [1].

PBMCs were cultured in RPMI-1640 (Biochrom AG, Berlin, Germany), supplemented with 100 U/mL penicillin (Gibco, USA) and 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany). Cells were adjusted to a concentration of 500.000 cells/mL/well on a 24-well plate. After settling for a few hours, different concentrations of ATP (0.05, 0.5, 5, 10 and 50 μM) were made in culture medium, added to the PBMCs for 24 and 48 h. All cell cultures were incubated at 37 °C in a water jacketed incubator and all cultures and analysis were developed in triplicates. We consider the maximum of 48 h a time for safe and reliable PBMC culture. This way, we analyzed our culture in 24 h (half the time) and in 48 h (maximum time).

2.10. MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water- soluble tetrazolium salt, which is converted to an insoluble purple formazan. Formazan crystals are impermeable to the cell membranes and therefore they accumulate in viable cells.

Cell viability was determined after 24 and 48 h by MTT 3-(4, 5-dimethy thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Briefly, cells were cultured in 96-well plates, and stained for 1 h at 37 °C with MTT reagent (10% concentration) and 5 mg/mL in phosphate-buffered saline (PBS, adjusted pH). Finally, mitochondrial dehydrogenases metabolized MTT to a purple formazan salt that was solubilized by the addition of 100 μL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 560 nm [33,34].

2.11. Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 (Prism 7.03, GraphPad Software, San Diego, CA, USA). Values are presented as the means ± standard error of the mean (SEM), unless otherwise stated. Normality was tested by the Shapiro-Wilk test. The differences between the groups in relation to the studied variables were evaluated through the analysis of unpaired *t*-test and one-way ANOVA. The results were presented as mean and standard deviation. The differences in the probability of rejection of the null hypothesis as being < 5% ($p < .05$) were considered statistically significant. Statistical significance was defined for p values of *$p < .05$, **$p < .01$ and ****$p < .001$.

3. Results

3.1. Clinical characteristics

We evaluated the clinical characteristics of the studied groups through a documented interview with each participant. The characteristics of our study groups are summarized in Table 1. In relation to gender, an increased incidence of CM was observed in men (55%) more than women (45%). The average age was similar between CT and CM groups (46,7 ± 16,6 and 56,1 ± 12,8 years old).

For our CM group, some body areas were more affected: lower extremities (35%), upper extremities (25%), trunk (15%) and head/neck (25%). Regarding risk factors for CM development, sun exposure was higher in CM group (60%) than in control group (55%), and both groups showed elevated percentage for Fitzpatrick Phototype I/II (100% for CM and 95% for CT). The main risk factors for the development of CM result from the combination of constitutional/genetic and environmental factors: skin types I/II Fitzpatrick classification, presence of multiple melanocytic nevi, presence of atypical or dysplastic nevi, history of melanoma or other skin cancer and mutations in genes.

Table 1
Clinical characteristics of studied CT and CM patients. The data were obtained through a documented interview with each participant.

	CT (n = 20)	*CM (n = 20)*
Age	46,7 (± 16,6)	56,1 (± 12,8)
Male (%)	55	65
Female (%)	45	35
Tumor location		
Lower extremities	–	7
Upper extremities	–	5
Trunk	–	3
Head/neck	–	5
Sun exposure (%)	55	60
Fitzpatrick skin classification:		
I / II (%)	95	100
III / IV / V (%)	5	0

CT: Control; CM: Cutaneous Melanoma.

3.2. Alteration on purinergic enzymes activity

To evaluate the purinergic enzymes alterations in melanoma, we first analyzed the hydrolysis of nucleotides ATP and ADP in lymphocytes, and ATP, ADP and AMP in platelets of CM and CT groups (Fig. 1).

The hydrolysis of ATP, ADP and AMP in platelets was significantly increased in CM patients when compared to CT group. As for the hydrolysis of ATP and ADP in lymphocytes, no significant differences were shown between the evaluated groups. These results suggest that platelets of CM patients are activated and represent a key factor about cancer cell extravasation.

3.3. Quantitative ATP determination in serum

In order to confirm the increase of purinergic enzymes activity and ATP consumption in extracellular medium, the extracellular ATP concentration was measured in the serum of CM and CT patients using luciferase/ luciferin reagent. The concentration of extracellular ATP was significantly decreased in CM group when compared to control group (Fig. 2) as expected.

3.4. Adenosine deaminase (ADA) activity

High hydrolysis of extracellular nucleotides causes a formation of large amounts of adenosine. Therefore, we evaluated ADA activity in platelets and lymphocytes of CM and CT groups (Fig. 3). As can be observed, ADA activity in platelets was significantly decreased in CM when compared to CT, but different results were observed in lymphocytes. ADA activity in lymphocytes was significantly increased in CM when compared to CT. These results indicate that (possibly activated) adenosine hydrolysis was inhibited in platelets, possibly promoting immunosuppression, because of its pro-cancer roles. Adenosine hydrolysis was increased in lymphocytes, probably due to its being a defense mechanism against high adenosine concentrations.

3.5. PBMCs culture

Given that high ATP levels and its accumulation can lead to an active signaling process with relevant pathophysiological implications on melanoma development and complications, and based on previous results with patients' analyses [10], we cultured the peripheral blood mononuclear cells of CM and CT patients. We used five doses of adenosine 5′-triphosphate disodium salt hydrate (ATP – 0.05, 0.5, 5, 10 and 50 μM) to treat cells for 24 and 48 h. We used increasing doses of ATP salt to evaluate the time and dose-dependent signaling effects. Fig. 4 shows ATP, ADP and AMP hydrolysis in CM and CT cells after 24 h of treatment with ATP.

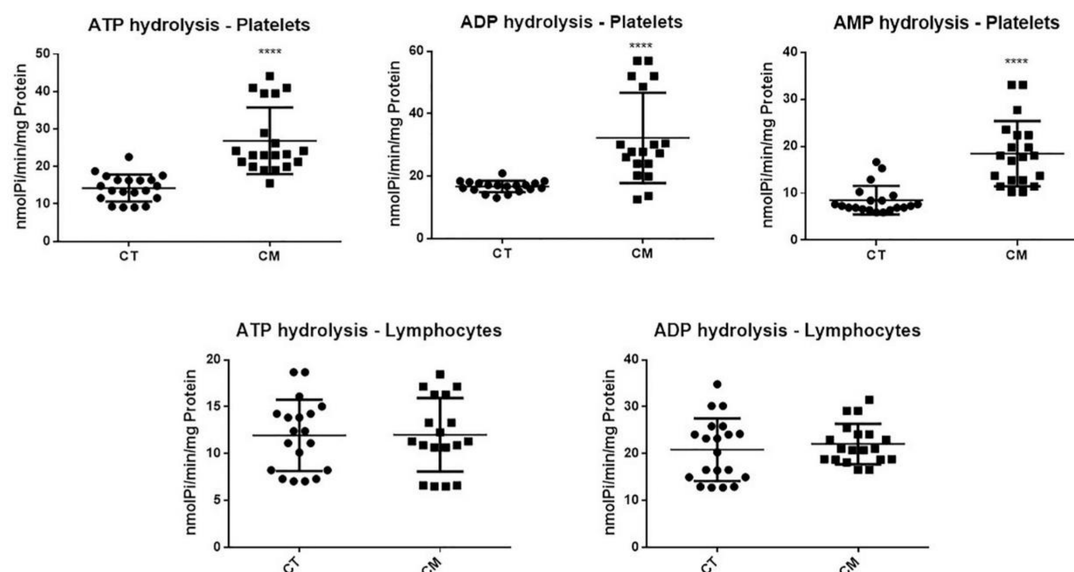

Fig. 1. ATP, ADP and AMP hydrolysis in platelets and lymphocytes in controls (CT) and melanoma patients (CM). E-NTPDase - hydrolyzing ATP and AMP and E-5'-Nucleotidase -hydrolyzing AMP. The assays were followed as described in materials. Data are presented as means ± SEM. ***Indicates a significant difference from the control group (Student's t test, $p < 0.0001$, n = 20).

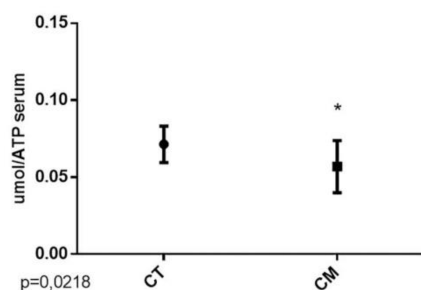

Fig. 2. Quantitative ATP determination in control (CT) and melanoma patients (CM). Extracellular ATP was determined as production of bioluminescence using a luciferin–luciferase reaction system (emission maximum ~560 nm at pH 7.8) through a commercial kit (Invitrogen®). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (Student's t test, $p < 0.05$, n = 20).

As can be observed in relation to ATP hydrolysis, when the cells were exposed to 0.05 μM of ATP, hydrolysis decreased only in CM cells when compared to CT cells. In ADP hydrolysis, no statistical difference was observed between groups. As for AMP hydrolysis, similarly to ATP, when the cells were exposed to 10 μM of ATP for 24 h, hydrolysis decreased significantly in CM cells when compared to CT cells.

Cells were similarly treated with ATP for 48 h (Fig. 5). ATP hydrolysis presented a significant decrease in CM group compared to CT group when cells were treated with 0.05 and 5 μM. In relation to ADP hydrolysis, no statistical difference was observed between the groups again, but AMP hydrolysis increased in CM when compared to CT cells when they were exposed to 10 μM of ATP.

Fig. 6 shows the extracellular ATP concentration in PBMCs culture. Similar to what was found in serum, the extracellular ATP concentration in PBMCs was significantly decreased on melanoma cells when compared to CT (both 24 and 48 h). The same occurred when CM cells were stimulated with 0,05 μM of ATP (for 24 and 48 h). Interestingly, in 5 μM and 50 μM concentrations of ATP treatment, the extracellular ATP concentration significantly increased in CM cells after 48 h of treatment.

In relation to ADA activity (Fig. 7), the results showed different changes in 24 and 48 h after ATP treatment. Fig. 7A shows the ADA activity after 24 h of treatment: we observed a significant decrease in CM cells in 0.5 μM, 5 μM, 10 μM and 50 μM groups when compared to CT cells. Interestingly, a similar result of ADA activity in platelets (Fig. 3) was observed: a decrease in ADA activity at different concentrations of ATP treatment.

Different responses to treatment with ATP during 48 h were observed in ADA activity (Fig. 7B). Although the activity in 0.05, 0.5 and 5 μM was significantly decreased in CM cells when compared to CT cells, higher doses of ATP (10 and 50 μM) showed increased ADA activity when compared to other groups.

