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Munique Mendonça Azevedo

Avaliação de Marcadores de Prognóstico no Melanoma

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Tese submetida ao Programa de Pós-Graduação em Patologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

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Aos meus queridos pais, Viviane e Wanderley, minha inspiração.

Aos meus anjos de quatro patas, meu refúgio.

Ao meu marido, Eduardo, com muito amor

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less".

— Marie Curie

SUMÁRIO

LISTA DE ABREVIATURAS

AJCC*: American Joint Committee on Cancer*

AL: acral lentiginoso

Ala: alanina

A-MnSOD: alelo variante Ala do polimorfismo da MnSOD

CAT: catalases

CEP: comitê de ética em pesquisa

CuZnSOD: superóxido dismutase dependente de cobre e zinco

cfDNA: *cell-free* DNA (DNA livre circulante)

DS: disseminativo superficial

EMT: transição epitélio-mesenquimal

EROs: espécies reativas de oxigênio

FDA: *Food and Drug Administration*

GSH: glutationa

GPX: glutationa peroxidase

IFN: interferon

IL: interleucina

INCA: Instituto Nacional do Câncer

ISCMPA: Irmandade Santa Casa de Misericórdia de Porto Alegre

LDH: lactato desidrogenase

LM: lentigo maligno

MM: melanoma maligno

MnSOD: superóxido dismutase dependente de manganês

MTS: *mitochondrial targeting sequence* (sequência de direcionamento mitocondrial

N: nodular

PRX: peroxirredoxinas

RGP: *radial growth phase* (fase de crescimento radial)

SNP: *single nucleotide polimorphism* (polimorfismo de nucleotídeo único)

SOD: superóxido dismutase

SOD2: superóxido dismutase 2

Th1: células T *helper* 1

Th2: Th1: células T *helper* 2

Tis: tumor *in situ*

TNF: fator de necrose tumoral

TNM: tumor, linfonodos regionais, metástases à distância

UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre

UV: ultravioleta

Val: valina

V-MnSOD: alelo variante Val do polimorfismo da MnSOD

VGP: *vertical growth phase* (fase de crescimento vertical)

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RESUMO DA TESE

Introdução:

O melanoma é altamente metastático, representando a causa mais comum de morte por câncer de pele em escala global. Sua classificação e prognostico são definidos pelo sistema de estadiamento da AJCC, o qual não é suficiente para acessar o risco de mortalidade para cada paciente individualmente devido ao caráter heterogêneo da doença, o que garante a busca por novos biomarcadores.

Objetivos:

Avaliar o estado-da-arte no que diz respeito à biomarcadores de prognóstico no melanoma, bem como analisar a associação entre a neoplasia e os seguintes marcadores: DNA livre circulante (*cfDNA*), citocinas inflamatórias e polimorfismo Ala16Val do gene SOD2 que codifica a enzima antioxidante superóxido dismutase dependente de manganês (MnSOD).

Material e Métodos:

Primeiramente, foi realizada uma revisão sistemática da literatura no que se refere a marcadores genéticos e epigenéticos com valor prognóstico no melanoma. Também, foram realizados dois estudos caso-controle onde foram incluídos pacientes com melanoma do Hospital Santa Rita e indivíduos saudáveis. No primeiro, foi realizada a quantificação dos níveis de *cfDNA* no plasma de casos e controles por meio do Kit Quant-iT™ PicoGreen® dsDNA (Invitrogen) e posterior leitura da fluorescência em fluorômetro. Para avaliar o *status* inflamatório dos indivíduos, a concentração de citocinas inflamatórias (IL-1, IL-6, TNFα, INFy and IL-10) foi medida. No segundo estudo, foi avaliada

a associação entre o melanoma e o polimorfismo Ala16Val do gene SOD2 através da genotipagem de pacientes com melanoma e indivíduos saudáveis. Para tanto, um fragmento do gene SOD2 contendo a região do polimorfismo foi amplificado com *primers* específicos seguido de clivagem com a enzima de restrição *Hae III*.

Resultados:

No artigo científico I, o texto completo de 25 artigos foi avaliado. A análise do perfil de microRNA e a expressão de mRNA associada ao status mutacional dos genes BRAF e NRAS foram as abordagens mais frequentes. No artigo II, níveis elevados de *cfDNA* e citocinas pró-inflamatórias foram observados nos pacientes com melanoma quando comparados aos controles. Por outro lado, os casos apresentaram níveis reduzidos da citocina anti-inflamatória interleucina 10 em relação aos indivíduos saudáveis. Após a excisão do tumor primário, houve redução significativa da concentração de *cfDNA* no sangue dos pacientes. Quanto ao artigo III, foi reportada a associação entre o polimorfismo da MnSOD e o risco de melanoma, a qual é influenciada por sexo e idade.

Conclusão:

Apesar de muito conhecimento ter sido acumulado no que se refere aos marcadores no melanoma, nenhum se mostrou eficiente para detectar o potencial de progressão da doença ainda em seus estágios iniciais. A implementação de biomarcadores sanguíneos pouco invasivos durante o acompanhamento clínico para identificar pacientes em alto risco de metástases é fundamental para melhorar o desfecho dos pacientes com melanoma.

1. INTRODUÇÃO

1.1 Melanoma Cutâneo

O melanoma é o tipo de câncer de pele com origem nos melanócitos e é bem definido histopatologicamente (Chin *et al.*, 1998). Esta doença pode se desenvolver tanto a partir de nevos pré-existentes (20 - 40% dos casos) quanto pode iniciar *de novo*, ou seja, não associado a nevos prévios (60 – 80% dos casos) (Eggermont *et al.*, 2014).

A etiologia do melanoma maligno (MM) é multifatorial. Entre os fatores etiológicos e predisponentes associados a esta neoplasia estão a exposição ao sol e a susceptibilidade genética, os quais podem explicar o aumento da incidência deste câncer na população mundial (Lens e Dawes, 2004).

A identificação visual do MM é acessível, uma vez que se desenvolve na superfície da pele. Entretanto, o desafio está na variabilidade das formas que as lesões podem apresentar durante as primeiras fases da doença, o que retarda o início do tratamento (Friedman *et al.*, 1985). Nos estágios iniciais, os quais apresentam maiores chances de cura, as lesões malignas podem mimetizar histologicamente as benignas. Nos anos 80, foi introduzido o critério ABCDE na prática clínica a fim de reconhecer as lesões de melanoma. Esse critério é baseado em características morfológicas do tumor, onde: **A** = assimetria; **B** = bordas irregulares; C = cor; **D** = diâmetro (> 6 mm); **E** = evolução (Herman, 2012) (figura 1). No entanto, o diagnóstico baseado neste

método tem sensibilidade de apenas 65 – 80%, além de exigir que muitas excisões desnecessárias sejam conduzidas uma vez que lesões benignas podem mimetizar melanomas na avaliação clínica (Grin *et al.*, 1990). Desta forma, a dermatoscopia – uma técnica diagnóstica não invasiva para observação de lesões pigmentadas *in vivo* – é capaz de melhorar a acurácia diagnóstica através da caracterização dos aspectos morfológicos dos tumores cutâneos (Rigel, 1997). Isso porque este método permite a visualização de estruturas pigmentares presentes na epiderme, junção dermoepidérmica, derme superior e vasos do plexo superior através de um sistema de lentes e filtros de aumento associados a uma fonte de luz (Pehamberger *et al.*, 1987).

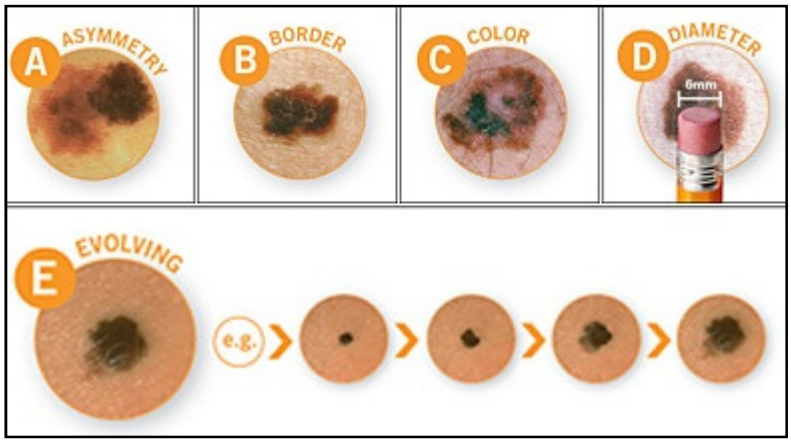

Figura 1. Ilustração demonstrando o critério ABCDE na detecção do melanoma. *Adaptado de* (Tsao *et al.*, 2015).

O melanoma cutâneo é classificado em quatro subtipos histológicos: disseminativo superficial (DS), lentigo maligno (LM), nodular (N) e acral lentiginoso (AL) (Clark *et al.*, 1969; Phan *et al.*, 2007) (figura 2). De acordo com a literatura, o subtipo disseminativo superficial é o mais freqüente (até 70 % dos casos de melanoma) e caracteriza-se por uma fase de crescimento radial

predominante (Clark *et al.*, 1986). O melanoma lentigo maligno surge com maior freqüência na face e cabeça, ou seja, áreas mais expostas ao sol (Langley e Sober, 1998). O segundo subtipo mais freqüente é o nodular. Clinicamente apresenta-se como pápula ou nódulo, os quais podem apresentam coloração escura ou amelanótica (Mcgovern *et al.*, 1973). Por último, o melanoma acral lentinoso é aquele que acomete as palmas das mãos, as plantas dos pés e as unhas.

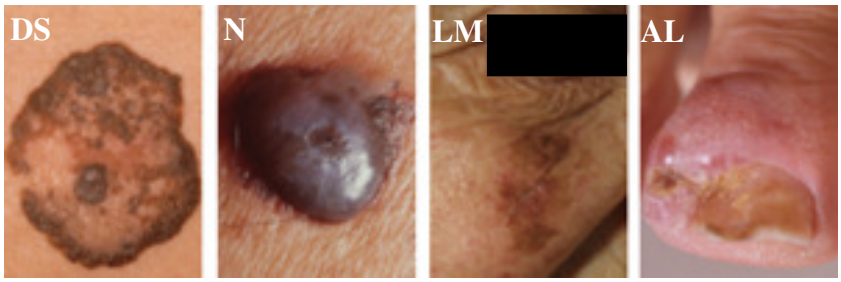

Figura 2. Subtipos histológicos do melanoma cutâneo. DS, disseminativo superficial; N, nodular; LM, lentigo maligno; AL, acral lentiginoso (imagem retirada e adaptada de http://www.jurmo.ch/work_heterogeneity.php, acessado em 26 de Novembro de 2015).

Os melanomas cutâneos passam por duas fases: fase de crescimento radial (RGP) e fase de crescimento vertical (VGP). Na primeira, o tumor pode invadir a epiderme e a derme papilar, mas a capacidade metastática está ausente. Durante a fase subsequente, ocorre a mudança para um estágio mais agressivo, onde metástases podem ocorrer. A transição entre a RGP e a VGP é marcada por alterações nas expressões gênicas, as quais são de grande interesse para estudos sobre a progressão da malignidade (Gallagher e Bergin, 2005).

Os principais fatores prognósticos do MM, segundo a classificação da *American Joint Committee on Cancer* (AJCC) 2009 (Balch *et al*., 2009), são baseados na classificação padrão TNM (tumor, linfonodos regionais, metástases à distância). A categoria T descreve o estado do tumor primário através de sua espessura de Breslow (mm), presença de ulceração e taxa mitótica (mitoses / mm^2). N significa o grau do envolvimento do linfonodo sentinela e M define a presença e localização de metástases (Balch *et al*., 2009) (tabela 1). A partir do sistema de classificação, foram designados quatro estágios clínicos e patológicos (tabela 2).

Tabela 1. Classificação TNM para melanoma.

Classificação			
T		**Espessura de Breslow (mm)**	***Status* da ulceração / mitoses**
	T*is*	NA	NA
	T1	≤ 1	a: sem ulceração e taxa mitótica < 1 / mm^2 b: com ulceração e taxa mitótica ≥ 1 / mm^2
	T2	1,01 – 2,00	a: sem ulceração b: com ulceração
	T3	2,01 – 4,00	a: sem ulceração b: com ulceração
	T4	> 4,00	a: sem ulceração b: com ulceração
N		**N° de linfonodos metastáticos**	
	N0	0	NA
	N1	1	a: micrometástases[a] b: macrometástases[b]
	N2	2 – 3	a: micrometástases[a] b: macrometástases[b] c: metástases em trânsito/satélites sem nodos metastáticos
	N3	4 ou mais	
M		**Sítio**	**LDH**
	M0	Sem metástases à distância	NA
	M1a	Metástases na pele, subcutâneas ou nodais	Normal
	M1b	Metástases no pulmão	Normal
	M1c	Outras metástases viscerais	Normal
		Qualquer metástase à distância	Elevado

T*is,* Tumor *in situ;* NA, não se aplica; LDH, Lactato desidrogenase.

[a] Micrometástases são diagnosticadas depois da biópsia do linfonodo sentinela; [b] Macrometástases são definidas como metástases nodais clinicamente detectáveis e confirmadas patologicamente. *Adaptado de* (Balch *et al*., 2009).

Tabela 2. Estadiamento do melanoma com base na classificação TNM.

	Estadiamento clínico[a]				Estadiamento patológico[b]		
	T	N	M		T	N	M
0	T*is*	N0	M0	0	T*is*	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Qualquer T	N > 0	M0	IIIA	T1-4ª	N1a	M0
					T1-4ª	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4ª	N1b	M0
					T1-4ª	N2b	M0
					T1-4ª	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Qualquer T	N3	M0
IV	Qualquer T	Qualquer N	M1	IV	Qualquer T	Qualquer N	M1

[a] Estaciamento clínico inclui o microestadiamento do melanoma primário e avaliação clínica / radiológica de metástases.

[b] Estadiamento patológico é o microestadiamento do melanoma primário e informações patológicas do linfonodo regional após linfadectomia parcial ou completa. Pacientes em estágios patológicos 0 ou IA são exceções, pois não necessitam de avaliação patológica de seus linfonodos. *Adaptado de* (Balch *et al*., 2009).