3.6. Cellular viability assay

In order to demonstrate that the five doses of ATP (0.05, 0.5, 5, 10 and 50 μM) did not exert toxic effects in the cells, we performed the MTT assay to determine cell viability. In summary, ATP treatment did not alter cell viability.

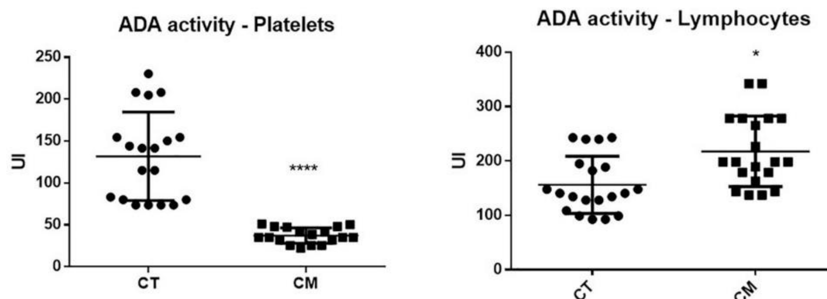

Fig. 3. ADA (Adenosine desaminase – hydrolyzing adenosine) in platelets and in lymphocytes of control (CT) and melanoma (CM) groups. The assay is based on the direct measurement of ammonia produced when adenosine deaminase acts in excess of adenosine. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol the ammonia per minute from adenosine at standard assay conditions. Data are presented as means ± SEM.*Indicates a significant difference from the control group (Student's t test, $p < 0.05$, $n = 20$). ***Indicates a significant difference from the control group (Student's t test, $p < 0.0001$, $n = 20$).

4. Discussion

This study clarifies the effects of ATP as an immunosuppressive signaling molecule in melanoma skin cancer. The extracellular ATP acts actively in tumor environments through its concentration and/or degradation rate for molecules with two or one phosphate – ADP and AMP [24]. By binding to their specific receptors, the purinergic signaling promotes regulatory T cell proliferation and immunosuppression [6,13,35,36].

We evaluated the activity of the purinergic system enzymes in platelets and an increased and effective extracellular ATP hydrolysis was observed, evidenced by a significant decrease on extracellular ATP levels and adenosine hydrolysis. To better understand this finding, for the first time we cultured the PBMCs of these patients and used ATP salt in these cells, given that previous studies demonstrated it is the cause of deleterious changes in melanoma, evidenced by the uncompensated inflammatory profile that it signals. [10,37–39]. The main and novel discovery of this study is that extracellular ATP as well as the ectonucleotidases activities have an important role in signaling the melanoma pathophysiology, with promising therapeutic prospects.

The increase of nucleotide hydrolysis in platelets (Fig. 1) and lower ATP levels in serum (Fig. 2) found in melanoma patients can be a

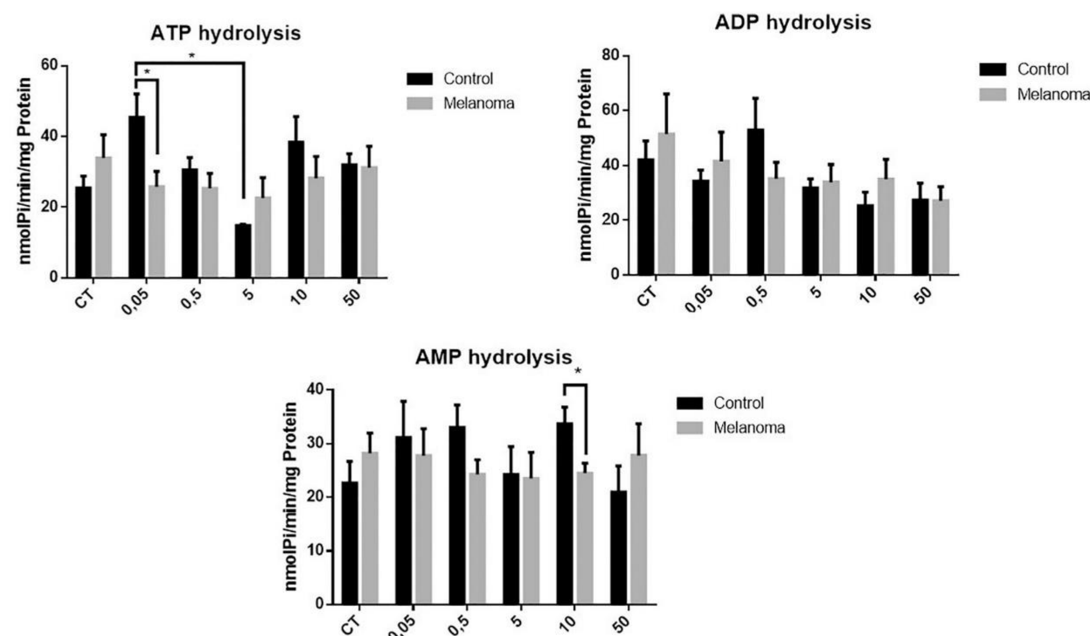

Fig. 4. Effects on ATP, ADP and AMP hydrolysis after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for 24 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$).

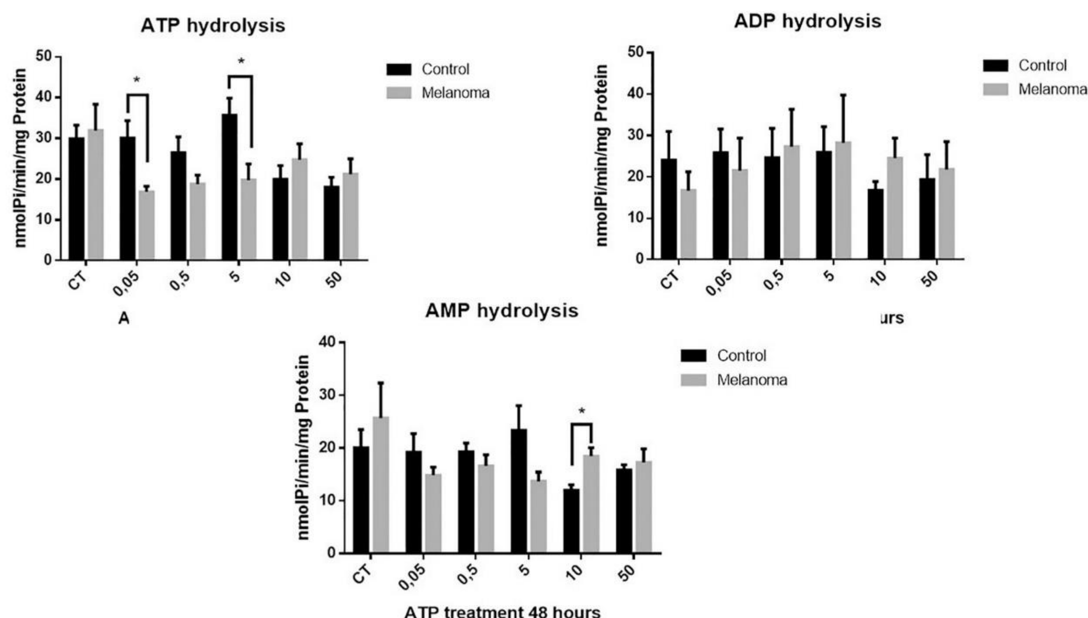

Fig. 5. Effects on ATP, ADP and AMP hydrolysis after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for 48 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$).

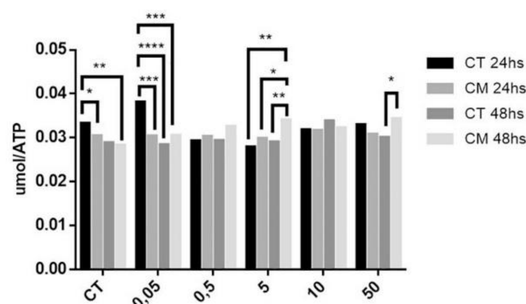

Fig. 6. ATP quantification in control (CT) and melanoma (CM) PBMCs cells after treatment with Adenosine Triphosphate (ATP) with 0,05, 0,5, 5, 10 and 50 μM) for 24 and 48 hours. The assay was followed as described in commercial kit (Invitrogen®). Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$). **Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.001$, $n = 20$). ***Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$).

possible mechanism of immunosuppression: high ATP hydrolysis could lead to the formation of large amounts of adenosine, therefore developing immunosuppression [24]. Interestingly, previous studies of our group with patients who underwent the surgical removal of melanoma showed a decrease of ATP and ADP hydrolysis in platelets and lymphocytes, as well as a decrease of AMP hydrolysis in platelets [10]. This difference between pre and post-treatment of melanoma patients can be a result of a chronic phase of purinergic signaling that can occur from days to months after treatment and lead to an extracellular ATP accumulation for extended periods [14,40], contributing to tissue damage and inflammation.

A study with breast cancer patients clearly demonstrated that hydrolysis of nucleotides by platelets is changed, with significant increase in NTPDase1 activity [41]. In lung cancer, an increase in E-5′-Nucleotidase activity was observed [9], and patients with thyroid cancer demonstrated a post-thyroidectomy increase in all purinergic system enzyme activities [42]. Furthermore, other types of cancer were studied [23,24,43–45] and displayed altered purinergic enzymes activity, what reinforces the importance of studying the mechanisms related to the purinergic system as well as diseases. Besides its immunosuppressive signaling effects, the increase in ATP hydrolysis can rise extracellular concentrations of adenosine, which is linked to tumor progression, chemotaxis, migration, invasion, and metastasis formation – one of the strongest immunosuppressive molecules [45]. Our results showed that in platelets, cells known for promoting metastasis dissemination [24], adenosine hydrolysis was decreased in CM patients, but in lymphocytes, known for immune functions [46], an increase in ADA activity was observed (Fig. 3). The high ADA activity seen in lymphocytes could be explained by the high concentrations of adenosine generated by the increased hydrolysis of ATP, ADP and AMP by platelets. Although the enzyme behavior is different in other diseases such as prostate cancer [24], the same augment in ADA lymphocytes was found in melanoma patients after surgical removal of the tumor, as well as sickle cell anemia and toxoplasmosis [47,48]. Therefore, we can suggest a stronger evidence of a possible immunosuppression caused by ATP signaling.

While immunosuppression caused by differences in ATP and adenosine levels acts in tumor progression [24,49], pharmacological modulation of nucleotide and nucleoside degrading enzymes in the tumor microenvironment can provide efficient means to revert this condition [49]. Thus, based on previous works [22,50–53], we cultured PBMCs from melanoma patients as well as control group and treated

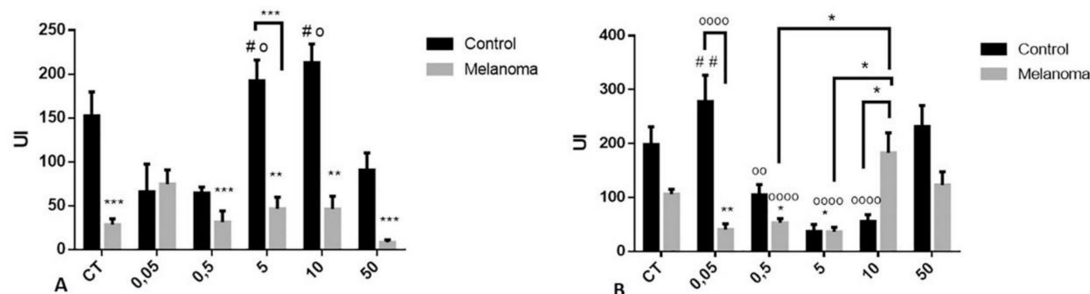

Fig. 7. Effects on ADA (Adenosine desaminase – hydrolyzing adenosine) activity after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for (A) 24 hours and (B) 48 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients (CM). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$). **Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.001$, $n = 20$). ***Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$); #Indicates a significant difference from the control melanoma group (One-Way ANOVA, $p < 0.05$, $n = 20$). ooIndicates a significant difference from the 0,05 CM group (One-Way ANOVA, $p < 0.001$, $n = 20$). ?Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$).

with different concentrations of ATP, in order to elucidate the possible signaling mechanism of this molecule through time and dose-dependent alterations in the purinergic system.

ATP concentrations were selected based on studies on cell lines and mice [10,22,23,51]. In general, it was observed that PBMCs had a similar behavior to the one found in platelets for both 24 (Fig. 4) and 48 h (Fig. 5). However, the enzymatic activity analysis revealed that even with higher ATP doses, cells metabolized adenine nucleotides less efficiently and presented low ATP, ADP and AMP hydrolytic activity in CM compared to CT cells. In human cervical cancer, lower ATP and ADP hydrolysis were observed in cell lines [54]. In this case, the signaling of ATP in these rates could not have any effects on the purinergic system enzymes activity.

It was possible to note that the ATP concentrations used caused diverse effects on cells, not often following a pattern. Therefore, we propose that PBMCs comprise a heterogeneous population that responds differently to extracellular ATP according to the level of P2X7 receptor present in the cell membrane and according the time of exposure [55]. Thus, we shed light on how melanoma cells respond to different extracellular ATP signaling effects.

Knowing that alterations on ATP concentrations lead to increased tumor growth and increased invasiveness of malignancies [56], we analyzed the extracellular ATP levels in cell culture (Fig. 6), and observed higher ATP metabolization in CM cells than CT cells. However, when adding ATP as a signaling molecule, there was an increase in ATP levels in melanoma cells (which can be observed at concentrations of 5 and 50 μM). This effect of high ATP levels in cancer cells was confirmed by a study with ATP infusion in patients with advanced non-small-cell lung cancer, where this molecule could reduce weight loss, increase muscle strength and improve the overall quality of life. These observations were confirmed by the incubation of whole human blood with low/medium ATP (100–500 mM), impairing LPS-stimulated IL-12 and IFNg secretion [57,58].

Studies with other cancer models tried to explain the signaling effects of this molecule. For example, when using breast cancer cells and bone migration of breast cancer cells [59]. However, in nasopharyngeal carcinoma cells the increase of extracellular ATP inhibited the growth and migration of this cell line [39]. This way, ATP released into the tumor microenvironment is considered a biochemical hallmark and could offer novel therapeutic prospects [24] through its signaling mechanism.

To understand the complete purinergic pathways, we performed the ADA activity of PBMCs culture for 24 and 48 h (Fig. 7) to investigate whether hydrolysis of extracellular adenosine formed by ATP degradation was altered after ATP stimuli. We found a different pattern of this enzyme activity depending on the dose and the time of exposure. We were able to note that ADA activity was decreased in cells of melanoma patients even in the control group (without ATP treatment). Except when the treatment dose was 10 μM for 48 h, all other CM groups showed lower ADA activity. These results are in accordance with

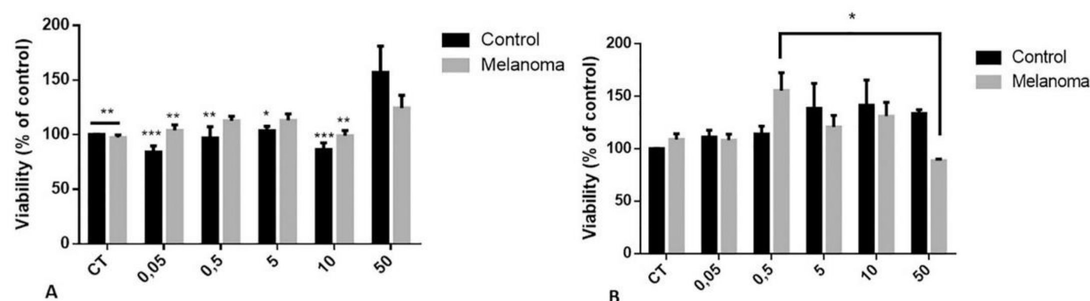

Fig. 8. Cell viability determined after 24 hours (A) and 48 hours (B) by MTT assay in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients (CM). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$). **Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.001$, $n = 20$). ***Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$).

the data obtained from the platelets of these patients, where an intense decrease of adenosine hydrolysis in the CM group was also observed in the CT group.

"The difference between time points means observed by different ATP concentrations in PBMCs could be explained by their P2 receptors binding and activation. Some reports on P2 receptors in cancer cells indicate that ATP in high concentrations might enhance cancer growth and contribute to malignancy [60]. Thus, our results showed that ATP presented different levels of activation of these receptors when cells to different salt concentrations.

Another possible explanation is that extracellular ATP is rapidly degraded to ADP, AMP and adenosine by ectonucleotidases, and these metabolites also modulate the cancer microenvironment by activation of purinergic or adenosinergic signaling. The enzymatic activity analysis revealed that our PBMCs metabolized adenine nucleotides according to different patterns when exposed to different concentrations of ATP and when compared to other tumoral cells [43,61–63]. In addition to all these effects, MTT assays showed that ATP treatment did not alter cell viability, so it was not toxic and did not kill the cells Fig. 8.

5. Conclusion

In summary, we showed for the first time important data about the purinergic signaling in the pathophysiology of *in vivo* melanoma and the effects of *in vitro* ATP signaling. Such understanding allows us to infer that ATP signaling exerts immunosuppressive effects and may modulate the purinergic signaling cascade in an upstream manner, thereby offering new avenues for drug therapies.

Conflicts of interest

None.

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5.3 MANUSCRITO:

Melanoma and oxidative stress: could ATP to be a key molecule?

MELANOMA AND OXIDATIVE STRESS: COULD ATP TO BE A KEY MOLECULE?

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- National Council for Scientific and Technological Development (CNPq).

ABSTRACT

Melanoma is characterized by insidious and rapid progression, causing approximately 80% of skin cancer deaths. It is associated with oxidative stress (OS) and release of inflammatory mediators such as extracellular adenosine 5'-triphosphate (ATP). Despite the fact that ATP shows a stronger immunosuppressive effect in melanoma skin cancer, its involvement in OS microenvironment is not well understood. Therefore, the aim of present study was to determine the oxidative stress parameters in response to various concentrations of ATP *in vitro* as well as to evaluate stress conditions *in vivo*. Forty patients with CM before and after treatment (20 individuals before - CMa and 20 individuals after - CMb) and 20 healthy subjects (the control - CT group) were included. We performed assays for thiobarbituric acid reactive substances and myeloperoxidase activity to evaluate oxidative parameters and measurements of non-protein thiols, protein thiols, glutathione oxidase and ascorbic acid to describe the antioxidant profile. We observed decreased oxidative parameters in melanoma patients treated with surgery and increases in some antioxidant parameters. In cell culture, ATP did not reverse OS or inhibit antioxidant defenses. In summary, for the first time, we showed that ATP possibly as activity involving OS in the peripheral blood mononuclear cells and the oxidative profiles of melanoma patients. Our data suggest that control of ATP levels may reduce cellular damage in melanoma patients.

Keywords: Oxidative profile; Skin cancer; Melanoma; Purinergic system.

1. *Introduction*

Cutaneous melanoma is a neoplasm that develops from malignant transformation of epidermal melanocytes; it is characterized by insidious and rapid progression, with heterogenic evolution among patients, and significant resistance to various therapeutic strategies [1]. The incidence of melanoma has been rising for the last 30 years worldwide; despite the fact that it represents only 4%–7% of skin cancers, melanoma causes approximately 80% of skin cancer deaths [2].

Malignant melanocyte transformation has been associated with oxidative stress (OS) [3]. OS is defined as an unbalance between increased exposure to oxidants and decreased antioxidant capacity; it plays an important role in the pathogenesis and progression of melanoma [4,5]. Oxidative injury of biomolecules (DNA, proteins and lipids) disrupts cells signaling, and depletes alternative sources of cellular energy, often culminating in cell death [6,7]. The intense formation of reactive oxygen species (ROS) that overwhelms enzymatic and non-enzymatic antioxidant protection in melanoma patients can trigger release of mediators such as extracellular adenosine 5'-triphosphate (ATP).

ATP is an extracellular signaling molecule that participates in several physiological processes [8–11]. Tumor microenvironments are rich in extracellular ATP [12–14] and its effects depends on both ATP concentration and the rate of ATP degradation to adenosine by ectonucleotidases. Substantial accumulation of this nucleotide reflects active signaling processes and triggers disease development [15]

We recently reported the effects of ATP in melanoma patients and in cell culture [10,16]. We found increased ATP concentrations even after surgical removal. In cell culture, we observed altered ATP signaling that exerted immunosuppressive effects and modulated the purinergic-signaling cascade. Based on these findings as well as based on the possible relationship between ATP and OS in melanoma development, we aimed in the present study to determine the effect of ATP in Peripheral blood mononuclear cells (PBMC) and to define the parameters of oxidative stress in melanoma patients.

2. *Materials and methods*

Chemical and equipment

Chemicals of the highest available purity were purchased from Sigma (St Louis, MO, USA) or Merck (Darmstadt, Germany) unless otherwise stated. Aqueous solutions were prepared using deionized, filtered water. The centrifuge was a Sigma 3k-16® refrigerated unit and the rotors were changed depending on the samples used.

Patients and samples

The subjects consisted of 40 patients with CM before and after treatment (20 individuals before - CMa and 20 individuals after - CMb) and 20 healthy subjects as the CT group; the CT was selected according to characteristics, age and gender of the CM group. Ten milliliters of blood were obtained from

each patient and were used for cell culture and biochemical analyses. The same procedure was carried out for the control group.