Nos estágios iniciais não-metastáticos do câncer, a cirurgia é a principal conduta para o tratamento. Quimioterapia e imunoterapia têm sido estudadas como outras formas de terapia. A quimioterapia tem seus efeitos limitados na sobrevivência geral dos pacientes devido a mecanismos quimioresistentes (La Porta, 2007; Rass e Hassel, 2009). Apenas uma pequena subpopulação de pacientes atingem benefícios duráveis a partir da imunoterapia (Qin *et al*., 2013).

1.2 Epidemiologia

Nas últimas cinco décadas, a incidência de melanoma vem aumentando de forma constante entre caucasianos (Erdmann *et al.*, 2013). Aproximadamente 250.000 novos casos são diagnosticados por ano no mundo e são responsáveis por cerca de 60.000 mortes (Internacional Agency for Research on Cancer. http://globocan.iarc.fr/Pages/burden_sel.aspx [acessado em 21 de novembro de 2015]).

Além de representar a causa mais comum de morte por câncer de pele em escala global (Siegel *et al.*, 2014), o melanoma atinge indivíduos no auge de suas vidas (idade média de 52 anos), quase uma década antes do surgimento da maioria dos tumores sólidos (Erdei e Torres, 2010).

A incidência aumentada dessa neoplasia pode ser atribuída a uma maior exposição à radiação UV do sol (Erdmann *et al.*, 2013) assim como a um melhor entendimento da doença, permitindo diagnósticos mais precisos e precoces (Lens e Dawes, 2004; Solomon *et al.*, 2004; Siegel *et al.*, 2014). Adicionalmente, autores sustentam que o longo período de latência entre a iniciação da carcionogênese do melanoma até a apresentação clínica pode se estender por décadas, fato este que ajudaria a explicar porque as suas taxas de incidência continuam aumentando em muitos países (Erdmann *et al.*, 2013).

Segundo o Instituto Nacional de Câncer (INCA), a incidência estimada da doença no Brasil para o ano de 2016 é baixa (3.000 casos novos em homens e 2.670 em mulheres), no entanto, a sua letalidade é elevada. Este

mesmo levantamento mostrou que as maiores taxas de incidência encontram-se na região sul do Brasil, sendo 8,02 casos para cada 100 mil homens e 7,06 casos para cada 100 mil mulheres no Rio Grande do Sul.

A patogênese do melanoma é influenciada pela interação de fatores genéticos e ambientais, uma vez que a incidência desta neoplasia é fortemente afetada pela cor da pele e localização geográfica (Balch, 2002). Deste modo, o melanoma é uma doença que acomete, predominantemente, caucasianos e, a sua incidência, é de 5 a 20 vezes menor entre populações de pele escura (Houghton e Polsky, 2002).

Atualmente, a grande maioria dos pacientes diagnosticados com melanoma é curada devido ao diagnóstico nos estágios iniciais da doença, o que permite intervenção cirúrgica prevenindo a progressão da doença (Erdei e Torres, 2010). Por outro lado, quando diagnosticado tardiamente, o melanoma pode atingir uma grande variedade de órgãos à distância. Como conseqüência, o diagnóstico nos estágios mais avançados da doença está associado a um mau prognóstico (Houghton e Polsky, 2002).

Pacientes com tumores primários espessos ou metástases linfonodais frequentemente desenvolvem metástases a distância e neste estágio, a média de sobrevida é de 6 a 8 meses (Cummins *et al.*, 2006) e a taxa de sobrevida em 5 anos é de apenas 15% (Finn *et al.*, 2012; Siegel *et al.*, 2013).

1.3 Metástases

O melanoma é conhecido pela sua rápida progressão, metástases e mau prognóstico. É responsável por mais de 80% das mortes relacionadas à neoplasias cutâneas (Miller e Mihm, 2006). Cerca de 1 em 5 pacientes (20%) com melanoma desenvolverão metástases (Siegel *et al.*, 2013).

O tumor tem capacidade de metastatizar muito cedo no curso da doença e por isto, reconhecer os pacientes em risco de progressão tumoral é crucial. Aproximadamente 5% dos pacientes que apresentam lesões finas (<1,0 mm de espessura); 25-40% com lesões de 2,0-4,0 mm; e 50-75% com tumores >4,0 mm de espessura desenvolverão doença metastática (Balch *et al.*, 2001).

As metástases tanto podem ser regionais quanto podem atingir órgãos distantes. Os sítios mais comuns das metástases regionais são: a pele nas imediações do tumor primário, o tecido subcutâneo e os linfonodos (Damsky *et al.*, 2010). Dependendo da distância entre a metástase e o tumor primário, as metástases de pele podem ser classificadas como satelitoses (mais próximas à lesão primária) ou metástase em trânsito (mais distantes do melanoma primário) (Balch *et al.*, 2009). O surgimento de metástases cutâneas é o melhor indicativo de disseminação linfática ou hematogênica da doença (Wolf *et al.*, 2004). Os órgãos preferenciais para ocorrência de metástases de melanoma à distância são: pele, pulmão, cérebro, fígado, ossos e intestino. Metástases pulmonares são prevalentes e, geralmente, o primeiro local onde surgem às metástases viscerais. Outros sítios metastáticos como ossos e intestino ocorrem mais tardiamente na progressão da doença (Damsky *et al.*, 2010).

Atualmente, é reconhecido que múltiplos mecanismos celulares estão desregulados na iniciação e progressão do tumor, como proteínas reguladoras do ciclo celular, apoptoses, transdução de sinais, adesão das células e digestão da matriz extracelular (Meyer *et al.*, 2012).

Em resumo, as etapas para a progressão tumoral de lesões oriundas de nevos displásicos incluem a conversão de uma lesão pigmentada para a forma de melanoma *in situ*, que cresce lateralmente na epiderme (fase de crescimento radial) (Haass e Smalley, 2009). Se não tratado adequadamente, o melanoma pode progredir para a fase de crescimento vertical, a qual está associada com invasão da derme e, frequentemente, com o aparecimento de metástases (Clark, 1991; Sondak *et al.*, 2001).

A perda da adesão celular, a repressão da expressão de E-caderinas e o aumento da motilidade da célula são fenômenos que em conjunto denominam-se “transição epitélio-mesenquimal (EMT)” (Their, 2002; Huber *et al.*, 2005). Tal mecanismo está envolvido na invasividade e metástase de MM. Os melanócitos estão aderidos à queratinócitos basais na epiderme através da expressão de E-caderinas (Hsu *et al.*, 1996). O escape inicial de células neoplásicas de seu local de origem está associado com alterações transcriptômicas e proteômicas de um perfil epitelial para mesenquimal. Sendo assim, moléculas típicas do epitélio como as E-caderinas são substituídas por N-caderinas do mesênquima o que aumenta a motilidade celular, culminando na invasão e migração dos melanócitos transformados para órgãos distantes (Poser *et al.*, 2001; Thiery, 2009).

O melanoma é frequentemente fatal quando a doença progride para o estágio metastático e geralmente é resistente às terapias disponíveis, além destes tratamentos possuírem limitações, custo elevado e desencadearem reações adversas (Slominski e Carlson, 2014).

A dacarbazina, o único agente quimioterápico aprovado pelo *Food and Drug Administration* (FDA) para o tratamento do melanoma metastático está associado com uma resposta de 7 a 12% e uma sobrevida global de 5,6 a 7,8 anos depois do início do tratamento (Chapman *et al.*, 1999).

Várias estratégias para estimular a resposta imune antineoplásica tem sido exploradas. Históricamente, altas doses de interleucina 2 (IL-2) e interferon α eram administrados para pacientes com melanoma metastático e para melanomas ressecados (estágios clínicos II e III) com alto risco de recorrência, respectivamente. Cerca de 15 a 20% dos pacientes com melanoma metastáticos que recebiam altas doses de IL-2 obtinham resposta terapêutica e 6 a 8% experimentavam remissão completa da doença por mais de três anos após o tratamento (Atkins *et al.*, 1999). No entanto, efeitos tóxicos agudos incluindo disfunção de múltiplos órgãos e comprometimento hemodinâmico são observados a partir da administração desta terapia (Schwartzentruber, 2001). Mais recentemente, o uso de ipilimumab, um anticorpo monoclonal que bloqueia o antígeno 4 de linfócito T citotóxico (CTLA4) nos linfócitos, tem sido associado com o aumento da sobrevida dos pacientes metastáticos (Hodi *et al.*, 2010). Outro anticorpo monoclonal que tem sido desenvolvido para o tratamento do melanoma avançado é o nivolumab, o qual tem como alvo terapêutico o receptor de morte programada 1 (PD-L1) e

seus ligantes, promovendo a regulação negativa da ativação das células T e assim, a imunidade antitumoral (Ott *et al.*, 2013). Ensaios clínicos fase I, II e III tem observado eficácia e segurança promissoras de nivolumab em comparação e associação com outras terapias (Johnson *et al.*, 2015).

Aproximadamente 40 a 60% dos melanomas cutâneos apresentam mutações no gene BRAF que leva a ativação constitutiva da via da MAP-quinase. Entre as mutações de BRAF, a mais freqüente, em 90% dos casos, é a V600E, a qual resulta na substituição do aminoácido glutamina pela valina no códon ¨600 (Chapman *et al.*, 2011). Os resultados de um ensaio clínico de fase III mostraram que o Vemurafenib, um inibidor específico do BRAF mutado, foi capaz de aumentar a taxa de sobrevida global e livre de progressão em pacientes com melanoma metastático em comparação com o grupo tratado com dacarbazina (Chapman *et al.*, 2011).

Embora, nos últimos anos, o tratamento para melanoma metastático tenha avançado significativamente, a média da sobrevida dos pacientes tratados é menor do que um ano (Eggermont *et al.*, 2014). Sendo assim, a detecção e o diagnóstico dos tumores nos estágios iniciais da doença, ainda constituem uma abordagem crucial para aumentar a taxa de sobrevivência e qualidade de vida do paciente (Geller *et al.*, 2007).

1.4 Biomarcadores no prognóstico do melanoma

Biomarcadores são definidos como indicadores de processos biológicos, condições patológicas ou respostas farmacológicas que podem ser

quantificados e avaliados, permitindo o diagnóstico de uma doença específica, o acesso ao prognóstico da doença ou o monitoramento da resposta ao tratamento (Colburn *et al.*, 2001).

É consenso na literatura que o sistema de classificação e prognosticação definido pelo *American Joint Committee on Cancer* (AJCC, 2009) (Balch *et al.*, 2010; Gershenwald *et al.*, 2010), não é suficiente para acessar o risco de mortalidade para cada paciente individualmente dentro de cada estágio (Friedman *et al.*, 2012).

Os principais preditores de desfecho são: espessura de Breslow, taxa mitótica, presença de ulceração e envolvimento do linfonodo sentinela (Balch *et al.*, 2001). No entanto, existe um subgrupo de lesões finas, não ulceradas e sem atividade mitótica que apresentam comportamento altamente agressivo e que escapam da identificação dos fatores histopatológicos de prognóstico (Abbas *et al.*, 2014).

O melanoma tem sido reconhecido, há vários anos, como sendo altamente heterogêneo, de tal modo que aproximadamente 5% dos tumores em estágio I desenvolvem metástases precocemente podendo levar o paciente a óbito (Gimotty *et al.*, 2005; Balch *et al.*, 2009), enquanto determinadas lesões espessas não exibem este comportamento (Ratzinger *et al.*, 2014).

Muitos são os esforços dispensados pelos pesquisadores para conhecer os mecanismos moleculares envolvidos na progressão do melanoma, bem como para o desenvolvimento de ferramentas que possam ajudar no

desenvolvimento de modelos com capacidade de predizer o curso provável da doença (Raskin *et al.*, 2013). Para tanto, diversos biomarcadores moleculares de tecido e sanguíneos tem sido investigados (Hoshimoto *et al.*, 2012; Jakob *et al.*, 2012; Xu *et al.*, 2012; Brunner *et al.*, 2013).

No momento, apenas a quantificação dos níveis de LDH sérico foi incorporada na rotina clínica como parte do sistema de estadiamento do melanoma (Hill *et al.*, 2015). Entretanto, estudos mostram que este marcador possui algumas limitações quanto à sua sensibilidade nos estágios iniciais do melanoma, porém, tem valor preditivo negativo para recidivas metastáticas (Hofmann *et al.*, 2009; Hofmann *et al.*, 2011).

Segundo (Saldanha *et al.*, 2016)), um dos maiores problemas enfrentados na busca por marcadores relevantes é que as macromoléculas são mal preservadas nas amostras de tecido de melanoma uma vez que, normalmente, todo o tecido tumoral é fixado em formalina e emblocado em parafina. Além do mais, a maioria dos estudos possui um tamanho amostral reduzido dificultando a inclusão dos biomaradores avaliados na prática clínica (El Hajj *et al.*, 2013).

A urgência na busca pelo desenvolvimento de biomarcadores complementares com potencial para o prognóstico específico do paciente com melanoma se justifica na medida em que poderia implicar na personalização de seu tratamento com melhoria no desfecho clínico (Schramm e Mann, 2011). Marcadores capazes de identificar pacientes com alto e baixo risco de desenvolver metástase poderiam fornecer informações adicionais, tais como

indicar aqueles com necessidade de monitoramento intensificado (Spatz *et al.*, 2010; Murali *et al.*, 2011; Murali *et al.*, 2012).

1.5 *Cell-free* DNA e inflamação

1.5.1 Liberação de DNA na corrente sanguínea

O DNA livre circulante (*cell-free* DNA ou cfDNA) é definido como moléculas de DNA extracelulares que ocorrem no sangue. O cfDNA pode ser detectado no plasma sanguíneo tanto de pacientes com câncer ou com outras doenças (p. e. processos inflamatórios severos) (Jiang e Pisetsky, 2005), quanto em pessoas saudáveis (Jung *et al.*, 2010).

A maior parte do DNA circulante apresenta-se no sangue como moléculas dupla-fita com peso molecular inferior ao DNA genômico e arranjadas em nucleossomos (Jung *et al.*, 2010). Entretanto, os mecanismos responsáveis pela liberação de cfDNA na circulação, em ambas condições fisiológica e patológica, ainda não são completamente entendidos.

Foi sugerido que, em indivíduos saudáveis, a presença de cfDNA ocorre devido a processos hematopoiéticos, onde as células liberam fragmentos de DNA de baixo peso molecular chamados de fração de DNA metabólico (Stroun *et al.*, 2001). O DNA metabólico pode formar um complexo com glicoproteínas que, na corrente sanguínea, atuaria como mensageiro, sinalizando funções entre células e tecidos (Gahan, 2006).