Experimental design

Patients with CM were selected according to the International Classification of Diseases (ICD) and were divided into two studied groups: before surgical removal or treatment (CMa) and after surgical removal (CMb) of the tumor. Control patients were those who had no acute or chronic pathology or CM history, in addition to having normal blood pressure and not undergoing any drug therapy. All subjects gave written informed consent. The Human Ethics Committee of the Federal University of the Southern Frontier approved the protocol under number 822.782. All analyses was performed in triplicate to ensure reliability.

Serum and PBMC separation

Blood samples were collected in tubes containing sodium citrate as anticoagulant for the preparation of plasma. The blood samples for the preparation of serum were collected in the absence of anticoagulant. Plasma and serum samples were obtained by separating supernatants with centrifugation at 2,500 g for 15 minutes. Mononuclear leukocytes were isolated from human blood collected with EDTA and separated on Ficoll-Histopaque density gradients as described by Böyum [17].

Protein determination

Protein levels were measured using the method of Bradford [19] with bovine serum albumin as the standard. This assay is based on the binding of the dye Coomassie Blue G-250 to protein and measuring the absorbance maxima at 595 nm.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMC were isolated from fresh blood from healthy participants and those with melanoma within 1–2 h after collection using density medium centrifugation and Ficoll-Paque PLUS (GE Healthcare Bio-Science, Darmstadt, Germany). Blood samples were collected in EDTA medium. Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS), carefully layered onto Ficoll-Paque PLUS, and centrifuged at 400 g for 40 min.

Separated PBMCs were carefully collected (2–4 ml), resuspended in 15 ml PBS, and centrifuged at 500 g for 15 min. The supernatants were removed, and the pellets were resuspended in 15 ml PBS and centrifuged at 500 g for 10 min [GE Healthcare info]. The supernatants were removed, and the pellets were resuspended in 1 ml RPMI medium (11.1 mM glucose, supplemented with 3% FBS, 50 units/ml penicillin, 50 g/ml streptomycin) [2].

PBMCs were cultured in RPMI-1640 (Biochrom AG, Berlin, Germany) supplemented with 100 U/ml penicillin (Gibco, USA) and 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany). Cells were adjusted to 500,000 cells/mL/well on 24-well plates. After settling for a few hours, various concentrations of ATP (0.05, 0.5, 5, 10 and 50 μM) were prepared in culture medium and were added to the PBMCs for 24 and 48 h. All cell cultures were incubated at 37 °C in a water jacketed incubator and all cultures and analyses were performed in triplicates. We considered the maximum of 48 h to be safe and reliable for PBMC culture. We analyzed our cultures at 24 h (half maximum) and at 48 h (maximum time).

Oxidative profile

Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS), according to Wachowicz and collaborators [20] with some modifications. The supernatants (0.2 mL) were added to reaction mixtures containing trichloroacetic acid 28% (v/v); alkaline solution of thiobarbituric acid (TBA) (0.1 mol/L) followed by heating at 95 °C. After cooling, readings were performed at 532 nm. The results were expressed as nmol MDA/mL.

The plasma activity of the pro-inflammatory enzyme myeloperoxidase (MPO) was measured spectrophotometrically according to Suzuki and collaborators [21]. In the assay, we used 3'-dimethoxybenzidine (DMB) as substrate as well as H_2O_2. The results were expressed as μM quinoneimine.

The quantification of thiol groupings, the classical biomarker of oxidative stress, was made according to Ellman [22] with adaptations. Results were expressed as μmol/NPSH/mL and μmol/PSH/mL.

GSH levels were determined using the technique described by Ellman [22], with potassium phosphate buffer (TFK) 1M at pH 7.4 and 5,5'-dithiobis acid (2-nitrobenzoic acid) (DTNB). The procedure was performed in an ice bath and readings made in a visible spectrophotometer at 412 nm. The results were expressed as mM GSH/mL plasma.

The determination of ascorbic acid serum status was performed using the direct method, with photometric analysis [23]. The method identifies the generation of an orange chromogen produced via the reaction of vitamin C with dinitrophenylhydrazine at 37 ° C, measured spectrophotometrically at 520 nm. The results were expressed as μmol/L ascorbic acid.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (Prism 7.03, GraphPad Software, San Diego, CA, USA). Values are expressed as means ± standard error of the mean (SEM) unless otherwise stated. Normality was tested using the Shapiro-Wilk test. Differences between groups, in relation to the variables of the study, were evaluated using one-way ANOVA. The results were expressed as mean and standard deviation. Differences in the probability of rejection of the null hypothesis being

less than 5% ($p < 0.05$) were considered statistically significant. Statistical significance was defined as p-values of $p < 0.05$.

3. Results

Clinical characteristics

The characteristics of our study groups are summarized in Table 1. We evaluated the clinical characteristics of the studied groups using a documented interview with each participant. The average age was similar between groups: CT: 46.7 ± 16.6 years; CMb: 56.1 ± 12.8 years old; and CMa: 48 ±12.5 years.

We observed a higher incidence of CM in men (65% and 60%) than in women (35% and 30%). The most common location for melanoma was in the lower and upper extremities. Less affected areas included the trunk and head/neck (35% of cases).

Patients with fair skin were at higher risk of melanoma than were those with darker skin. The Fitzpatrick skin typing system was created in 1975 to predict skin reactivity; it has been used worldwide to estimate the risk of skin cancer [24]. We found a high percentage of skin types I/II according to the Fitzpatrick classification – higher than 98% in both groups.

TABLE 1 HERE

Antioxidant and oxidative profiles

Because malignant melanocyte transformation is associated with OS and high ATP levels, and because its accumulation triggers an active signaling process with relevant pathophysiological implications for melanoma development and complications [4,10,16], we determined the oxidant and antioxidant profiles in melanoma patients; we also cultured PBMC cells from melanoma and CT patients before and after tumor removal. We used five doses of adenosine 5′-triphosphate disodium salt hydrate (ATP - 0.05, 0.5, 5, 10 and 50 µM) to treat or expose the cells for 24 and 48 hours.

The Fig. 1a shows MDA production using the TBARS assay. We analyzed serum of CT (controls), melanoma patients before treatment (CMb) and melanoma patients after surgery (CMa). We observed significantly lower MDA levels in CMa than in the other groups ($p = 0.0016$). In cell culture, we evaluated the effects of ATP on MDA production after exposure at different concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (Fig. 1b) and 48 hours (Fig. 1c), in control (CT) and melanoma cells. For these parameters, no differences were observed.

FIGURE 1 HERE

We measured myeloperoxidase activity *in vivo* and *in vitro*. We found significantly lower myeloperoxidase activity in the CMa group than in the other groups ($p = 0.0145$) (Fig. 2a). In terms of

myeloperoxidase activity after treatment with ATP at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (Fig. 2**b**) and 48 hours (Fig. 2**c**), there were no differences between groups.

FIGURE 2 HERE

In terms of antioxidant parameters, the results were notable. Quantified *in vivo,* we found higher levels of NPSH (Fig. 3a) and PSH (Fig. 4a) in CMa than in CT ($p = 0.0004$ and $p < 0.0001$, respectively).

FIGURE 3 HERE

With respect to the effects of ATP at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (Fig 3b/Fig. 4b) and 48 hours (Fig 3c/Fig. 4c) in PBMCs of control (CT) and melanoma patients, no significant difference was observed between the groups.

FIGURE 4 HERE

There were no significant differences in levels of GSH in CT, CMb and CMa patients (Fig. 5a). Similar to the *in vivo* results, GSH determination after treatment with ATP (Fig. 5b and 5c) also showed no significant differences between the groups.

FIGURE 5 HERE

We measured ascorbic acid levels in serum and in PBMC cells. We found higher levels of ascorbic acid in the CMb group than in the CT and CMa groups ($p = 0.0035$). Over 25 hours of treatment (Fig. 6b), no significate alterations were observed. Interestingly, ascorbic acid levels after treatment with ATP showed differences over 48 hours of treatment at 5, 10 and 50 µM. At these concentrations of ATP, ascorbic acid levels were significantly lower in melanoma patients than in CT (Fig. 6c).

FIGURE 6 HERE

4. Discussion

We recently reported that ATP was an immunosuppressive signaling molecule in melanoma [10]. We demonstrated alterations of ATP signaling *in vitro* by purinergic system, altering the evolution and aggressiveness of melanoma. In the present study, we investigated the oxidative stress parameters of ATP at various concentrations *in vitro* as well as evaluating stress condition *in vivo*. To the best of our knowledge, this type of experiment has not yet been reported.

We selected ATP because of its extracellular role during melanoma development. We previously reported [10] that, on the one hand, ATP showed stronger immunosuppressive effects in melanoma; however, its involvement in OS microenvironment was not well understood. The present study was motivated by the notion that melanoma is characterized by oxidant/antioxidant imbalance, leading to OS [4].

OS and ROS play crucial roles in melanoma pathophysiology [25,26]. The effects of OS may be mediated by peroxidation of membrane lipids, or by interaction of ROS with functional thiol/disulfide groups [5,27]. On the basis of this reasoning, we measured peroxidation of membrane lipids in melanoma patients before and after treatment and controls and we found lower MDA levels in treated patients (Fig. 1a), but no differences when PBMC cells were incubated with ATP at various concentrations (Fig. 1b and 1c).

Our results suggest that treatment may decrease the peroxidation of membrane lipids and that ATP in the concentrations we used did not alter this status. Confirming that treatment decreased MDA production, th Sander et al. [28] reported significantly elevated MDA levels in malignant tissues of melanoma patients. They were the first group to identify a correlation between melanoma and MDA in human skin *in vivo*. Similar to our findings, Gadjeva et al. [29] documented significant increases in plasma MDA in melanoma patients; however, they also showed that plasma MDA levels decreased after surgery (removal of melanoma tissues) suggesting that melanoma tissue is a significant ROS producer.

Having noted these important data, we measured activity of myeloperoxidase (MPO), another oxidant parameter, and we observed similar results to those of TBARS: decreased quinoneimine levels in treated patients (Fig. 2a), but no differences when the PBMC cells were incubated with ATP at various concentrations (Fig. 2b and 2c). Liu et al. [30] reported increased MPO activity in B16F10 tumor cells. In their study, MPO-expressing myeloid-derived cells functioned as anti-tumor components of the cellular innate immune response during early melanoma progression in a NF-κB-dependent manner.

TBARS and MPO values were higher in the retinas of eyes with choroidal melanoma, but declined significantly with increasing distance from the tumor [31]. We therefore hypothesized that oxidative stress would decrease in melanoma patients after surgical treatment and that ATP would not have stressor or protective action in these cells.

In addition to evaluating OS, we also performed non-enzymatic antioxidant analyses, including quantification of thiol groupings, measurement GSH levels and determination of ascorbic acid. Notably, NPSH (Fig. 3a) and PSH (Fig. 4a) levels were significantly higher in CMa than in CMb and CT; however, cell culture showed that ATP did not have stressor or protective action, as expected.

This result was also reported by Bernardes et al. [32]. They evaluated the relationship between systemic OS and Breslow thickness in 43 patients with cutaneous melanomas and showed that thiol levels were higher in patient samples than in controls. Other studies in humans demonstrated the presence of elevated levels of antioxidant molecules in melanoma cells [28,33]. This was confirmed by studies conducted in animals or cell lines [32,34–36], suggesting that moderate ROS levels have a pro-tumorigenic role [4].

Despite the fact that ROS react with most biomolecules, their major targets are unsaturated lipids and intracellular thiols. Oxidation results in alterations in protein structure and function, representing a

versatile and robust defense system against biochemical perturbations caused by OS [37]. Because of high inter-individual variation, possibly caused by a varying genetic backgrounds of the subjects, GSH activity did not show any variation between the groups, and still less by the addition of ATP in PBMCs (Fig. 5).

This is an important result, because GSH is a natural protective mechanism for prevention of oxidative damage and promotion of cell survival [4]. One explanation for this result could be the possible low expression of glutamate-l-cysteine ligase catalytic subunit (GCLC), a key factor of GSH synthesis in these patients. Another study [38] investigated this relationship in malignant melanoma cell lines and concluded that GCLC levels correlated with better 5-year overall survival.

Another important natural protective molecule in prevention of oxidative damage is ascorbic acid [39]. We demonstrated that, in CMb, ascorbic acid levels higher than those of other groups. This confirmed our hypothesis that OS decreases in melanoma patients after surgical treatment, because prior to treatment, cancer causes high OS levels.

Ascorbic acid is an essential vitamin in humans [39]. Its antitumor activity has been studied extensively in numerous *in vitro* and *in vivo* studies with both human and animal tumors. Correlations were found between tumor ascorbic acid levels and longer disease-free survival [40]. In agreement with the results of our study, Wagner et al. [41] recommended using intravenous ascorbic acid levels as an adjuvant to IL-2 treatment of melanoma.

We demonstrated that ATP at concentrations of 5, 10 and 50 μM decreased ascorbic acid levels in melanoma PBMC cells at 48 hours of treatment. This suggests that ATP, in addition to exerting immunosuppressive effects [10] also has a damage effect, decreasing antioxidant levels that might contribute to oxidative stress.

Similar to our study, other studies showed the deleterious effects of ATP *in vitro*. For example, ATP appeared to prime neutrophils for functional responses to various inflammatory mediators, as indicated by increased production of ROS [8,42]. Extracellular nucleotides have also been shown to stimulate ROS generation and were shown to enhance LPS-induced NO production in mouse macrophages, probably via P2X7 receptor activation [43–45].

5. *Conclusion*

ATP promotes oxidative damage in PBMC cells of melanoma patients by inhibiting antioxidant defenses. There is reduced oxidative stress in melanoma patients after surgical removal. This suggests that ATP levels in melanoma patients could increase oxidative stress. Taken together, our results suggest that ATP levels are controlled, cellular oxidative damage in melanoma could be reduced.

6. *Conflicts of interest:* none

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Table 1: Clinical characteristic of CT and CM patients before and after treatment.

	CT (n = 20)	***CMb*** (n = 20)	***CMa*** *(n = 20)*
Age	46.7 (±16.6)	56.1 (±12.8)	48 (±12.5)
Male (%)	55	65	60
Female (%)	45	35	30
Tumor location			
Lower extremities	Not applicable	7	11
Upper extremities	Not applicable	5	3
Trunk	Not applicable	3	4
Head/neck	Not applicable	5	2
Fitzpatrick skin classification:			
I / II (%)	95	100	98
III / IV / V (%)	5	0	2

CT: Control; CMb: Cutaneous Melanoma before treatment; CMa: Cutaneous Melanoma after treatment.

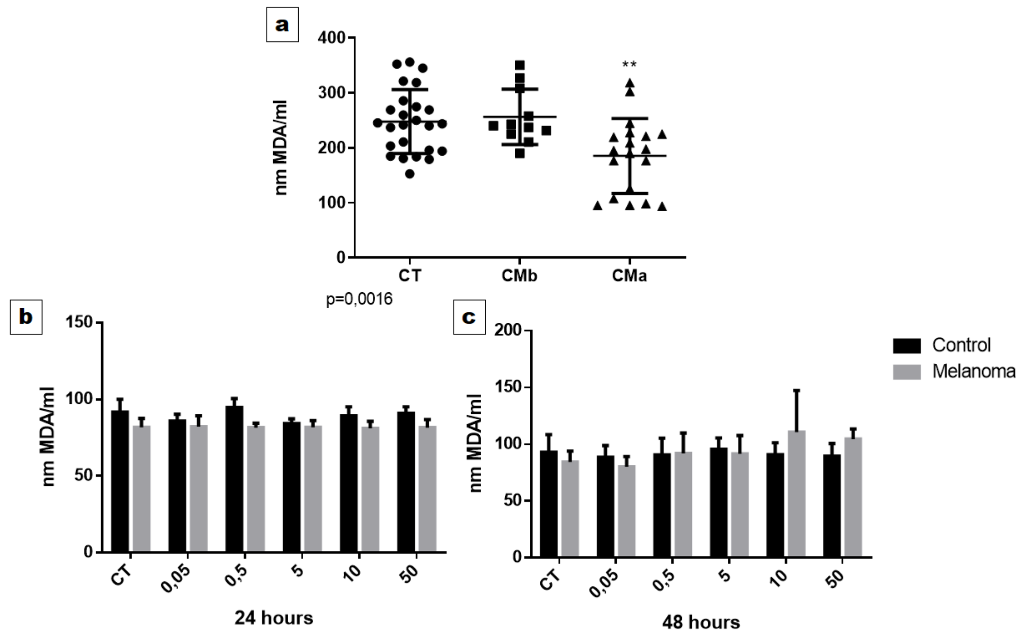

Fig.1: MDA levels by TBARS assay. MDA production in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) (**a**). Effects MDA levels after treatment with adenosine triphosphate (ATP) at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (**b**) and 48 hours (**c**), in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assays were performed as described in methods. Data are presented as means ± SEM. **Indicates a significant difference from the CT and CMb group (a: one-way ANOVA; b and c: two-way ANOVA, $p < 0.05$, $n = 20$).

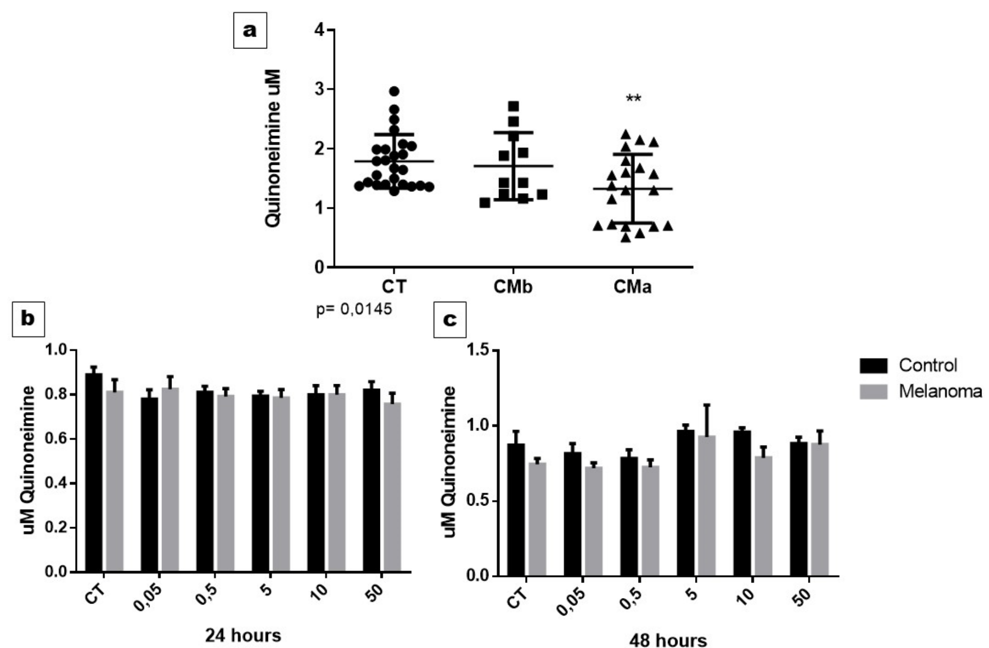

Fig. 2: Myeloperoxidase activity *in vivo* and *in vitro*. Myeloperoxidase activity in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) (**a**). Myeloperoxidase activity after treatment with adenosine triphosphate (ATP) at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (**b**) and 48 hours (**c**), in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assays were performed as described in methods. Data are presented as means ± SEM. **Indicates a significant difference from the CT and CMb group (a: one-way ANOVA; b and c: two-way ANOVA, $p < 0.05$, $n = 20$).

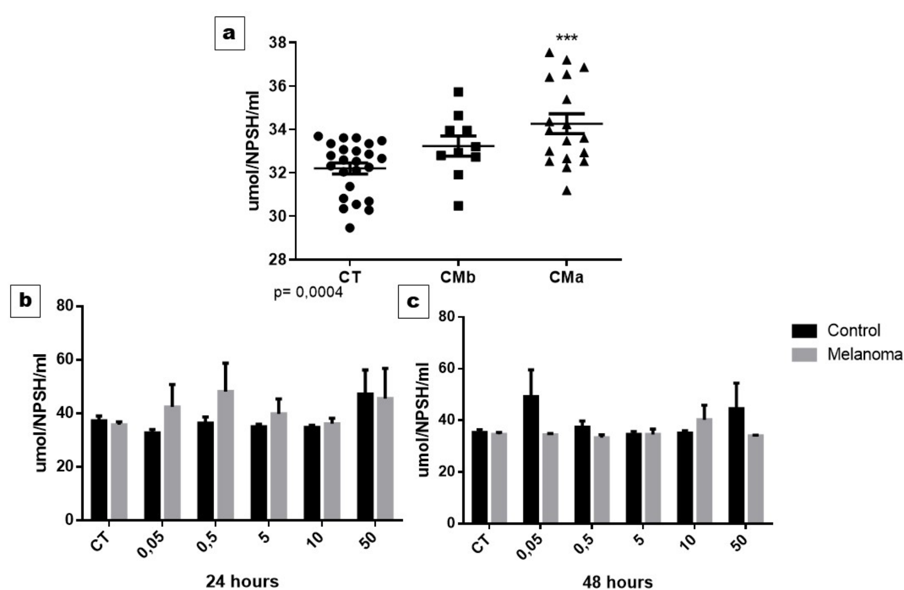

Fig. 3: Non-protein thiols (NPSH) quantitation *in vivo* and *in vitro*. NPSH in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) (**a**). NPSH after treatment with adenosine triphosphate (ATP) at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (**b**) and 48 hours (**c**), in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assays were performed as described in methods. Data are presented as means ± SEM. ***Indicates a significant difference from the CT and CMb group (a: one-way ANOVA; b and c: two-way ANOVA, p <0.001, n = 20).