Além dos processos normais de liberação de cfDNA na circulação, portadores de câncer possuem vias adicionais de liberação desse tipo de DNA extracelular. São elas a apoptose e a necrose, processos que ocorrem com freqüência em células tumorais com taxas elevadas de proliferação celular (Li *et al.*, 2003). Células mortas em excesso levam à saturação do processo de fagocitose pelos macrófagos e fragmentos de DNA degradado são liberados na circulação, o que acaba por elevar os níveis de cfDNA no sangue (Stroun *et al.*, 2001).

Alternativamente, uma baixa parcela de cfDNA origina-se da lise de células neoplásicas frágeis que se desprendem do tumor e entram na corrente sanguínea durante a disseminação do tumor. No entanto, este processo é dependente do tipo de câncer (Stroun *et al.*, 2000).

Concentrações mais elevadas de cfDNA freqüentemente são encontradas em pacientes com câncer em relação àqueles saudáveis (Van Der Vaart e Pretorius, 2010). Sendo assim, vários estudos atribuem valor prognóstico à quantificação de DNA livre no sangue (Jung *et al.*, 2004; Pathak *et al.*, 2006; Ren *et al.*, 2006; Catarino *et al.*, 2008; Yoon *et al.*, 2009; Kamat *et al.*, 2010; Ulivi e Silvestrini, 2013; Spindler *et al.*, 2015). Em pacientes portadores de câncer de mama (Catarino *et al.*, 2008), fígado (Ren *et al.*, 2006), pulmão (Yoon *et al.*, 2009), ovário (Kamat *et al.*, 2010) e próstata (Jung *et al.*, 2004), altas concentrações mostraram-se – na maioria dos casos – como fatores indicativos para um pior desfecho quanto à sobrevida global ou livre de doença.

1.5.2 O papel da inflamação no câncer

Evidências sugerem que pelo menos 20% de todas as neoplasias surgem associadas a infecções e inflamações crônicas e, até mesmo aqueles tumores que não se desenvolvem como conseqüência destes processos, exibem extensos infiltrados inflamatórios com a expressão de níveis elevados de citocinas no microambiente tumoral (Coussens e Werb, 2002; Grivennikov e Karin, 2010).

Citocinas inflamatórias são pequenas proteínas mediadoras que desempenham papel-chave na resposta imunológica. São secretadas principalmente por linfócitos e macrófagos e possuem efeito específico na interação e comunicação entre células. Durante a resposta inflamatória, as citocinas influenciam na atividade, diferenciação, proliferação e sobrevivência de células imunológicas, bem como regulam a produção e atividade de outras citocinas que podem aumentá-la (citocinas pró-inflamatórias) ou reduzi-la (citocinas anti-inflamatórias) (Oliveira *et al.*, 2011). São exemplos de citocinas pró-inflamatórias (resposta Th1) as interleucinas 1 e 6 (IL-1 e IL-6), fator de necrose tumoral alfa (TNF-α) e interferon gama (IFN-γ). Entretanto, a resposta persistente e exacerbada de citocinas pró-inflamatórias pode levar a danos nos tecidos-alvo. Sendo assim, citocinas anti-inflamatórias, tais como IL-4, IL-10 e IL-13, podem regular a resposta imunosupressora, minimizando os efeitos indesejáveis da inflamação (Curfs *et al.*, 1997; Lin, E. *et al.*, 2000).

As citocinas podem atuar nas células que as secretam (ação autócrina), em células adjascentes (ação parácrina) ou, menos comumente, em células

distantes (ação endócrina) (Lin, E. *et al.*, 2000). Além disto, estas proteínas apresentam redundância nas atividades que desempenham, significando que funções similares podem ser estimuladas por citocinas diferentes. Geralmente são produzidas em cascata, à medida que uma citocina estimula a célula-alvo a produzir citocinas adicionais (Zhang e An, 2007).

Uma vez que as citocinas não podem ser agrupadas de acordo com as suas células de origem ou sua função biológica, elas são classifcadas em interleucinas (IL; numeradas da IL-1 a IL-35), fatores de necrose tumoral (TNF), quimiocinas (citocinas quimiotáticas), interferons (IFN) e fatores de crescimento mesenquimal (Curfs *et al.*, 1997). Além disso, as citocinas podem ser classificadas em tipo I (p. e. TNF e IFN-γ), as quais estão envolvidas na resposta imunológica Th1 (T *helper* 1) e tipo II (p. e. IL-4, IL-10 e IL-13), envolvidas na resposta imune Th2 (T *helper* 2) . A resposta Th1 é mediada por citocinas pró- inflamatórias expressas por células T CD4+ que induzem as células do sistema imune a atuarem contra infecções e malignidades (Dranoff, G., 2004; Smyth *et al.*, 2004). Já a resposta Th2, que também é mediada por citocinas produzidas pelas células T CD4+, promove imunidade humoral contra tumores e/ou desvio do sistema imune para uma resposta não protetora (tolerância) (Dranoff, Glenn, 2004; Smyth *et al.*, 2004).

As citocinas podem estar envolvidas tanto na ativação de mecanismos imunes que limitam o crescimento tumoral, quanto atuar como fatores de crescimento e sobrevivência de células pré-malignas (Karin e Greten, 2005), uma vez que são capazes de estimular a angiogênese, auxiliar na progressão

tumoral e metástases e também, manter a inflamação promotora do tumor (Karin, 2006; Yu *et al.*, 2009; Grivennikov e Karin, 2010).

As citocinas são produzidas pelo estroma do hospedeiro e por células do sistema imune em resposta a moléculas secretadas pelas células cancerígenas ou como parte do processo inflamatório que freqüentemente acompanha o crescimento do tumor. Além disto, as células malignas também contribuem secretando citocinas no ambiente tumoral (Smyth *et al.*, 2004). Neste ambiente, os níveis e a composição do conjunto de citocinas sofrem flutuações nos vários estágios do desenvolvimento do tumor (Smyth *et al.*, 2004). Essas proteínas podem atuar de modo a desempenhar um efeito aditivo, sinérgico ou antagonista. Portanto, é a integração destes efeitos na resposta antitumoral que determinará o desfecho geral (Lin, Edward *et al.*, 2000).

De acordo com a literatura, três cenários são possíveis no que se refere ao envolvimento de células imunitárias na patogênese tumoral. Densos infiltrados intratumorais de linfócitos nos estágios iniciais da tumorigênese estão fortemente correlacionados com freqüências reduzidas de metástases e maior sobrevida em vários tipos de câncer (Clark *et al.*, 1989; Clemente *et al.*, 1996; Naito *et al.*, 1998; Nakano *et al.*, 2001; Zhang *et al.*, 2003), indicando que a resposta do hospedeiro é capaz de atenuar a progressão da doença (Dunn *et al.*, 2002). Alternativamente, a inflamação crônica pode aumentar o risco de transformação maligna quando não resolvida (Ames *et al.*, 1995) e, portanto, a resposta imune do hospedeiro pode promover o desenvolvimento tumoral (Coussens e Werb, 2002). Por último, estudos mostram que certas amostras de tumores, particularmente aquelas obtidas durante o estabelecimento da fase

disseminativa da doença, não apresentam infiltrado de células imunológicas, indicando que estas neoplasias podem permanecer indetectáveis pelo sistema imune (Ochsenbein *et al.*, 2001).

O melanoma é um tipo de câncer fortemente associado com processos inflamatórios devido à secreção de altos níveis de citocinas pelas células tumorais (Richmond *et al.*, 1988; Claffey *et al.*, 1996; Amiri e Richmond, 2005; Leslie e Bar-Eli, 2005). A imunogenicidade do melanoma é atribuída aos seus antígenos, tais como: MART-1, gp100, gp75 e tirosinase (Hussein, 2005).

Apesar da tentativa do hospedeiro de eliminar a doença, a qual é representada através da resposta imune massiva contra antígenos do melanoma, é rara a sua regressão espontânea mediada pelo sistema imunológico (Tikoo e Haass, 2015). De fato, evidências sugerem que as citocinas secretadas pelas células do melanoma (p.e. interleucina 8, GROα, GROβ, GROγ, entre outras) exercem controle autócrino durante o crescimento e promoção da angiogênese no microambiente tumoral (Richmond *et al.*, 1988; Claffey *et al.*, 1996; Amiri e Richmond, 2005; Leslie e Bar-Eli, 2005).

1.6 Polimorfismo da enzima MnSOD

1.6.1 Espécies reativas de oxigênio e o estresse oxidativo

O termo espécies reativas de oxigênio (EROs) compreende moléculas derivadas de oxigênio com capacidade de receber elétrons extras e que podem oxidar outras moléculas (Cross *et al.*, 1987). As EROs contém um ou mais

elétrons desemparelhados, o que faz com que estas moléculas ou elementos moleculares se tornem altamente reativos na busca por estabilização eletrônica (Halliwell e Gutteridge, 2015). As três formas primárias de EROs são: ânion radical superóxido ($O^{\cdot-}_2$), derivado da redução de um único elétron de oxigênio; peróxido de hidrogênio (H_2O_2), que resulta da conversão de duas moléculas superóxido em uma molécula de ERO não-radical e uma molécula de água através de enzimas superóxido dismutases; radical hidroxila ($^{\cdot}OH$), formado por meio da reação de Fenton, na qual o peróxido de hidrogênio é reduzido pela doação de um elétron de Fe^{2+} (Sullivan e Chandel, 2014) (figura 3).

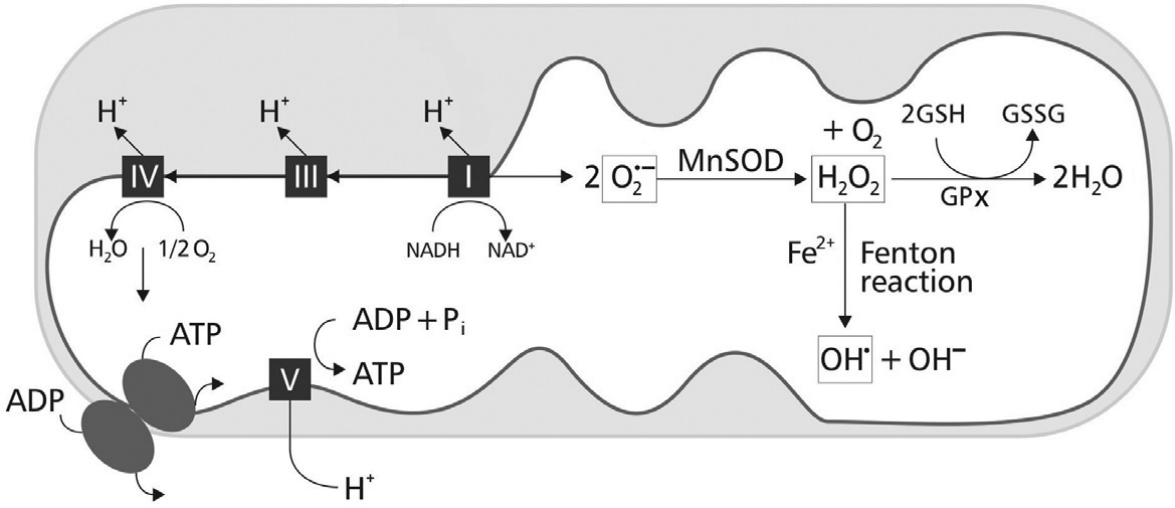

Figura 3. Produção de EROs na mitocôndria. NADH coleta elétrons de moléculas de oxigênio (O_2). Os elétrons fluem através da cadeia mitocondrial de transportes de elétrons para síntese de ATP. Uma pequena quantidade de O_2 sofre uma redução incompleta dando origem ao ânion superóxido ($O^{\cdot-}_2$), a primeira espécie reativa de oxigênio dos organismos aeróbicos. Posteriormente, a enzima superóxido dismutase dependente de manganês (MnSOD) dismuta $O^{\cdot-}_2$ em peróxido de hidrogênio (H_2O_2) e O_2; H_2O_2 é ainda neutralizado pela glutationa peroxidase (GPX) em H_2O com glutationa (GSH) como substrato. Igualmente, H_2O_2 pode ser convertido no radical

hidroxila ($^{\bullet}OH$) através da reação de Fenton com ferro (Fe^{2+}). *Adaptado de* (Edeas, 2011).

EROs são produzidos durante o metabolismo aeróbico normal como produto de diversos processos celulares, principalmente na cadeia de transportes de elétrons na mitocôndria, bem como por agentes tóxicos ambientais (p.e. radiação, poluição do ar e xenobióticos) (Sosa *et al.*, 2013). Certos níveis de EROs são indispensáveis a medida que atuam como mediadores em processos celulares, tais como, diferenciação, controle da progressão do ciclo celular, apoptose e imunidade (Gonzales *et al.*, 1984; Abdel-Aziz e El-Naggar, 1997). No entanto, a produção excessiva de espécies reativas de oxigênio – tanto por questões patológicas quanto fisiológicas – acima da capacidade do sistema antioxidante de defesa do organismo, pode acabar resultando em estresse oxidativo (Ambrosone *et al.*, 1999).

As espécies reativas de oxigênio reagem com diferentes alvos intracelulares, incluindo lipídios, proteínas e DNA. Sendo assim, o estresse oxidativo pode resultar na quebra da molécula de DNA, na peroxidação de lipídios, na modificação de proteínas, na ruptura de membranas e no dano mitocondrial (Schwartz *et al.*, 1993; Emerit, 1994). Freqüentemente, essa interação provoca danos às macromoléculas, o que pode resultar na morte das células, mutação ou carcinogênese (Ambrosone *et al.*, 1999).

Evidências sugerem que as EROs estão envolvidas em vários estágios do desenvolvimento em múltiplos passos do câncer (Marnett, 2000). O principal mecanismo pelo qual as EROs contribuem para a carcinogênese é através de

mutações e alterações nas expressões gênicas geradas a partir de lesões ao DNA provenientes do estresse oxidativo (Feig *et al.*, 1994; Cooke *et al.*, 2003). O radical hidroxila reage com todos os componentes da molécula de DNA, danificando tantos as bases púricas e pirimídicas quanto a desoxirribose (Halliwell e Gutteridge, 2015). A lesão oxidativa do DNA mais proeminente é a 8-Hidroxiguanina (8-OH-G), representando o primeiro passo nos processos de mutagênese, carcinogênese e envelhecimento (Kasai e Nishimura, 1991). Durante a replicação celular, 8-OH-G pode causar a transversão de GC→TA pelo pareamento incorreto com uma adenina (Cheng *et al.*, 1992; Moriya, 1993; Kamiya *et al.*, 1995).