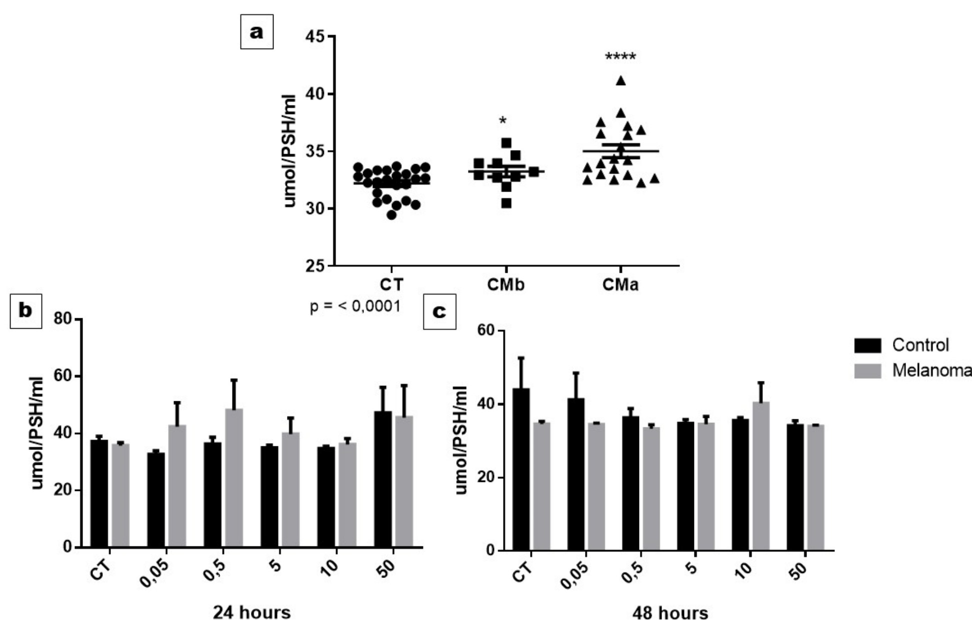

Fig. 4: Protein thiols (PSH) quantitation *in vivo* and *in vitro*. PSH in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) (**a**). PSH after treatment with adenosine triphosphate (ATP) at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (**b**) and 48 hours (**c**), in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assays were performed as described in methods. Data are presented as means ± SEM. ****Indicates a significant difference from the CT (a: one-way ANOVA; b and c: two-way ANOVA, p <0.001, n = 20).

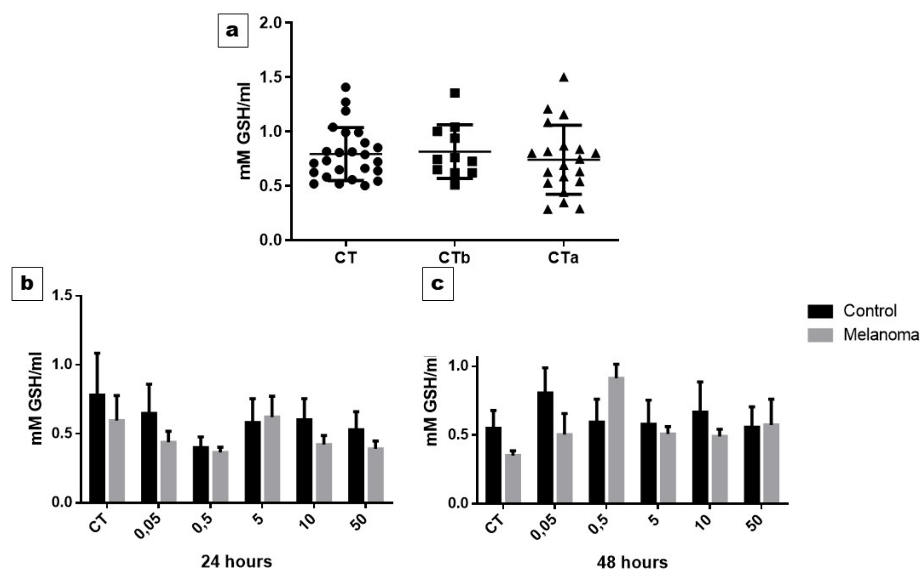

Fig. 5: GSH determination. Quantitation of GSH in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) (**a**). GSH determination after treatment with adenosine triphosphate (ATP) at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (**b**) and 48 hours (**c**), in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assays were performed as described in methods. Data are presented as means ± SEM. a: one-way ANOVA; b and c: two-way ANOVA, $p < 0.001$, $n = 20$.

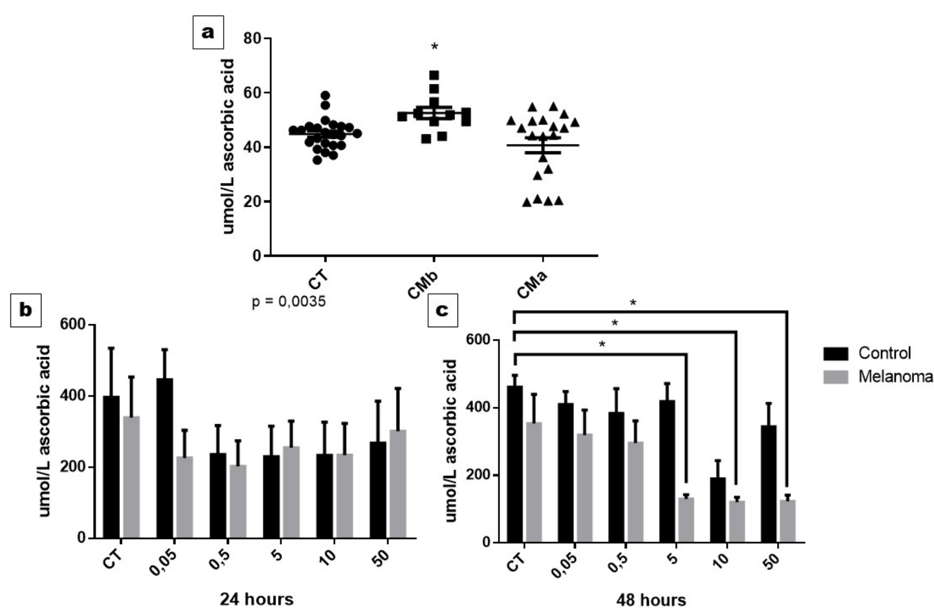

Fig. 6: Ascorbic acid assay in serum and PBMC cells. Quantitation in CT (controls), melanoma patients before treatment (CMb) and melanoma patients after treatment (CMa) (**a**). Ascorbic acid levels after treatment with adenosine triphosphate (ATP) at various concentrations (0.05, 0.5, 5, 10 and 50 µM) for 24 hours (**b**) and 48 hours (**c**), in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assays were performed as described in methods. Data are presented as means ± SEM. *Indicates a significant difference from the CT group (a: one-way ANOVA; b and c: two-way ANOVA, $p < 0.05$, $n = 20$).

5. DISCUSSÃO

Os dados apresentados nesta tese demonstram que o ATP possui efeitos imunomodulares no câncer de pele do tipo melanoma, podendo acelerar o curso da doença dependendo da sua concentração e taxa de degradação. As modificações observadas neste estudo podem refletir as alterações do microambiente tumoral, ao passo que podem justificar a alta agressividade deste tipo de câncer.

No contexto mundial, cerca de 20% dos indivíduos com melanoma (qualquer subtipo) desenvolvem metástases e, para os diagnosticados com doença metastática, o prognóstico é desfavorável, com uma sobrevida média de apenas 6 a 9 meses (DA ROCHA DIAS et al., 2013). No Brasil os maiores índices de incidência de melanoma estão na região Sul (MORENO, MARCELO et al., 2017; MORENO; CONTE; MENEGAT, 2015), mais especificamente na região oeste do estado de Santa Catarina, razão pela qual escolhemos essa área geográfica para estudo.

Em nossos estudos (MANICA et al., 2018; MÂNICA et al., 2019) encontramos dados epidemiológicos que merecem destaque. A média de idade dos pacientes com melanoma foi de 51,4 anos com predomínio no sexo masculino em relação ao sexo feminino. Nossos resultados validam o que é encontrado na literatura (BATTISTI et al., 2009; MORENO; CONTE; MENEGAT, 2015), e que consagra adultos jovens como os mais susceptíveis para o melanoma. Ainda não se sabe ao certo o motivo pelo qual o melanoma mostra a tendência de aumento das taxas de diagnóstico na meia-idade, pois seria de se esperar uma maior ocorrência em faixas etárias mais avançadas, em virtude do efeito cumulativo da radiação solar e deterioração do sistema imune.

Em relação as regiões anatômicas onde o melanoma apresentou maior incidência no grupo estudado, pode-se observar que as mais afetadas foram as que ficavam cronicamente expostas ao sol: membros inferiores, membros superiores e tronco. Isso pode ser justificado pelo aumento da exposição solar nestes locais e falta de conscientização e/ou medidas de prevenção. Em concordância com esses dados, outros estudos realizados na região oeste do estado de Santa Catarina (MORENO et al., 2012; MORENO; CONTE;

MENEGAT, 2015) mostraram uma maior incidência de melanoma nos membros superiores e inferiores nas mulheres e no tronco, cabeça e pescoço dos homens.

No que se refere à classificação do tumor, a grande maioria dos casos foram classificados como melanoma extensivo superficial, seguido do melanoma nodular e menos incidente, mas não menos importante o lentigo maligno. O melanoma extensivo superficial é mais comum em indivíduos caracterizados como fototipos I e II (pessoas de pele e olhos claros e com muitos nevos) ou que tenham casos de melanoma na família, características frequentes na população estudada (INCA, 2018).

Os estudos apresentados nesta tese confirmam o envolvimento do ATP extracelular na fisiopatologia do melanoma, através de experimentos *in vivo* e *in vitro,* em comparação com um grupo controle pareado por sexo, idade, características fenotípicas e história de exposição solar.

Nosso grupo de pesquisa vem demonstrando as alterações no sistema purinérgico, principalmente nas ectoenzimas, em diversas doenças e no câncer (BAGATINI et al., 2018; BALDISSARELLI et al., 2018; BATTISTI et al., 2013; CARDOSO et al., 2015; DO CARMO ARAÚJO et al., 2005; LUNKES et al., 2003; SCHETINGER et al., 2007; ZANINI et al., 2012). Contudo, a relação do ATP extracelular como uma molécula chave neste processo de recaída, mestástases ou até mesmo no desenvolvimento primário de um melanoma, ainda não tinha sido explorada.

A liberação extracelular de ATP e sua consequente hidrólise para ADP, AMP e adenosina, tem sido relatada como fator determinante para progressão de cânceres, pois tais moléculas desempenham um papel fundamental na regulação da inflamação e da homeostase nos tecidos através da ativação dos receptores purinérgicos (BASTID et al., 2015). Verificamos em nossa pesquisa que nos pacientes com melanoma, as enzimas do sistema purinérgico apresentaram uma modificação da sua atividade mesmo após a excisão cirúrgica do tumor, provocando um aumento dos níveis de ATP extracelular.

A literatura aponta que o ATP extracelular desempenha um papel preponderante na inflamação da pele, atuando como um amplificador da resposta imune através da geração de outros mediadores inflamatórios que recrutam leucócitos para o local da lesão (ANTONIOLI et al., 2018). Sendo

assim, evidenciamos um perfil inflamatório descompensado nestes pacientes, podendo ser a causa de alterações deletérias mesmo após a remoção cirúrgica do melanoma.

Em relação ao perfil inflamatório, encontramos um aumento de IL-2, IL-4, IL-6, TNF-α e IFN-γ nos pacientes com melanoma após a remoção cirúrgica do tumor, o que pode indicar um prognóstico desfavorável, pois tais citocinas estimulam a proliferação de células endoteliais, migração e angiogênese, que são importantes fatores para o crescimento e metástases de melanomas (YURKOVETSKY et al., 2007).

Ademais, observamos que os pacientes com melanoma apresentaram um aumento nos níveis de IL-10 bem como um aumento da atividade ADA em plaquetas e linfócitos. Como a adenosina e a IL-10 são moléculas anti-inflamatórias, podemos inferir que estas moléculas estão mediando uma resposta imunossupressora com o objetivo de proteger os tecidos da inflamação instalada.

Aliados a estes dados, realizamos uma investigação em pacientes com melanoma antes de qualquer tratamento ou da remoção cirúrgica do tumor. Observamos um aumento da hidrólise dos nucleotídeos ATP, ADP e AMP nas plaquetas dos pacientes com melanoma e uma menor quantificação do ATP extracelular nestes pacientes, ou seja, a alta hidrólise deste nucleotídeo pode levar à formação de grandes quantidades de adenosina, desenvolvendo assim imunossupressão (STAGG; SMYTH, 2010).

Nesse sentido para clarear os efeitos do ATP como molécula imunossupressora e sinalizadora, cultivamos as PBMCs desses pacientes e usamos ATP em diferentes concentrações nessas células. No geral, a análise da atividade enzimática revelou que as PBMCs dos pacientes com melanoma metabolizaram os nucleotídeos de adenina diferentemente quando expostos as concentrações de ATP, ou seja, encontramos diferentes atividades enzimáticas dependendo da concentração de ATP que estavam expostas e dependendo do tempo de exposição (24 ou 48 horas).

Em outros estudos realizados com diferentes células tumorais, também foi possível observar que o ATP possui atividade sinalizadora de acordo com sua liberação para o tecido extracelular e sua concentração (BASTID et al., 2015; RITEAU et al., 2012). Seu papel como mediador pró-inflamatório o caracteriza

como potencial agente que favorece a interação tumor-hospedeiro (FANG; TIAN, 2017). Sendo assim, a partir deste experimento, concluímos que o ATP exerceu efeitos imunossupressores no melanoma através da modulação da cascata de sinalização purinérgica e sabendo que a inflamação geralmente está associada ao estresse oxidativo, nosso último estudo buscou entender a relação do ATP no perfil oxidativo de PBMCs dos pacientes com melanoma.

Como mencionado, a atividade do ATP extracelular nas concentrações de 0,05 a 50 µM apresentou efeito imunossupressor nas células dos pacientes com melanoma e também demonstrou favorecer a geração de estresse oxidativo. Este ambiente imunossupressor e rico em EROS favorece o desenvolvimento de mestástases rapidamente, caraterística muito comum nos melanomas (CALLAHAN; FLAHERTY; POSTOW, 2016).

Nosso estudo mostrou que o ATP possivelmente exerce ações de dano oxidativo em pacientes com melanoma através da redução de defesas antioxidantes, pois observamos uma diminuição dos níveis de vitamina C na cultura de PBMC exposta ao ATP. Tal fato é de suma importância, pois sabe-se que um nível elevado de estresse oxidativo em melanomas aumenta sua agressividade (HAMBRIGHT et al., 2015).

Também demonstramos que há uma diminuição de alguns marcadores de estresse oxidativo em pacientes com melanoma após a remoção cirúrgica do tumor, sendo um bom indicativo da efetividade do processo cirúrgico.

Por meio da nossa investigação, verificamos que altas concentrações de ATP no microambiente tumoral podem favorecer a agressividade do melanoma. Aliado a isso, Fang e Tian (2017) concluíram que o ATP tem a capacidade de estimular a invasão de diferentes linhagens de cânceres humanos em testes i*n vitro*. (FANG; TIAN, 2017)

Diante do exposto, nota-se que o melanoma tem efeitos profundos nos pacientes acometidos e esta tese nos mostra que o ATP extracelular pode ser uma molécula chave nesse processo de desenvolvimento, recaídas ou metástases. Pode-se perceber a estreita relação entre processos inflamatórios e danos oxidativos na fisiopatologia do câncer de pele tipo melanoma. A análise destes resultados apresenta grande relevância para o contexto médico, visto que, a incidência e a mortalidade crescente exigem um esforço centrado

na prevenção primária, bem como nos estudos e pesquisas acerca deste câncer.

6 CONCLUSÃO

- Observamos uma diminuição da hidrólise dos nucleotídeos nos pacientes com melanoma após a remoção cirúrgica do tumor em contraste de aumento quando foram avaliados pacientes com melanoma antes da retirada cirurgica. As enzimas demonstraram expressão similar em pacientes com melanoma e controles.
- Tais achados nos levaram a concluir que as modificações observadas na atividade das enzimas promoveram um aumento de ATP extracelular, confirmado pela sua quantificação podendo servir como papel chave no estudo do estadiamento de melanomas.
- Os pacientes com melanoma após remoção cirurgica do tumor apresentaram um ambiente inflamatório descompensado, evidenciado pelo aumento das interleucinas IL-2, IL-4, IL-6, TNF, IFN-Y, IL-17A, IL-10 e aumento da atividade da ADA.
- Em relação aos parâmetros de estresse oxidativo em pacientes antes e após tratamento cirúrgico, foi observado uma diminuição dos danos oxidativos e um aumento das defesas antioxidantes após a cirurgia, confirmando a efetividade deste tipo de intervenção para lesões localizadas.
- O ATP extracelular pode estar exercendo efeitos sinalizadores, imunossupressores e oxidante na cultura de PBMCs dos pacientes com melanoma.

Em conjunto, esses resultados nos permitem inferir que o ATP é uma importante mólecula envolvida no processo de melanomagênese e progressão tumoral. As alterações evidenciadas neste trabalho podem ajudar a elucidar mecanismos desconhecidos envolvidos na fisiopatologia do melanoma, bem como, ser uma ferramenta na prática clínica para monitoramento e através do controle dos seus níveis, melhorar o prognóstico para pacientes acometidos por esse câncer de alta letalidade e agressividade.

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APÊNDICE

Apêndice 1: TERMO DE CONSENTIMENTO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

O Curso de Enfermagem da Universidade Federal da Fronteira Sul, está desenvolvendo um projeto de pesquisa intitulado**:** Avaliação de Enzimas que Degradam Nucleotídeos e Nucleosídeos de Adenina em Pacientes com Melanoma Cutâneo, através da mestranda Aline Mânica - Universidade Federal da Fronteira Sul - Rua General Osório, 413D, CEP: 89802-210. Bairro Jardim Itália, Chapecó SC, que tem por objetivo estudar dosagens bioquímicas em algumas enzimas de pacientes da cidade de Chapecó – SC, que tiveram diagnóstico de melanona cutâneo. O projeto de pesquisa justifica-se pelos elevados fatores de risco presentes, visto que os hábitos de vida da população, a exposição solar intermitente sem a devida proteção e as características étnicas, são também desfavoráveis: uma maioria de habitantes de pele clara expostos a radiação solar vários meses por ano.

Os voluntários participantes da pesquisa permitirão uma coleta de sangue. Todo o material utilizado para a coleta será descartado adequadamente. Em caso de acidente de coleta, os pacientes poderão desenvolver leve inflamação local ou pequena/discreta hemorragia, neste caso serão atendidos e receberão os cuidados necessários no local. As amostras serão tratadas de acordo com os protocolos experimentais estabelecidos.

A participação neste estudo é livre e voluntária, sendo que não haverá nenhuma forma de compensação financeira ou custos para o participante. No entanto, a participação no estudo garante a realização de exames laboratoriais, como forma de devolutiva a participação no projeto de pesquisa como: hemograma, perfil lipídico e glicídico e parâmetros inflamatórios. Esses exames serão realizados e entregue gratuitamente para os voluntários com orientações em caso de alguma alteração patológica, sendo estes encaminhados para atendimento especializado.

A recusa na participação não leva a nenhum prejuízo ou comprometimento dos cuidados de saúde aos pacientes. A qualquer momento durante a realização da

pesquisa e após finalização da mesma os participantes poderão esclarecer qualquer dúvida através dos contatos disponibilizados abaixo.

Pelo presente Termo de Consentimento Livre e Esclarecido, declaro que estou de acordo em participar deste projeto de pesquisa, livre de qualquer constrangimento, pois fui informado de forma clara e detalhada dos objetivos e dos procedimentos que serão realizados. Fui igualmente informado da garantia de receber respostas a qualquer dúvida que ainda puder ter sobre assuntos relacionados com a pesquisa por meio do contato com o pesquisador e/ou E-mail, (vide endereço abaixo), e da liberdade de retirar meu consentimento a qualquer momento, sem que haja prejuízo de qualquer ordem.

Os dados coletados ficarão sob-responsabilidade do pesquisador e os mesmos serão utilizados apenas para fins científicos, garantindo o anonimato dos sujeitos durante todas as fases da pesquisa e mesmo após finalização da mesma.