1.6.2 Estresse oxidativo no melanoma

No que se refere às neoplasias dermatológicas, a radiação ultravioleta (UV) é um fator de risco bem-estabelecido que pode contribuir na geração de dano oxidativo e, conseqüentemente, no processo de carcinogênese (Nishigori *et al.*, 2004; Sander *et al.*, 2004). EROs são geradas indiretamente após a absorção de raios UVA (320-400 nm) pelos cromóforos das células da pele (Brash, 1997; Kielbassa *et al.*, 1997). Os raios UVB (280-320 nm), em uma menor proporção, também são capazes de induzir o estresse oxidativo (Kvam e Tyrrell, 1997).

No melanoma, sabe-se que espécies reativas de oxigênio estão presentes em todas as fases do seu desenvolvimento e que há um desequilíbrio no estado redox dos pacientes (Kvam e Tyrrell, 1997; Gadjeva *et al.*, 2008). Sander *et al.* (2003) demonstram a expressão excessiva de enzimas

antioxidantes em biópsias de pacientes com melanoma quando comparadas aos tecidos adjacentes ao tumor, aos nevos melanocíticos benignos e aos indivíduos controle. Adicionalmente, os autores concluíram que o aumento do estresse oxidativo encontrado nas células de melanoma poderia danificar os tecidos que circundam o tumor e então, auxiliar na sua progressão metastática.

1.6.3 Detoxificação enzimática de espécies reativas de oxigênio

Devido ao efeito deletério das EROs, diversos mecanismos de defesa evoluíram para proteger as células. Estes mecanismos incluem os sistemas de reparo, as enzimas antioxidantes (superóxido dismutases, SOD; catalase, CAT; e glutationa peroxidase, GPX) e pequenas moléculas “scavengers”, tais como a glutationa (GSH) (Gadjeva *et al.*, 2008).

Na pele, a modulação enzimática dos níveis de EROs é realizada pela superóxido dismutase dependente de cobre e zinco (CuZnSOD), superóxido dismutase dependente de manganês (MnSOD) e catalase, as quais mantém o balanço redox dentro das células (Sander *et al.*, 2003). Entre estas enzimas, a MnSOD tem despertado interesse, uma vez que localiza-se na mitocôndria e é a primeira linha de defesa contra os radicais superóxido produzidos como produto da fosforilação oxidativa oriunda da exposição aos raios UV e de processos inflamatórios (Poswig *et al.*, 1999).

Na mitocôndria, a MnSOD atua convertendo EROs em peróxido de hidrogênio, o qual é catalisado em água, na sequência, pela catalase e pela glutationa peroxidase (Wallace, 1999; Li *et al.*, 2005). Além do mais, tem sido

demonstrado que a MnSOD tem capacidade de atuar como supressor tumoral, possivelmente modulando as vias apoptóticas e proliferativas *in situ*. (Oberley e Buettner, 1979; Li *et al.*, 1998). A expressão aumentada desta enzima foi observada na supressão do fenótipo maligno de células tumorais, até mesmo no melanoma (Church *et al.*, 1993).

A enzima MnSOD é codificada pelo gene superóxido dismutase 2 (SOD2), um gene de cópia única que contém cinco éxons espaçados por quatro introns e está localizado no cromossomo 6q25.3 (Wan *et al.*, 1994). O éxon 2 (códon 16) do gene SOD2 abriga um polimorfismo de nucleotídeo único (SNP), ocorrendo a substituição de T (timina) por C (citosina). Extensivamente estudado, este polimorfismo denominado Ala16Val, resulta na troca do aminoácido valina (Val, GTT) pelo aminoácido alanina (Ala, GCT) (Rosenblum *et al.*, 1996).

A substituição de aminoácidos ocorre na porção N-terminal da seqüência que serve para direcionar o transporte da proteína para dentro da mitocôndria (*mitochondrial targeting sequence*, MTS), uma vez que o precursor da enzima é sintetizado no citoplasma (Pfanner e Geissler, 2001). Portanto, sugere-se que este polimorfismo altere a estrutura secundária da proteína implicando na eficiência do transporte da MnSOD para o interior da mitocôndria (Rosenblum *et al.*, 1996). O alelo variante Ala (A-MnSOD) permite uma importação mais eficiente da enzima para a matriz mitocondrial, devido à sua tradução em uma cadeia de aminoácidos precursora da MnSOD com estrutura em alfa-hélice, o que resulta em uma maior atividade enzimática quando comparado ao alelo Val (V-MnSOD), organizada em um a estrutura beta-folha que encontra dificuldade

em atravessar os poros da membrana interna da mitocôndria, sendo parcialmente destruída no proteossoma (Sutton *et al.*, 2003) (figura 4). Desse modo, sugere-se que indivíduos portadores do genótipo homozigoto AA (Ala/Ala) possuem maior atividade da MnSOD do que os homozigotos VV (Val/Val) (Sutton *et al.*, 2003).

Embora ainda muito controversa, diversos estudos sugerem haver associação entre a susceptibilidade ao câncer e o genótipo AA (Ambrosone *et al.*, 1999; Cheng *et al.*, 2005; Li *et al.*, 2005). Como, por exemplo, no câncer de mama, onde o risco de desenvolvimento da doença está aumentado em duas vezes, aproximadamente, nos indivíduos AA (Mitrunen *et al.*, 2001; Cai *et al.*, 2004; Slanger *et al.*, 2006), porém o genótipo VV pode estar correlacionado com tumores com maior potencial metastático (Bica *et al.*, 2010). De modo semelhante, foi demonstrada a associação entre o polimorfismo do SOD2 e o câncer de próstata, onde o alelo Ala estava associado com um risco maior a essa neoplasia (Woodson *et al.*, 2003). Já no caso das neoplasias cutâneas, não foi observada associação significativa entre o genótipo AA e o risco de melanoma, carcinoma basocelular e carcinoma epidermóide (Han *et al.*, 2007) De acordo com nossa busca na literatura, até o momento, o estudo de Han *et al* (2007) foi o único que avaliou a associação entre o polimorfismo Ala16Val do gene SOD2 no melanoma.

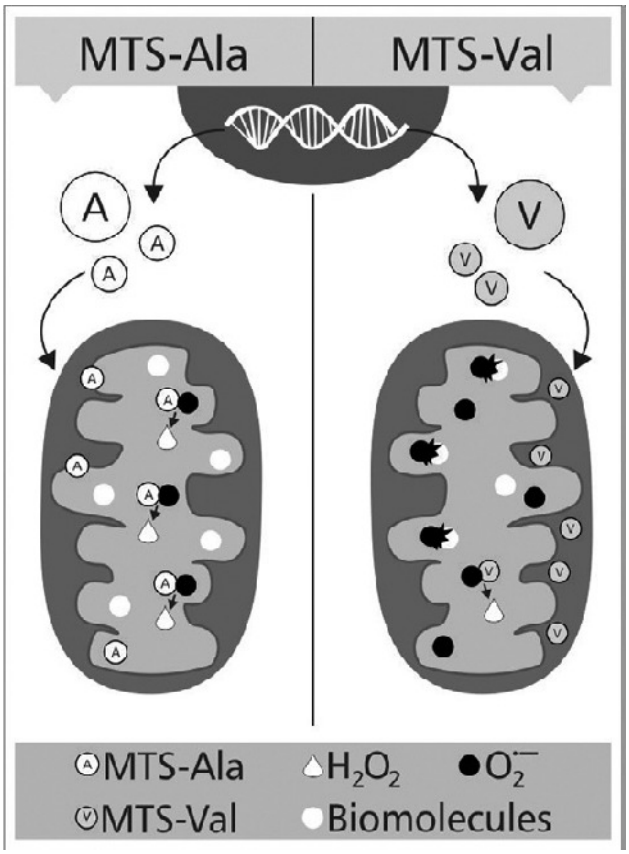

Figura 4. Conteúdo de MnSOD na mitocôndria de acordo com os diferentes precursores originados do polimorfismo da enzima: alelo variante Ala e alelo variante Val. Enquanto que o precursor da MnSOD Ala-variante (MTS-Ala) é corretamente transportado através das duas membranas mitocôndrias, o precursor Val-variante (MTS-Val) é parcialmente contido na membrana interna da mitocôndria. O aumento do precursor da MnSOD contendo o alelo Ala é traduzido em uma detoxificação mais eficiente do superóxido, enquanto que o precursor Val permite o acúmulo de EROs na mitocôndria. *Adaptado de* (Kurmi *et al*., 2013).

1.7 Justificativa

Dada a natureza agressiva do melanoma, é grande o esforço despendido por investigadores na buscar por biomarcadores moleculares para complementar o diagnóstico e prognóstico de pacientes em risco potencial de desenvolver metástases (Larson *et al.*, 2009). A identificação de tais pacientes ainda nos estágios iniciais da doença poderia beneficiá-los por meio da complementação do tratamento padrão com terapias adjuvantes, além de um acompanhamento mais rigoroso.

Atualmente, os principais fatores prognósticos aceitos na prática clínica são a espessura de Breslow, a taxa mitótica, a presença de ulceração e o status do linfonodo sentinela (Balch *et al.*, 2009; Abbas *et al.*, 2014). Entretanto, sabe-se que tumores semelhantes – clínico e histopatologicamente – em indivíduos diferentes podem ter comportamento distinto quanto à progressão da doença.

A avaliação dos níveis de cfDNA no sangue de pacientes com melanoma, bem como de seu status imunológico, vem de encontro a esta tendência de identificação precoce de pacientes com mau prognóstico. Além de estabelecer a melhor estratégia terapêutica, é importante que estes marcadores possam auxiliar na identificação de recidivas e metástases sub-clínicas, permitindo o seu tratamento imediato e o aumento da sobrevida (Larson *et al.*, 2009).

Quanto ao estresse oxidativo no melanoma, sabe-se que as espécies reativas de oxigênio geradas como conseqüência da incidência de radiação UV sob a pele têm um papel importante no processo de carcinogênese (125, 126). No entanto, até onde temos conhecimento, apenas um artigo se propôs a avaliar a associação do polimorfismo da MnSOD no melanoma e nenhuma associação foi observada. Sendo assim, consideramos que esta questão ainda não foi completamente elucidada e merece atenção.

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2. OBJETIVOS

2.1 Objetivo geral

Avaliar o potencial prognóstico dos níveis de DNA livre circulante (*cf*DNA) como marcador relacionado à inflamação e do polimorfismo da superóxido dismutase dependente de manganês (MnSOD) em pacientes com melanoma.

2.2 Objetivos específicos

a) Determinar a associação entre os níveis de *cf*DNA e o melanoma; através da comparação entre casos (pacientes com melanoma) e controles (indivíduos saudáveis);

b) Avaliar o impacto do tratamento cirúrgico nos níveis de *cf*DNA;

c) Analisar a associação entre a concentração de *cfDNA* e as características histopatológicas da doença, bem como fatores de risco reconhecidos para o melanoma;

d) Validar o status inflamatório dos pacientes com melanoma por meio da quantificação de citocinas pró-inflamatórias e anti-inflamatórias;

e) Determinar a freqüência alélica e genotípica dos alelos Ala e Val do gene SOD2 na população em estudo e comparar estas freqüências entre casos e controles;

f) Correlacionar o genótipo da MnSOD aos fatores prognósticos descritos pela AJCC e fatores de risco para melanoma.

3. ARTIGOS CIENTÍFICOS

3.1 Artigo científico I: "Genetic and epigenetic biomarkers in melanoma prognosis: a last five years update"

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Genetic and epigenetic biomarkers in melanoma prognosis: a last five years update

Short title: Update on melanoma biomarkers

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DECLARATIONS OF INTEREST

The authors declare that they have no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the present work.

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ABSTRACT

Cutaneous melanoma is an aggressive malignancy due to its propensity to metastasize and intrinsic resistance to treatment. Melanoma is responsible for over 80% of deaths from skin cancer and its incidence is rising faster than any other cancer worldwide. Early-stage melanoma is frequently curable, in contrast, when the disease reaches stage IV, the prognosis is poor. Predicting the behaviour of melanoma in individual patients can be difficult once it has long been recognized as a highly heterogeneous disease. Currently, the general guideline for prognostication is AJCC (American Joint Committee of Cancer) staging system, which is based on clinical and histological parameters. Despite many attempts to identify complementary molecular markers to stratify patients into risk-subgroups in each AJCC, to date, none of them are recommended in clinical assessment. In this review, we analyzed the last five years state-of-the-art in genetic and epigenetic biomarkers in melanoma prognosis. Twenty-five publications have been analyzed. The majority comprises evaluation of microRNA profile, following by studies witch compare mRNA expression of different biomarkers with BRAF and NRAS mutational status. Significant insights into melanoma progression have been achieved through genetic and epigenetic analysis, however, further studies are necessary to validate and incorporate prognostics molecular biomarkers in clinical practice.

KEY WORDS: cutaneous melanoma; biomarkers; prognosis; genetic; epigenetic

INTRODUCTION

Cutaneous melanoma represents a highly aggressive malignancy that accounts for only about 4% of all dermatological cancers, but it is responsible for the deaths of more than 80% of skin cancer patients [1]. Globally, the incidence of melanoma has risen faster than any other cancer in white populations in the last years [2,3]. This increase may be due to the tendency for early detection and surveillance, but also to the intense sun exposure [4-6].

Despite early-diagnosed primaries tumors can be cured by surgical excision, once spread to distant sites occurs the prognosis is poor [7]. Melanoma can metastasize very early in the disease process. Approximately 5% of patients with thin lesions (<1.00 mm thick), 25–40% presenting intermediary thickness (lesions between 2–4 mm) and 50–75% with thick lesions (> 4.00 mm thick) develop metastatic disease and die within 10 years [8]. The median overall survival (OS) in stage IV (metastatic disease) is 8–18 months [9] and only 15% live more than 5 years [10].

Treatment options for advanced melanoma are historically limited because it is considered a refractory disease [11]. The recent development of new therapies such as MAP-Kinase inhibitors and the immune checkpoint antibodies (anti-CTLA-4, anti-PD1/PDL1) [12-14] have prolonged life for a few months, but they have some limitations and the prognosis still poor [15].

The staging system proposed by American Joint Committee on Cancer [9] is currently used for melanoma classification, prognostic prediction and

management of patients. The system is based in histopathological features such as Breslow thickness, presence of ulceration and mitotic rate, as well the presence of nodal and/or systemic metastases to classify patients with primary melanoma into stages I/II and individuals with regional/distant metastases into stages III/IV, respectively. Also, serum LDH levels have been taken into account to stratify patients into the AJCC Stages [9].

However, predicting the behaviour of melanoma in individual patients can be difficult due to the disease heterogeneity [16,17]. There are a small subset of tumors exhibiting aggressive behaviour that are not identified by any of AJCC predictors, which limit the capability to screening and early treatment to patients at higher risk of metastasis [18,19]. This warrants the development of complementary biomarkers with prognostic significance to stratify patients into risk-subgroups in each AJCC stage as an attempt to improve outcomes [7]. Various studies have sought to assess molecular biomarkers of melanoma progression, but to date, none of them are recommended in clinical assessment [20].

Molecular biology of melanoma is complex and over 85,000 coding mutations have been reported [21]. Over the last years, melanoma research has been focused in underlying genetic alterations involved in acquisition of metastatic potential by tumor cells [22]. In this regard, determining genetic expression profiles could be utilized to evaluate risk of disease progression and patient survival in primary melanoma [23-25]. In addition to genetic, epigenetic alterations recently have provided new insights regarding to the melanoma progression [26].

In this review, we focus on studies published in the last 5 years that evaluated genetic and epigenetic biomarkers with predictive value for melanoma prognostication.

METHODOLOGY

An active virtual search of medical literature has been conducted on studies evaluating studies on genetic and epigenetic biomarkers for melanoma, published in the period between 2012 and 2016. We searched MEDLINE and COCHRANE LIBRARY using different combinations of the following descriptors: "Melanoma", “Melanomas”, “Malignant Melanoma”, “Malignant Melanomas”, "Biological Markers", “Biomarkers”, “Genetic Marker”, “DNA Markers”, “DNA Marker”, "Prognosis", “Prognoses” and “Prognostic”.

Initially, sorting of articles was conducted based on title followed by abstract and finally full texts were reviewed if they were categorized as relevant reports. We did not restrict the language in our research.

Studies were included if they met the following criteria for eligibility: having human beings as research subjects; having cutaneous melanoma tissue or blood sample from melanoma patient as biological sample; being an experimental study, suitable for the evaluation of prognostic biomarker; using molecular methods to access genetic and epigenetic alterations. Studies restricted to mucosal or uveal melanoma and in vitro reports, as well as studies whose purpose was to search for potential therapeutic targets or diagnosis biomarkers, have been excluded. The eligibility criteria were assessed

independently by 2 reviewers, and in case of disagreement, a third researcher was consulted.

RESULTS

This review summarizes the current existing genetic and epigenetic biomarkers in cutaneous melanoma patients. A total of 726 publications were retrieved from our electronic search. Of these, 80 abstracts were considered relevant and full texts were reviewed in detail. By the end of the review 25 studies met our inclusion criteria (Figure 1).

Figure 1 here.

In the last 5 years, many studies evaluating candidate biomarkers for melanoma prognostication have been conducted. Most of these markers are implicated in molecular pathways involved in cell proliferation and survival, including mutations in mitogen-activated protein kinase (MAPK) and cell cycle regulators [27]. However, until now, none of them have been recommended for stratification and management of melanoma patients in clinical practice.

In our research, several genetic and epigenetic biomarkers with prognostic significance have been identified and are listed in Table 1 [7,28-51].

Table 1 here.

DISCUSSION

Malignant melanoma is an aggressive skin cancer due to its rapid progression, propensity to metastasize, poor prognosis and intrinsic resistance to treatment. The incidence of melanoma is rising worldwide, being a major public-health problem [52]. Due to the disease heterogeneity, further biomarkers to stratifying melanoma patients and estimating probability of disease progression and survival are needed. Complementary biomarkers with specific prognostic potential could allow for more focused clinical surveillance of patients with increased risk of disease progression. However, until today, there are no validated molecular prognostic markers in melanoma [53].

Despite many attempts to identify complementary biomarkers predicting clinical outcome in melanoma, the majority of them is not robust enough and their utility in routine clinical practice still limited. Therefore, further studies are necessary in order to provide new biomarkers with predictive value.

Combinations of two or more biomarkers to predict prognosis have gained increasing importance in this field and many studies comprising in this review presented multiple biomarkers assays [33-35,43,47-49]. However, some studies still focusing on *BRAF* and *NRAS* polymorphism assessment or making comparisons between the mutational status of this ongenes and microRNA levels or gene expression, for example [36,38,40,46].

Furthermore, we could notice in this review that microRNA expression profile is rising as a promising tool for melanoma prognostication

and, in the last years, the number of publications using this biomarker in the attempt to predict melanoma outcome has been increased [7,45-51].

In addition, we have compiled the markers categories with greatest representation in this search for genetic and epigenetic biomarkers in melanoma prognosis.

MAPK pathway

The mitogen activated protein kinase (MAPK) pathway is one of the most important signaling pathways in regulation of cell proliferation and survival [38]. Mutations in upstream factors such as RAS and RAF lead to a constitutive activation of the MAPK pathway, giving growth advantage to cells in various cancers [54]. A majority of cutaneous melanomas show mutations in genes which encode proteins of MAPK signaling pathway and thus acting in regulation of melanocytes proliferation [55].

Indeed, approximately 40-50% of melanoma cases exhibiting *BRAF* mutations and 15-20% with mutated *NRAS* [56,57] . *BRAF* is a member of the RAF family of enzymes, which plays a critical role in mediating the cellular effects of growth factor signals through receptors on the cell surface to the nucleus [38].

The most prevalent *BRAF* mutation, accounting for ~85% *BRAF* mutations, is *V600E*, a T to A nucleotide transversion which results in a valine to glutamic amino acid substitution [58,59]. This single mutation implicates in a

growth factor-independent proliferation of malignant cells by increasing the catalytic activity of B-Raf [60].

Recent evidences suggest the value of testing *BRAF* mutational status in melanoma to access patient prognosis. Moreau *et al.* [38] reported that patients with *BRAF*-mutated melanoma had worse overall survival and distant metastases-free survival than wild-type *BRAF* melanoma patients [38]. In addition, a meta-analysis showed that *BRAF* mutation is a risk factor for patient survival in melanoma by increasing the risk of mortality in 1.7 times (95% confidence interval, 1.37 to 2.12) [61].

Besides *BRAF* mutations, the protocongene *NRAS* is susceptible to genetic alterations which also activate the MAPK pathway. This gene codes for a guanine triphosphate (GTP)-binding protein and mutations at hotspot in exon 2 (codon 61) lead to loss of GTPase activity and to a permanently activated conformation of RAS [62,63].

Regarding prognostic value of *NRAS*, Jakob *et al.* [40] provided the first evidence that it could be a biomarker for melanoma outcome independent of current validated prognostic markers. The results of this analysis pointing to a significantly shorter survival from patients diagnosed in stage IV of melanoma with an activating mutation in the *NRAS* gene than patients without a mutation in either the *BRAF* or the *NRAS* genes [40].

MicroRNA profile

Over the last few years, there is growing interest in evaluating the prognostic potential of epigenetic biomarkers in the search for melanoma outcome predictors. Epigenetic alterations, defined as changes in gene expression without modifications in DNA sequence, have been implicated in the pathogenesis of various human cancers, including melanoma [18]. These events are potentially reversible, occur at greater frequency than genetic mutations and they consist of DNA methylation, histone modifications and non-coding RNA [26,64]. Regarding DNA methylation in melanoma, hypomethylation of large areas across genome, for example, is recognized as the responsible to increased expression of genes normally silenced during development [65].

Another epigenetic mechanism becoming the focus in melanoma research is microRNA (miRNA), playing an important role in the regulation of protein expression [60]. MicroRNAs are a large family of short non-coding single-stranded RNAs (approximately 22 nucleotides), evolutionarily conserved, that negatively regulate gene expression at the posttranscriptional level [66] and act in cell differentiation, metabolism, and apoptosis [60]. Gene regulation occurred by base pairing within the 3'-untranslated region of messenger mRNA of its target gene to specify degradation or repression of translation [66]. Many studies currently demonstrated the miRNAs implication in tumorigenesis process – targeting oncogenes and tumor supressors [67] – as well their relevance in melanoma progression [68]. miRNAs can be deregulated and

change their expression profiles in tissue and/or blood samples upon development and progression of cancer, leaving specific signatures [69,70].

The evidence thus far shows that miRNAs could represent a potential prognostic biomarker in melanoma, since they have technical advantages like stability, being resistant to RNase digestion [71], and they are not susceptible to degradation in FFPE samples because of their very small size [3]. Actually, the miRNA most differentially expressed between normal melanocytes and primary melanomas from patients identified is miR-211, which is encoded by an intronic region of TRPM1, a candidate suppressor of melanoma metastasis [72,73].

CONCLUSIONS

In the last decades, there was an advance concerning molecular investigations of melanoma biology. Biomarkers in oncology that provide useful tools of molecular biology to characterize cancer signatures are crucial to personalized treatment. Development of a prognostic assay that could identify with a high risk of relapse will be highly valuable for melanoma management and beneficial for melanoma patients. Significant insights into melanoma progression have been achieved through genetic and epigenetic analysis, however, further studies are necessary to validate and incorporate prognostics molecular biomarkers in clinical practice.

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LEGEND OF TABLE AND FIGURE

Table 1. Genetic and epigenetic biomarkers in melanoma.

Figure 1. Flowchart representing the process of publications selection based on elegibility criteria.

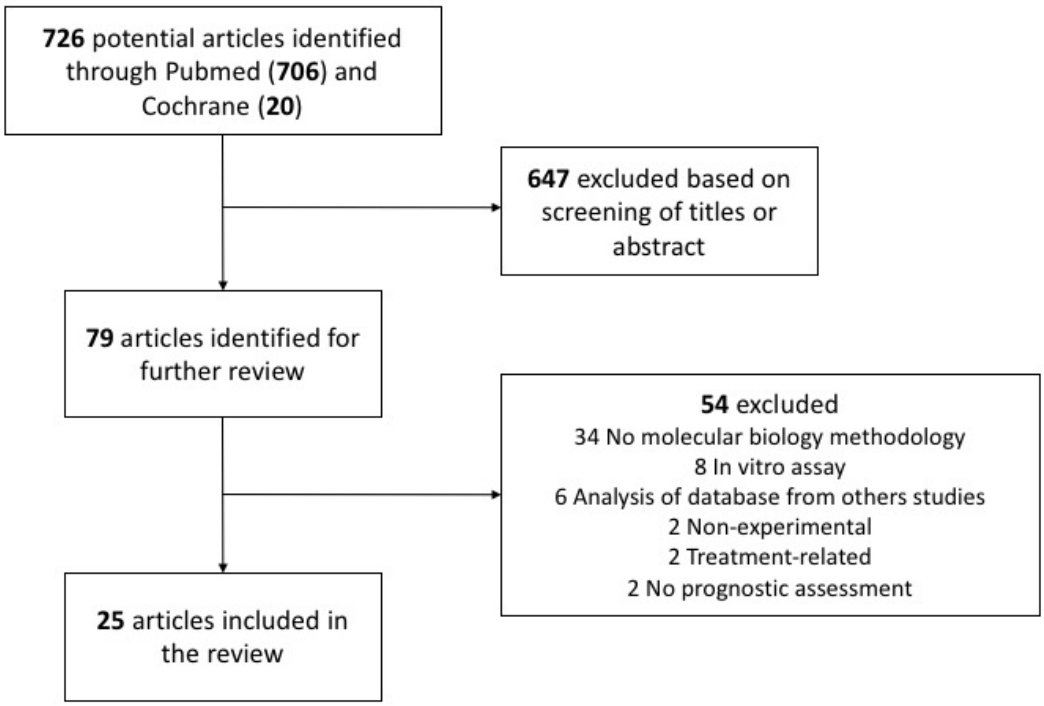

Figure 1. Flowchart representing the process of publications selection based on elegibility criteria.

Table 1. Genetic and epigenetic biomarkers in melanoma.