Ciente e de acordo com o que foi anteriormente exposto, eu ____ estou de acordo em participar nesta pesquisa, rubricando a primeira página e assinando a segunda página deste consentimento. Uma via ficará de posse do pesquisador e a outra com o participante.

Chapecó/ SC, ____de_______201_.

____

Assinatura

____

Aline Mânica/Pesquisadora/(49) 9166-1637 ou (55) 9990 – 2460
Rua Marechal J. Bormann, 61 E. Centro, Chapecó SC CEP: 89.802-200
Universidade Federal da Fronteira Sul. Rua General Osório, 413D, CEP: 89802-210. Bairro Jardim Itália, Chapecó SC

Margarete Dulce Bagatini/Coordenadora (49) 9911-4126
Comitê de Ética em Pesquisa (CEP) da Universidade Federal da Fronteira Sul
Avenida General Osório, 413-D, Jardim Itália, Ed. Mantelli, 3º andar.
CEP: 89.802-210 - Chapecó-SC.
Fone: (49) 2049-1478
E-mail: cep.uffs@uffs.edu.br

Apêndice 2: Fichas de coleta para pacientes com melanoma e controles

FICHA DE COLETA: PACIENTES COM MELANOMA CUTÂNEO

Nome:
RG:____________________
Data de nascimento: __/__/__
Telefone: ________________
Sexo: ( ) F ( ) M
Endereço:__________________________
____
____
____
Há quanto tempo foi diagnosticado o melanoma?
____
Qual o tipo e localização do melanoma:
____
___

Toma medicamentos? Sim ( ) Não ( )
Se sim, quais são?_______________________________
____

Já fez transfusão de sangue? Sim ( ) Não ( )
Você fuma? Sim ( ) Não ( )
Ingere bebidas alcoólicas frequentemente? Sim ( ) Não ( )
Tem ou teve alguma das doenças abaixo?
- Problemas cardíacos? Sim ( ) Não ( )
- Pressão alta? Sim ( ) Não ( )
- Anemia? Sim ( ) Não ( )
-Hepatite? Sim ( ) Não ( )
- Diabetes? Sim ( ) Não ( )
- Depressão? Sim ( ) Não ( )
-HIV? Sim ( ) Não ( )
-Outras?______________________________

Tem exposição solar freqüente?
Sim ( ) Não ( )
Cor da pele:
( )Branca ( )Morena ( )Negra

Profissão:

Data coleta: ___/___/___

FICHA DE COLETA: PACIENTES SAUDÁVEIS - CONTROLES

Nome:
RG:____________________
Data de nascimento: __/__/__
Sexo: ( ) F ( ) M
Telefone: ______________________
Endereço:__________________________
____
____
____

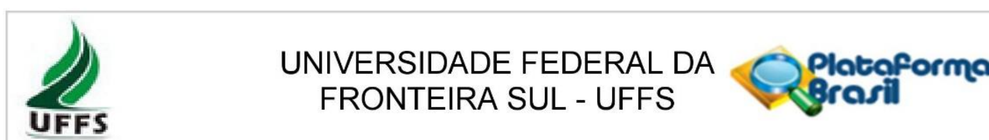
Toma medicamentos? Sim ( ) Não ( )
Se sim, quais são?
Já fez transfusão de sangue? Sim ( ) Não ( )
Você fuma? Sim ( ) Não ( )
Ingere bebidas alcoólicas frequentemente? Sim ( ) Não ( )
Tem ou teve alguma das doenças abaixo?
- Problemas cardíacos? Sim ( ) Não ( )
- Pressão alta? Sim ( ) Não ( )
- Anemia? Sim ( ) Não ( )
-Hepatite? Sim ( ) Não ( )
- Diabetes? Sim ( ) Não ( )
- Depressão? Sim ( ) Não ( )
-HIV? Sim ( ) Não ( )
Outras?______________________________

Tem exposição solar freqüente?
Sim ( ) Não ( )
Cor da pele:
( )Branca ( )Morena ( )Negra

Profissão:
____

Data coleta: ___/___/___

ANEXO: APROVAÇÃO COMITE DE ÉTICA



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de Enzimas que Degradam Nucleotídeos e Nucleosídeos de Adenina em Pacientes com Melanoma Cutâneo.

Pesquisador: Margarete Dulce Bagatini

Área Temática:

Versão: 3

CAAE: 33702814.5.0000.5564

Instituição Proponente: UNIVERSIDADE FEDERAL DA FRONTEIRA SUL - UFFS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 822.782

Data da Relatoria: 19/10/2014

Apresentação do Projeto:

TRANSCRIÇÃO DO RESUMO:

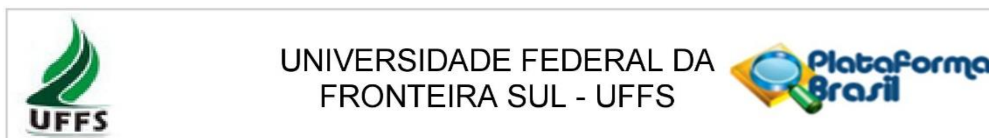
O Melanoma Cutâneo (MC) é a neoplasia maligna com maior crescimento na última década, tornando-se um problema de saúde pública. No Brasil, as maiores taxas de incidência estão na região sul, devido aos hábitos e as características da população. Vários fatores podem estar envolvidos neste tipo de câncer inclusive o sistema purinérgico. O objetivo deste estudo será avaliar as enzimas que degradam nucleotídeos e nucleosídeos de adenina em plaquetas e linfócitos de pacientes com MC. Serão selecionados 60 pacientes, com diagnóstico de MC, com idade entre 30 e 60 anos e 60 indivíduos saudáveis do mesmo sexo e na mesma faixa etária. Será realizada uma coleta de sangue, através de punção venosa para determinação dos parâmetros enzimáticos e da agregação plaquetária. O estudo do MC é de fundamental importância para diminuir a mortalidade e reduzir os gastos públicos, já que a frequência de novos casos está dobrando a cada década e os índices aumentando em pessoas jovens.

Objetivo da Pesquisa:

TRANSCRIÇÃO DOS OBJETIVOS PRIMÁRIO E SECUNDÁRIO:

Objetivo Primário:

Endereço: Avenida General Osório, 413d - Ed. Mantelli da UFFS
Bairro: CENTRO **CEP:** 89.802-265
UF: SC **Município:** CHAPECO
Telefone: (49)2049-1478 **E-mail:** joseane@uffs.edu.br



Avaliar os marcadores do sistema purinérgico e enzimas que degradam nucleotídeos e nucleosídeos de adenina em plaquetas e linfócitos de pacientes com Melanoma Cutâneo (MC).
Objetivo Secundário:
• Analisar a atividade das enzimas E-NTPDase, E-NPP, E-5'-Nucleotidase e ADA em plaquetas e linfócitos de pacientes com MC e em pacientes controles. • Verificar a expressão das enzimas E-NTPDase e E-5'-Nucleotidase em plaquetas e linfócitos da população estudada. • Quantificar o nível sorológico dos nucleotídeos ATP, ADP e AMP, e do nucleosídeo adenosina em ambos os grupos.

Avaliação dos Riscos e Benefícios:
Os riscos estavam adequadamente descritos no TCLE. Foi solicitado que a informação fosse escrita no campo "Riscos e Benefícios" da plataforma brasil. Atendido.
Riscos e benefícios: de acordo.

Comentários e Considerações sobre a Pesquisa:
A pesquisadora atendeu às solicitações do CEP e o trabalho apresenta elevada relevância social e científica.

Considerações sobre os Termos de apresentação obrigatória:
De acordo. Ver parecer consubstanciado do CEP número 816.552.

Recomendações:
Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:
A pesquisadora atendeu às solicitações do CEP.

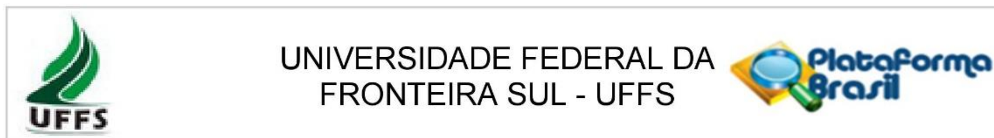
Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Considerações Finais a critério do CEP:
Prezado (a) Pesquisador(a)

A partir desse momento o CEP passa a ser corresponsável, em termos éticos, do seu projeto de pesquisa – vide artigo X.3.9. da Resolução 466 de 12/12/2012.

Fique atento(a) para as suas obrigações junto a este CEP ao longo da realização da sua



pesquisa. Tenha em mente a Resolução 466 de 12/12/2012 principalmente, os artigos XI.1 e XI.2 itens c) ao h) , a Normativa 001/2013 e o Capítulo III da Resolução 251/1997.

A página do CEP-UFFS apresenta alguns pontos no documento "Deveres do Pesquisador" acessível no Link http://www.uffs.edu.br/images/proppg/Deveres_do_pesquisador_CEP.pdf

Atente:

1) No prazo máximo de 6 meses, a contar da emissão deste parecer consubstanciado, obedecidos os 20 dias antes da reunião do CEP do mês correspondente aos 6 meses, deverá ser enviado um relatório parcial a este CEP (via Plataforma Brasil) referindo em que fase do projeto a pesquisa se encontra (exceto se a pesquisa estiver totalmente finalizada, pois, neste caso, deverá ser enviado o relatório final). Veja modelo na página do CEP, no item "6) Documentos a serem anexados à Plataforma Brasil" no subitem " 6.1) Obrigatórios " . A cada 6 meses novo relatório parcial deverá ser enviado até que seja enviado o relatório final.
2) Qualquer alteração que ocorra no decorrer da execução do seu projeto e que não tenha sido prevista deve ser imediatamente comunicada ao CEP para que possa ser avaliada e as medidas adequadas possam ser tomadas. O não cumprimento desta determinação acarretará na suspensão ética do seu projeto. Após um projeto ter sido aprovado, alterações devem ser solicitadas na forma de EMENDA.
3) Além do relatório semestral, a qualquer momento o CEP poderá solicitar esclarecimentos sobre a sua pesquisa – vide artigos X.1.3.b), X.3.6 e XI.2.e)
4) Ao final da pesquisa deverá ser encaminhado o relatório final.
OBS: Os relatórios deverão ser enviados utilizando-se da opção "enviar notificação", na "Plataforma Brasil".
Em caso de dúvida: (1) contate este CEP pelo telefone 20491478, das 8:00 às 12:00 e das 14:00 às 17:00 (2) contate a Plataforma Brasil pelo telefone 136, opção 8 e opção 9, solicitar ao atendente suporte Plataforma Brasil das 08h às 20h, de segunda a sexta, (3) Contate a "central de suporte "da plataforma Brasil no canto superior direito da plataforma e cujo atendimento é online.

Boa pesquisa!