Biomarker	Description	N	Findings	Reference
mRNA expression				
KRT9	Structural constituent of cytoskeleton	44	Protective gene; expression correlated with OS.	Brunner *et al.* [28]
DCD	Peptidase activity	44	Protective gene; expression correlated with OS.	Brunner *et al.* [28]
PIP	IgG binding	44	Protective gene; expression correlated with OS.	Brunner *et al.* [28]
SCGB1D2	Lipophilin gene	44	Protective gene; expression correlated with OS.	Brunner *et al.* [28]
SCGB2A2	Secretoglobin	44	Protective gene; expression correlated with OS.	Brunner *et al.* [28]
COL6A6	Potentially involved in tumor cell invasion	44	Protective gene; expression correlated with OS.	Brunner *et al.* [28]
KBTBD10	Potentially involved in tumor cell invasion	44	Risk gene; expression inversely correlated with OS.	Brunner *et al.* [28]
ECRG2	Inhibits tumor cell invasion	44	Risk gene; expression inversely correlated with OS.	Brunner *et al.* [28]
HES6	Might act as a regulator of melanoma cell differentiation	44	Risk gene; expression inversely correlated with OS.	Brunner *et al.* [28]
MCAM/MUC18	Cell adhesion	175	Significantly associated with poor prognosis and death, regardless of the AJCC stages; positive expression associated with melanoma progression.	Rapanotti *et al.* [29]
HMGA2	Transcription factor	421	Transcription factor HMGA2 is up-regulated in primary and metastatic melanoma compared with normal skin; HMGA2 is independently associated with OS, DFS and DMFS.	Raskin *et al.* [30]
MLANA	Melanoma cell marker	382	Expressed in 45% of patients who experienced disease recurrence.	Reid *et al.* [31]
ABCB5	Melanoma cell marker	382	Expressed in 49% of patients who experienced disease recurrence. Co-expression of *MLANA* and *ABCB5* produced a positive predictive value of 56% and a negative predictive value of 72%.	Reid *et al.* [31]
TYRP1	Pigment-related	104	*TYRP1/S100B* ratio correlated with reduced DFS and OS, increased Breslow thickness and presence of ulceration of primaries.	El Hajj *et al.* [32]
		84	Median mRNA expression was lower for those stage III patients with good outcome compared with those with poor outcome.	Falkenius *et al.* [33]
GAPDHS	Glycolytic gene	84	A significant difference was observed in GAPDHS expression between short- and long-term survivors (stage III patients); the median mRNA ratio between long- and short-term survivors was 0.19.	Falkenius *et al.* [33]
GAPDH	Glycolytic gene	84	Median mRNA expression was lower for those stage III patients with good outcome compared with those with poor outcome (the median mRNA ratio between long- and short-term survivors was 0.65).	Falkenius *et al.* [33]
PKM2	Glycolytic gene	84	Median mRNA expression was lower for those stage III patients with good outcome compared with those with poor outcome (the median mRNA ratio between long- and short-term survivors was 0.78).	Falkenius *et al.* [33]
Triple-marker: Tyrosinase, GP-100 and MART-1	Melanocyte-related	165	Patients with SLN presenting positivity for the 3 markers had significantly poor MSS and DFS when compared to those with positivity for 1 or 2 markers or PCR negativity.	Ito *et al.* [34]
PER 1	Clock gene	32	*PER1* showed significantly decreased levels in melanoma tumors when compared with adjacent nontumorous skin tissues. The magnitude of decrease in mRNA level was 39%.	Lengyel *et al.* [35]
PER2	Clock gene	32	*PER2* showed significantly decreased levels in melanoma tumors when compared with adjacent nontumorous skin tissues. The magnitude of decrease in mRNA level was 40%.	Lengyel *et al.* [35]
CLOCK	Clock gene	32	*CLOCK* showed significantly decreased levels in melanoma tumors when compared with adjacent nontumorous skin tissues. The magnitude of decrease in mRNA level was 48%. Reciprocal association was found between CLOCK mRNA levels in tumor and Breslow thickness.	Lengyel *et al.* [35]
CRY1	Clock gene	32	*CRY1* expression showed reciprocal associations with Breslow thickness and ulceration.	Lengyel *et al.* [35]
CXCR4	Chemokine receptor activity	87	Correlation between elevated *CXCR4* expression and the presence of BRAF mutation; *CXCR4* mRNA was significantly lower in AJCC stage 2 compared with stage 1; absence of a brisk host response was associated with *CXCR4* mRNA expression.	Mitchell *et al.* [36]
Genetic polymorphism				
KIR	Immune protection	387	*KIR2DL3/C1* genotype as a protective marker for melanoma patients and for patients with SLN metastasis; *KIR2DL1(+)/S1(-)/C2C2* genotype associated with susceptibility to melanoma and SLN metastasis.	Campillo *et al.* [37]
BRAF	Oncogene	105	Death occurred in 83.3% (OS of 1.4 years) and 60.3% (OS of 2.8 years) of stage III patients with and without BRAF mutations, respectively.	Moreau *et al.* [38]
TERT promoter	Telomerase RNA reverse transcriptase activity	300	*TERT* promoter mutations were associated with histopathological features of tumor; patients with TERT mutations had shorter DFS than patients without those mutations; poor MSS compared to those without TERT mutations.	Nagore *et al.* [39]

Table 1. (*continued*)

Biomarker	Description	N	Findings	Reference
NRAS	Oncogene	677	Patients with *NRAS* mutations had a median survival of 8.2 months from stage IV diagnosis (wild type patients = 15.1 months); *NRAS* mutations are associated with decreased OS independently of age, sex, metastases category and LDH level.	Jakob *et al.* [40]
DNA methylation				
CDH11	Cell-cell adhesion	28	Increased values either in *CDH11* or age lead to a shorter expected time to death in metastatic melanoma patients.	Kaehler *et al.* [41]
BASP1	Membrane bound	28	Increased BASP1 values seem to correlate with time to death in metastatic melanoma patients.	Kaehler *et al.* [41]
TNFRSF10D	Member of the TNF-receptor superfamily	68	*TNFRSF10D* DNA methylation status significantly associated with poor OS and RFS.	Ratzinger *et al.* [42]
LINE-1	Retrotransposable element	113	*LINE-1* U index (hypomethylation) level was significantly higher in melanoma patients than that of normal skin and nevi, and was elevated with increasing AJCC stage; associated with DFS and OS in stage I/II patients.	Hoshimoto *et al.* [43]
AIM1	Tumor-suppressor gene	92	*AIM-1* promoter hypermethylation was found in higher frequency in metastatic than in primary melanoma; associated with DFS and OS in stage I/II patients.	Hoshimoto *et al.* [43]
NT5E	5'-nucleotidase activity	52	Relapse with metastatic disease is more common in primary melanomas lacking *NT5E* methylation.	Wang *et al*. [44]
microRNA profile				
miR-10B	N/A	79	Increased expression of miR-10B (3.7-fold) in thick primaries melanomas with metastasis (cases) compared with thick primaries melanomas non metastatic (controls) and a trend of increasing expression between primaries tumors and their matched metastases.	Saldanha *et al.* [7]
Cell-free miRNAs	N/A	126	Several miRNAs were down-regulated in late stage melanoma patients (e.g. miR-200c-3p, -204-5p, -182-5p, -301a-3p) while others were up-regulated (e.g. miR-211-5p, -193b-3p, -720, -205-5p).	Margue *et al.* [45]
miR-142-3p	N/A	84	Biomarker expression >1.5-fold in good compared with poor prognosis patients; expression was significantly lower in patients with stage IV compared with stage III of the disease.	Tembe *et al.* [46]
miR-142-5p	N/A	84	Biomarker expression >1.5-fold in good compared with poor prognosis patients	Tembe *et al.* [46]
miR-150-5p	N/A	84	Biomarker expression >1.5-fold in good compared with poor prognosis patients; reduced expression in BRAF mutation positive tumors.	Tembe *et al.* [46]
miR-200a	N/A	23	Correlation between tumor thickness and loss of expression of miR-200a, miR-200c and miR-203, and their progressive loss in a series of matched primary tumors and associated metastases suggests a possible association with disease progression.	van Kempen *et al.* [47]
miR-200c	N/A	23	Correlation between tumor thickness and loss of expression of miR-200a, miR-200c and miR-203, and their progressive loss in a series of matched primary tumors and associated metastases suggests a possible association with disease progression.	van Kempen *et al.* [47]
miR-203	N/A	23	Correlation between tumor thickness and loss of expression of miR-200a, miR-200c and miR-203, and their progressive loss in a series of matched primary tumors and associated metastases suggests a possible association with disease progression.	van Kempen *et al.* [47]
miR-150	N/A	283	Expression combined with stage separated patients by RFS and Os and improved prediction of recurrence over stage alone.	Fleming *et al.* [48]
miR-30b	N/A	283	Expression combined with stage separated patients by RFS and Os and improved prediction of recurrence over stage alone.	Fleming *et al.* [48]
miR-15B	N/A	283	Expression combined with stage separated patients by RFS and Os and improved prediction of recurrence over stage alone; serum levels significantly increased over time in recurrent patients.	Fleming *et al.* [48]
miR-425	N/A	283	Expression combined with stage separated patients by RFS and Os and improved prediction of recurrence over stage alone.	Fleming *et al.* [48]
miRNA signature: miR150, miR-15b; miR-199a-5p, miR33a and miR-424	N/A	130	Melanoma patient's classification into high and low recurrence risk groups with significant separation of RFS.	Friedman *et al.* [49]
miR-106b	N/A	129	High miR-106b expression was correlated with Breslow thickness, tumor ulceration and advanced clinical stage; patients with shorter 5-years OS showed high miR-106b expression than those with low expressions.	Lin *et al.* [50]
microRNA profile: miR-9, miR-145, miR-150, miR-155 and miR-205	N/A	41	The combination of miR-9, miR-145, miR-150, miR-155 and miR-205 was capable to distinguish the patients with metastasis from those without it.	Shiiyama *et al.* [51]

OS = overall survival, DFS = disease-free survival, DMFS = distant metastases-free survival, SLN = sentinel lymph node, MSS = melanoma-specific survival, RFS = recurrence-free survival, N/A = not applicable.

3.2 Artigo científico II: "Cell-free DNA as an inflammatory marker in post-surgical monitoring of melanoma patients"

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Cell-free DNA as an inflammatory marker in post-surgical monitoring of melanoma patients

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Cell-free DNA as an inflammatory marker in post-surgical monitoring of melanoma patients

Introduction: Cell-free DNA (cfDNA) is extracellular DNA detectable in blood plasma and increased levels have been reported in cancer patients. **Material and Methods:** A case-control study was conduct to determine potential association between cfDNA levels and melanoma. A total of 136 individuals (68 cases and 68 controls) had the plasmatic cfDNA concentration measured by an ultra-sensitive fluorescent dye and spectrofluorometer. In order to validate chronic inflammatory state of melanoma patients, the levels of proinflammatory cytokines (IL-1, IL-6, TNF-α, IFN-γ) and anti-inflammatory cytokine (IL-10) were also quantified. **Results:** Plasma cfDNA and proinflammatory cytokines levels were significantly higher in pre-surgical patients group compared with controls. In contrast, levels of IL-10 are higher in controls than in cases. A cutoff point was established and the individuals were categorized in high levels of cfDNA (≥ 46 ng/mL) and low levels of cfDNA (< 46 ng/mL) groups. Among melanoma patients, 23.5% of the subjects presented cfDNA high levels, while in healthy control, only 2.8% showed. Furthermore, high levels of cfDNA trended to be significantly associated with distant metastasis. There was a significant reduction of about 76% of cfDNA levels after tumor excision. **Conclusion:** cfDNA can be a potential biomarker for melanoma inflammatory status and a tool for help in post-surgical monitoring.

Keywords: Cell-free DNA – Biomarker – Inflammatory cytokines – Post-surgical follow-up – Prognostic factors

Cell-free DNA as an inflammatory marker in post-surgical monitoring of melanoma patients

Introduction

Cutaneous melanoma, arising from the transformation of melanocytes, represents the most aggressive skin cancer with rapid progression, intrinsic resistance to treatment and high mortality rate (1). The incidence of melanoma has increased over the past decades, faster than all other cancers (2), potentially due to improved surveillance, but also to increased sun exposure (3). When diagnosed early, melanoma can be cured by surgical excision. However, approximately 1 in 35 patients develop metastatic tumors and only 15% of those survive for 5 years (4, 5).

Concerning prognosis, the main clinical and histopathologic predictors of outcome are Breslow thickness, presence of ulceration, mitotic rate, as well as sentinel node status (6). However, melanoma has long been recognized as a highly heterogeneous disease which, in some cases, may show an unpredictable course (7, 8). For example, there is a subset of early-stage tumors exhibiting high-risk behavior that are not identified through histological prognostic factors in primary tumor (9). Despite many advances in melanoma molecular knowledge, the identification of patients at risk of developing advanced disease remains a valid medical issue that warrants the search for biomarkers with prognostic significance.

Cell-free DNA (cfDNA) is represented by fragments of double-stranded DNA that left the nucleus of the cells and is present in plasma, serum and others body fluids (10). Although mechanisms of cfDNA release into the blood stream are not completely elucidated, the most common hypothesis is that it arises from dead or dying immune cells (11). Evidences suggest that cfDNA

levels are elevated in both cancer patients (12-15) and in various non-malignant pathological conditions, such as chronic inflammatory diseases or tissue trauma (16, 17), compared to healthy individuals. Some studies even suggest the clinical relevance of this biomarker for the diagnosis, prognosis and monitoring of cancer (18).

Hypothetically, large amounts of cfDNA are expect in cancer patients as result of increased apoptosis and necrosis process, characteristic of tumor cells turnover, leading to an overload of phagocytic cells responsible for clear such debris (19, 20). Therefore, inflammatory processes could be triggered by tumoral areas, which exhibit chronic necrosis and apoptosis events. In consequence, waves of cell death are observed in cancer patients releasing cfDNA fragments to blood stream (21-23).

In this context, which cfDNA could be consider a marker related to inflammatory conditions and melanoma is a highly immunogenic cancer associated with massive immune cell infiltration, we performed a case-control study to determine potential association between cfDNA levels and melanoma. We also quantified the levels of proinflammatory cytokines, interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ) and levels of IL-10, an anti-inflammatory cytokine, in order to validate chronic inflammatory state of melanoma patients. Additionally, we evaluated the impact of surgical treatment on cfDNA levels and the association with disease progression.

Clinical significance

- Cell-free DNA levels are higher in melanoma patients than healthy individuals;

- Proinflammatory cytokines levels are increased in melanoma cases compared to controls;
- Anti-inflamatory cytokine level are reduced in melanoma cases compared to controls;
- Cell-free DNA levels dropped after surgery in melanoma patients;
- Surgical process decreased inflammatory background of melanoma patients and cfDNA can be a potential tool for help in post-surgical monitoring.

Methods

Study population

The study was approved by two Ethical Board Committees (Universidade Federal de Ciências da Saúde de Porto Alegre and Santa Casa de Misericordia de Porto Alegre, Porto Alegre, Brazil) where the study was conducted (approval number 465145 and 436339, respectively). Written informed consent was obtained from all subjects included in the study.

A case-control epidemiological study was conducted in order to evaluate the association between cfDNA levels and melanoma. One hundred thirty-six subjects were included, 68 cases and 68 healthy controls, sex and age matched. The cases were selected from patients who had received, for the first time melanoma diagnosis, and who had applied for treatment at the Melanoma Nucleus of Hospital Santa Rita (Santa Casa de Misericordia de Porto Alegre) between the years 2013 and 2015. Healthy subjects were selected from academic community.

In order to exclude co-morbidities that could affect cfDNA levels, such as chronic inflammatory, autoimmune diseases and recent trauma, the medical records of all subjects were checked and also blood tests and imaging exams were analyzed. The patients compared with healthy subjects were identified as pre-surgical group.

In addition, a complementary analysis was conducted to evaluate the cfDNA levels of patients that reached a year of follow-up after the surgical treatment, designated as the post-surgical group. From 68 subjects enrolled in the study, 61 returned and participated of this second analysis.

The histological sections of all patients were reviewed by the institution's senior pathologist. Histophatological data from primary melanoma tumor were obtained from pathology report of biopsy and patients' medical records. Information from exposition to main risk factors for melanoma (sunburn episodes, familiar and personal melanoma history, phenotypic characteristics and skin's phototype) was collected through standardized questionnaire.

Blood sample collection

A total of 5mL of peripheral blood was collected from the study population in a sterile tube containing EDTA. The blood collection was carried out one single time in individuals of the control group. Among melanoma patients, two blood collections were performed: the first before the surgical procedure (pre-surgical group), meaning the primary tumor had not been completely excised yet at this point; and a second one year after surgical treatment (post-surgical group). In order to separate the blood plasma, the samples were centrifuged at 3.000 rpm

for 10 minutes and stored at -80^{o}C until the quantification of cfDNA levels be run.

Quantitative analysis of cfDNA

cfDNA analysis was performed similar to described in Cichota*et al.*(24). Briefly, the commercial kit Quant-iT™ PicoGreen® dsDNA (Invitrogen) was used to quantify cfDNA. The Quant-iT™ PicoGreen® dsDNA is an ultra-sensitive fluorescent stain which has affinity for double-stranded DNA solutions, detecting and quantifying even low concentrations (from 25 pg/ml of DNA). The stain dilution was made according to the instructions of fabricant and the reactions were performed in 96 well plates. On each plate were added 10µL of plasma, 10 µL of fluorescent stain and 80 µL 1X TE.Calf thymus DNA was used for internal control of reaction. After 5 minutes, cfDNA concentration was reading on spectrofluorometer (SpectraMax® M2e Multimode Microplate Reader) with 480nm excitation and 520nm emission. Each sample was quantified on triplicate and the mean values were calculated to obtain cfDNA final concentration.

Inflammatory cytokines quantification

To validate the inflammatory state of melanoma patients, cytokine levels (IL-1, IL-6, TNFα, INFy and IL-10) were quantified using the ELISA capture, according to the manufacturer's instructions (Abcam's kit, Cambridge, MA, USA).

Statistical Analysis

The data was analyzed using SPSS 23.0 software (Chicago, USA). To compare case and control groups, initially a Kolmogorov-Smirnov analysis was

performed indicating non-normal cfDNA distribution. For this reason we compared quantitative values between case and control groups by Mann-Whitney test and we presented results as mean ± standard deviation (SD), median and 95% confident interval (CI). From results obtained we performed a percentile distribution analysis and categorized our sample by 75th percentile value. Categorical analysis were performed by Chi-square or Fisher's exact test and results presented by relative frequencies (%). From univariate analysis the potential influence of sex and age was evaluated by multivariate analysis of logistic regression (Backward Wald method). A complementary analysis compared the cfDNA levels between pre and post-surgical moment by paired-Student *t* analysis. The statistical significance was established as $P < 0.05$.

Results

Cases and controls included in the study were caucasian, aged with 54.46 ± 14.84 and 55.62 ± 16.36 years old, respectively. In both cases and controls individuals, 51.5% (35/68) were men and 48.5% (33/68) were women. The baseline clinical characteristics referring to the primary tumor from melanoma patients are described in Table 1.

Table 1 here.

Table 2 shows the median concentration of cfDNA and proinflammatory cytokines in samples of melanoma patients without previous treatment and healthy controls. Plasma cfDNA levels were significantly higher in pre-surgical patients group (42.61± 32.96 ng/mL) compared with controls (17.36 ± 10.64 ng/mL) (P<0.005). In the same way, levels of proinflammatory cytokines are significantly increased in melanoma patients when compared to healthy

individuals, except IL-10, which higher measures were find in controls than in cases (P<0.001).

Table 2 here.

As cfDNA and cytokines distribution did not present a normal pattern, additional percentile analysis was performed. A cutoff point was determined from the percentile distribution analysis comparing the cfDNA levels of cases and controls. The percentiles analysis showed differences between groups in 75th cfDNA value, equivalent to 46 ng/ml. From these data, the samples were categorized in high levels of cfDNA group (≥ 46 ng/mL) and low levels of cfDNA group (< 46 ng/mL) and were statistically compared.

Among melanoma patients, 23.5% of the subjects (16/68) presented cfDNA levels above the cutoff point, while in healthy control, only 2.8% (2/68) (X^2_1 = 13.219; P <0.001) showed. Additionally, the multivariate logistic regression analysis (Backward Wald method) showed that the association between melanoma and high levels of cfDNA (≥ 46 ng/mL) was independent of sample gender and age.

High levels of cfDNA trended to be significantly associated with distant metastasis (X^2_1 = 3.878; P = 0.089). Metastasis to distant organs were more frequent in group of melanoma patients with cfDNA levels ≥ 46 ng/mL than group with lower levels of cfDNA, 18.8% and 3.9% respectively (table 3).

Table 3 here.

Concerning to the others prognostic (Breslow thickness, mitotic rate, presence of ulceration and SLN status) and risk factors analyzed (previous skin cancer, another prior cancer, familial melanoma, skin phototype, eye color,

sunburn episodes and childhood freckles), no significant relationship with high and low cfDNA levels were identified.

Finally, when comparing average cfDNA levels before (42.61 ng/mL ± 32.96) and after primary tumor complete excision (27.13ng/mL ± 11.68), there was a significant reduction of these levels after surgical treatment (t_3 = 24.2; P < 0.05). Furthermore, a significant difference was found between cfDNA concentration melanoma patients samples collected one year after surgery and control group (Figure 1).

Figure 1 here.

The impact of surgery on cfDNA was evaluated by percentage of cfDNA found in post-surgical group in comparison with pre-surgical group (100%). The mean of cfDNA dropped 76.9% indicating that surgical process decreased inflammatory background of melanoma patients.

Discussion

The results of the present investigation indicate that blood cfDNA levels quantified by Picogreen dye are increased in melanoma patients prior surgery. Moreover, was observed a lowering in cfDNA values when patients were submitted to tumor excision. For our best knowledge, this is the first report with association between cfDNA levels and melanoma patients pre and post-surgery.

The presence of cfDNA in human plasma was described, for the first time, over six decades ago (25). Nonetheless, only in 1977 the presence of abnormally high levels of cfDNA was identified in the blood stream of cancer patients compared with healthy controls (26). Since then, the potential

diagnostic, prognostic and monitoring significance of cfDNA has been demonstrated for various types of cancer (27-29).

The progression in the isolation of cfDNA was only possible due to the development of sensitive molecular procedures using specific fluorescent dyes and PCR techniques (30). Nowadays, cfDNA quantification is easy, low cost and minimally invasive technique sensible enough to detect few quantities of DNA released in the blood.

Corroborating our results, plenty of studies have been demonstrating increased levels of cfDNA in cancer patients compared with those with non-malignant disease (31-33). In addition, we showed that there is association between increased levels of cfDNA and melanoma, regardless of age or sex. It is believed that DNA release in the blood of patients with malignant disease is associated with neoplastic cellular death process in tumoral environment (34). Associated with necrosis and apoptosis, inflammation process can also coexist in tumors, leading to an elaboration of cytokines and inflammatory mediators (21-23).

Prior evidences reported that melanoma is strongly associated with the inflammatory process, which can be observed by high levels of proinflammatory cytokine secretion (35). For this reason, we quantified proinflammatory cytokines IL-1, IL-6, TNF-α, IFN-γ and IL-10in the pre-surgical group to confirm the inflammatory background of melanoma patients. Actually, our results showed higher levels of proinflammatory cytokines, and lower levels of IL-10, an anti-inflammatory cytokine, in case group than control. Corroborating our findings, Wiguna and Walden (36) showed that the expression of IL-10 gene is lower in melanoma metastases than normal skin.

It is well established that inflammatory process is mediated by a balance between proinflammatory (Th1) and anti-inflammatory (Th2) cytokines expressed by CD4+ T cells (37). In this way, significant association or with proinflammatory cytokines or anti-inflammatory cytokines may indicate that cfDNA levels could be an emergent marker of melanoma inflammatory status. In fact, subjects with higher cfDNA levels (> 46 ng/mL) showed a significant association with lower IL-10 levels.

Potential association between cfDNA levels and other clinical aspects of melanoma diseases was also evaluated, but no statistically significant was found. However, our data showed a tendency of association between higher cfDNA levels and distant metastasis development in patients' follow-up during one year. Unfortunately, statistical analysis was not significant probably due to a low number of patients including in our study and/or the short follow-up time that the patients were observed. In the literature, it is recognized that distance metastasis also has the potential to collaborate with increasing levels of cfDNA in individuals (38).

We are conscious that number of patients was low affecting the analysis of results. However, melanoma patients with a recent diagnosis, without prior treatment or with other non-inflammatory diseases and dysfunctions are very hard to be selected. For this reason, we believe that our results could be considered epidemiologically relevant.

Finally, after surgery for tumor excision the cfDNA plasmatic levels of melanoma patients decreased significantly, although it has not reached a concentration as low as those present in healthy individuals. Therefore, it is probable that cfDNA levels decrease after the surgical treatment is

consequence of local inflammation reduction. The decrease of cfDNA levels after anticancer therapies has already been reported in the literature (34) and it has been observed that the maintenance of high levels of this biomarker may suggest the presence of residual tumor cells (39).

All this results combined propound that the quantification of cfDNA has the potential to be consider an emergent marker of melanoma inflammatory status, as well as its potential to become an additional monitoring method for surgical success of melanoma patients.

Conclusion

The cfDNA proved to be a significant biomarker for melanoma inflammatory status regardless of sex and age, as well as its potential as a complementary test in the postoperative monitoring of the patients. In this manner, changes in cfDNA can be traced by repeated blood samples during patient's follow-up allowing for early relapse detection. The major challenge is melanoma management is the lack of an efficient marker for detecting disease progression at an early stage and/or for monitoring patients' treatment response. The employing of non-invasive blood biomarker assays during follow-up allow for monitoring of disease dynamic associated with treatment effectiveness is crucial in order to improve patients' outcome.

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Declaration of interest

The authors declare that they have no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the present work.

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Figure captions

Figure 1: Comparison of cfDNA levels between cases at pre-surgical moment and after one year from surgery and healthy controls. The surgical treatment decreased cfDNA levels in melanoma patients, leading to concentration almost as low as those observed in controls.

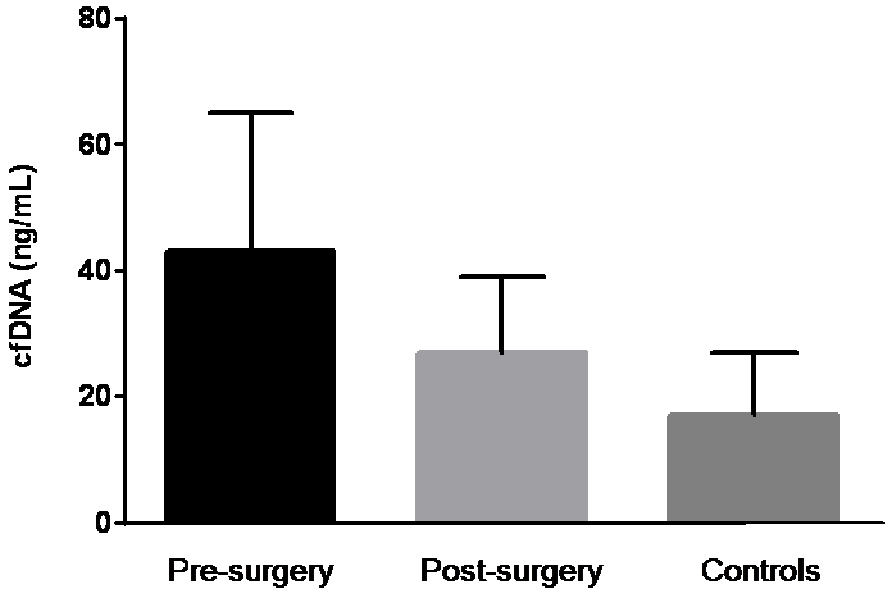

Table 1. Clinicopathological features from primary tumor of melanoma patients.

Variable	*N*	%
Tumor Site		
Head/neck	11	16.2
Trunk	36	52.9
Upper limb	12	17.7
Lower limb	9	13.2
Histological subtype		
Superficial spreading	51	75
Lentigo maligna	7	10.3
Nodular	7	10.3
Acral lentiginous	2	2.9
Desmoplasic	1	1.5
Clark's level		
I	16	23.6
II	13	19.1
III	17	25
IV	20	29.4
V	2	2.9
Breslow thickness		
T*is*	16	23.5
≤ 1 mm	32	47.1
1.01 - 2 mm	12	17.6
2.01 - 4 mm	3	4.4
> 4 mm	5	7.4
Presence of ulceration		
Yes	8	11.8
No	60	88.2
Mitotic rate		
Mean ± SD	1.63 ± 2.51	
Satellitosis		
Yes	2	2.9
No	66	97.1

T*is* = tumor *in situ*; SD = standard deviation

Table 2. Comparison of cfDNA and inflammatory cytokines levels from melanoma patients before surgical treatment (cases) and healthy controls.

Inflammatory markers	Cases (*N* = 68)			Controls (*N* = 68)			*P* value*
	Mean ± SD**	Median	95% CI (Lower-Upper)	Mean ± SD**	Median	95% CI (Lower-Upper)	
cfDNA (ng/μL)	42.61 ± 32.96	34.94	34.44 – 52.16	17.36 ± 10.64	14	14 – 19.3	< 0.005
IL-1 (pg/mL)	175.92 ± 51,27	166	161.34 – 190.49	101.45 ± 16.69	100	97.3 – 105.62	< 0.001
IL-6 (pg/mL)	185 ± 72,69	180	185 – 226.31	126.64 ± 14.81	129	122 –130.84	< 0.001
TNFα (pg/mL)	221.38 ± 71.6	212	201.03 – 241.72	144.5 ± 26.6	149	137.44 – 152.31	< 0.001
INFγ (pg/mL)	277.24 ± 101.5	294	248.39 – 306.08	200.26 ± 37.16	213	189.64 – 210	< 0.001
IL-10 (pg/mL)	32.3 ± 12.12	33.5	28.85 – 35.74	67.2 ± 22.65	59.5	59.5 – 73.68	< 0.001

* Mann-Whitney test was used in comparisons.
**SD = standard error.

Table 3. Association of melanoma risk and prognostic factors with cfDNA levels.

Variable	N	cfDNA level (%)		*P* value
		≥ 46 ng/mL	< 46 ng/mL	
Previous skin carcinoma				0.58
Yes	10	0	19.2	
No	58	100	80.8	
Another prior cancer				0.064
Yes	3	0	3	
No	65	35	30	
Familial melanoma				0.835
Yes	14	18.8	21.2	
No	54	81.3	78.8	
Skin phototype I/II				0.722
Yes	45	62.5	67.3	
No	23	37.5	32.7	
Eye color				0.788
Light	36	50	53.8	
Dark	32	50	47.1	
Sunburn episodes				0.184
Childhood	10	25	11.5	
Another periods of life	58	75	88.5	
Childhood freckles				0.897
Yes	13	7.7	92.3	
No	55	27.3	72.7	
Presence of ulceration				0.346
Yes	9	6.3	15.4	
No	59	93.8	84.6	
SLN status				0.933
Positive	4	14.3	13	
Negative	26	85.7	87	
Metastasis				
Yes	5	18.8	3.9	0.089
No	63	81.3	96.1	
Breslow thickness				0.634*
Mean ± SD	68	1.04 ± 1.31	1.27 ± 2.46	
Mitotic rate				0.934*
Mean ± SD	68	1.66 ± 2.55	1.60 ± 2.51	

SLN = sentinel lymph node; SD = standard deviation; *Statistical test: Student's *t* test (the others variables were analyzed with Chi-square test); Statistical significance considered when $P < 0.05$.

3.3 Artigo científico II: "Gender and age-specific association between manganese superoxide dismutase polymorphism (Ala16Val-SOD2) and melanoma risk"

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Gender and age-specific association between manganese superoxide dismutase polymorphism (Ala16Val-SOD2) and melanoma risk

Short title: Ala16Val-SOD2 polymorphism and melanoma risk

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Abstract

Reactive oxygen species (ROS) inadequately detoxified may result in oxidative stress, which is associated with cancer development and progression, including skin carcinogenesis. Enzymatic detoxification is one of the defense mechanisms to protect cells against ROS. We evaluated the association

between melanoma and the functional polymorphism of SOD2 (Ala16Val-SOD2), which encodes the manganese superoxide dismutase (MnSOD). The polymorphism was determined in 62 melanoma patients and 500 healthy subjects. Genotyping from blood of cases and controls was carried out through PCR-RFLP method using specific primers and *Hae III* restriction enzyme. The genetic frequencies of Ala16Val-SOD2 polymorphism were in Hardy-Weinberg equilibrium. Allele frequencies were A = 0.55 and V = 0.45 in the control group and A = 0.56 and V = 0.44 in melanoma group. Comparison between genotypes showed a higher frequency of AA in melanoma patients when compared to healthy subjects. For the first time in literature, our results provide evidences about an increased melanoma risk associated with SOD2 polymorphism. However this association is not independent of sex and age, meaning that women and elderly carrying AA genotype are more susceptibility to melanoma.

Keywords: Oxidative stress, antioxidant enzymes, Ala16Val polymorphism, melanoma risk

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Gender and age-specific association between manganese superoxide dismutase polymorphism (Ala16Val-SOD2) and melanoma risk

Introduction

Cutaneous melanoma, arising from abnormal proliferation of melanocytes, is an aggressive and lethal form of skin cancer due to its propensity to metastasize and intrinsic resistance to treatment [1]. Although melanoma represents only 4% of all dermatological neoplasias, it is responsible by 75% of skin cancer-related deaths [2]. The disease can be cured by surgical removal when diagnosed in early stage. However, only 14% of metastatic melanoma patients survive for 5 years [2]. Currently, the American Joint Committee on Cancer (AJCC) staging system, based on histopathological features of primary tumor, is widely used to predict melanoma prognosis [3]. Patients are classified into four stages according Breslow thickness, presence of ulceration, extent of nodal involvement for primary and locally metastatic melanoma, metastasis to distant organs and serum lactate dehydrogenase (LDH) level [3].

In the last decades, the incidence of melanoma is increasing worldwide, faster than any other malignancy [4]. This increase can be explained both by changes in diagnostic criteria and early detection due to improved surveillance, as by increased sun exposure [5]. Consider the major etiological factor in skin cancer, the ultraviolet radiation [UVR] of the sun can directly damage DNA or generate reactive oxygen species (ROS) [6].

ROS, such as superoxide anion radical ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($^{\bullet}OH$), are generated during normal aerobic metabolism or exposure to toxicants [7,8]. ROS inadequately detoxified may result in oxidative stress which is associated with cancer development and progression, including skin carcinogenesis [9,10,11,12]. Concerning to the melanoma, ROS are present in all stages of its development and systemic and tumor redox deregulation has been reported in patients [13-16].

ROS are known to react with macromolecules inducing DNA oxidative damage which can result in cell death or mutations [17]. One of the defense mechanisms to protect cells from ROS includes detoxifying enzymes [15]. Manganese superoxide dismutase (MnSOD), a family of superoxide dismutases(SODs), is an endogenous antioxidant enzyme that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide in the mitochondria [18]. This enzyme is encoded by the superoxide dismutase (SOD2) gene (locus 6q25.3). A polymorphism occurs at codon 16 of SOD2 gene where a single nucleotide change from T to C leading to valine-to-alanine substitution (Ala16Val)[19]. The Ala variant allows more efficient SOD2 uptake into the mitochondria and generates more active SOD2 compared with the Val variant [32, 33]. Despite extensively researched, Ala16Val-SOD2 polymorphism association with cancer remains controversial. Several studies have suggested increased cancer risk associated with the AA [Ala/Ala] genotype, contrary to might be predicted [22-26].

As far as we know, there are few studies evaluating SOD2 polymorphism in melanoma [27]. Therefore, in this study, we investigated the association between the Ala16Val-SOD2 polymorphism and melanoma. In addition, we

evaluated the potential association between this genetic variant and melanoma clinicopathological prognostic factors, as well as the relationship with phototype and sunburn episodes related with risk factors on melanoma.

Methods

Study population

A case-control study was conducted in order to evaluate the association between MnSOD polymorphysm and melanoma. A total of 562 subjects were included, from which 62 were melanoma patients and 500 were healthy subjects.

Eligible for inclusion as cases in the present study were patients diagnosed with melanoma who applied for treatment at the Melanoma Nucleus of Hospital Santa Rita (Porto Alegre, Brazil) between the years 2013 and 2015. Medical records of patients were checked and blood tests and imaging exams were analyzed to exclude possible co-morbidities and other cancer diagnosis prior to the date of blood draw. Histological sections from biopsies, surgery and sentinel lymph nodes were analyzed by the institution’s senior pathologist. Information about the primary tumor was recollected from pathology report of biopsy and patients' medical records. Information from exposition to main risk factors for melanoma was collected through standardized questionnaire.

Control group was composed by healthy volunteers from an academic community and were paired by sex and age with cases. Controls were eligible for selection if they had no pathologic condition such cancer, chronic

inflammation and recent trauma. Institutional review board approved the study. Written informed consent was obtained from all subjects included in the study.

DNA extraction and Ala16Val-SOD2 polymorphism genotyping

Peripheral blood was collected from all participants by venous puncture and was stored at -20˚C until analysis. Genomic DNA from blood leukocytes was extracted using a salting-out method [28]. Cases and controls were genotyped as described by Bica *et al* [29]. Briefly, the method is based in a Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism (PCR-RFLP). Initially, a fragment of the human SOD2 gene (110bp) was amplified with primers 5'-ACCAGCAGGCAGCTGGCGCCGG-3' (sense-strand) and 5'-GCGTTGATGTGAGGTTCCAG-3' (antisense-strand). The PCR reaction was carried out in a total volume of 50 µL containing 5 µL 10X PCR buffer, 1.25 µL 10mM dNTPs, 1 µL 25mM $MgCl_2$, 0.5 µL Taq polymerase (Gibco Inc, Co.), 10 ng genomic DNA, 0.4 pM each primer and enough ddH2O for complete the reaction volume. PCR reaction consisted of an initial denaturation at 95 ºCfor 5 min followed by 35 cycles at 95 ºC for 1 min, 61 ºC for 1 min and 30 s of extension at 72 ºC. An additional final extension of 7 min at 72ºC was performed at the end of final cycle. Amplicons (10 µL) were digested with specifc endonuclease *Hae III* (15 U, Gibco. Inc, Co.)for 6 h at 37˚C. Digestion products were analyzed by electrophoresis in 4% agarose gel stained with ethidium bromide. Different patterns of restriction are observed in agarose gel electrophoresis according to genotype: AA (2 fragments; 23 and 85 bp); AV (3 fragments; 23, 85 and 110 bp); and VV (110 bp).

Statistical analysis

The data was analyzed using SPSS 23.0 software (Chicago, USA). Student's t test was carried out to compare quantitative baseline variables between case and control subjects, while Chi-square (χ^2) was performed to compare categorical variables between the groups. The allele frequencies were estimated by the gene-counting method. Chi-square analysis was used to estimate the Hardy–Weinberg equilibrium. The allelic and genotype frequencies were compared among groups using chi-square or the Fisher's exact test. The association between prognostic/risk factors of melanoma and Ala16Val-SOD2 polymorphism was evaluated by ANOVA or chi-square test. The statistical significance was established as $P< 0.05$.

Results

Clinicopathological features of melanoma cases are listed in Table 1. Melanoma patients and healthy individual included in the study were Caucasian and there were no signifcant differences in age and sex in both groups. Table 2 shows baselines characteristics of the study population and Ala16Val-SOD2 genotype frequencies.

Table 1 here.

The genetic frequencies of Ala16Val-SOD2 polymorphism were in Hardy-Weinberg equilibrium. Allele frequencies were A = 0.55 and V = 0.45 in the

control group and A = 0.56 and V = 0.44 in melanoma group. Comparison between genotypes showed a higher frequency of AA in melanoma patients when compared to healthy subjects.

Table 2 here.

However, the association between AA frequency and melanoma was influenced by sex and age. Women with AA genotype were at higher melanoma risk ($P< 0.05$; OR = 2.49; 95% CI = 1.15 – 4.41). Similarly, elderly (≥ 60 years old) homozygote AA were more susceptible to melanoma development ($P< 0.001$; OR = 4.0; 95% CI = 1.79 – 8.97) (Figure 1 and 2).

In addition, the association between tumoral aggressiveness at the time of diagnosis and Ala16Val-SOD2 polymorphism was evaluated in melanoma patients. There was no significant association between genetic polymorphism and Breslow thickness ($P = 0.51$), mitosis rate ($P = 0.89$), presence of ulceration ($P = 0.53$) or metastasis ($P = 0.88$). Moreover, no significant interaction was observed between this polymorphism and patients' phototype ($P = 0.88$) or sunburn history ($P = 0.88$).

Figure 1 here.

Figure 2 here.

DISCUSSION

The results of the present case–control study indicate that the genotypes frequencies of Ala16Val-SOD2 polymorphism were different when melanoma

patients were compared to controls. A significant association was found between AA genotype and melanoma risk. Nevertheless, this association is not independent of sex and age, meaning that women and elderly carrying AA genotype are more susceptibility to melanoma. To our knowledge, this is the first report in medical literature to suggest an association of melanoma and SOD2 polymorphism.

Previous results described by Han *et al* in skin cancer have shown no significant association between Ala16Val-SOD2 polymorphism and the risk of each type of skin cancer, including melanoma. Similar to our finding, they did not observed significant interaction between this polymorphism and sunburn history, well known as an etiological factor in dermatological neoplasias[27, 30].

Ultraviolet radiation can induce photooxidative damage in the skin and it is an important issue since sun exposure has been increasing [5, 31]. The oxidative stress induced by inflammatory response to UV exposure causes damage to cellular components and changes in pattern of gene expression leading to skin pathologies such as cancer [12].

Concerning antioxidant mechanism, manganese superoxido dismutase (MnSOD) is considered the first line of defense against reactive oxygen species produced by oxidative phosphorylation upon UV radiation and inflammation [32]. Evidences suggest that SOD activities are altered in blood of melanoma patients, indicating oxidative stress associated to the disease [15].

Several epidemiological studies have been evaluating the association between the functional polymorphism Ala16VAL-SOD2 and cancer risk [33]. It has been postulated that this polymorphism, which alter the secondary structure of MnSOD, affect the efficiency of mitochondrial transport of the enzyme [19]. In

fact, Ala variant allows more efficient MnSOD uptake into the mitochondria than Val variant, suggesting that AA homozygous may have higher SOD2 antioxidant activity, which could suppress carcinogenesis process [20, 21, 33]. However, this hypothesis is controversial in the literature, with an increase in the number of studies that have observed association between Ala allele and cancer risk [23,34]. Bica *et al* in a case-control study found that the risk of breast cancer was increased in men and women who carried AA genotype, as well as in prostate cancer, when compared to controls[29].

Our results also suggest that AA genotype significantly increase the baseline risk of melanoma in women and elderly. Although confirmation of this study is needed due to the small number of melanoma patients included, these findings suggest an important role of Ala16Val-SOD2 polymorphism in melanoma susceptibility related to sex and age. Therefore, complementary studies with a larger sample size are needed to confirm these results.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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TABLE AND FIGURES LEGENDS

Table 1. Clinicopathological features of the case group ($n = 62$).

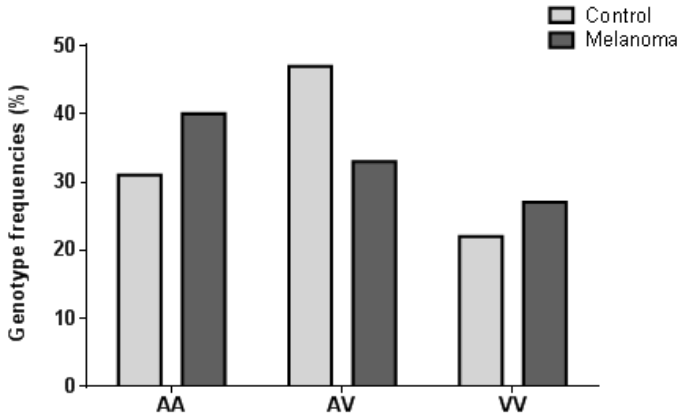
Table 2. Baseline characteristics and Ala16Val-SOD2 genetic frequencies of melanoma patients and controls.

Fig.1 Genotype frequencies comparison between cases and controls. (A) Significant differences between genotypes frequencies were observed when cases were compared to controls ($P < 0.05$); Ala16Val-SOD2 polymorphism was significantly associated with melanoma risk, but this association was not independent of (B) sex and (C) age. Women and elderly carrying AA genotype are more susceptibility to melanoma.

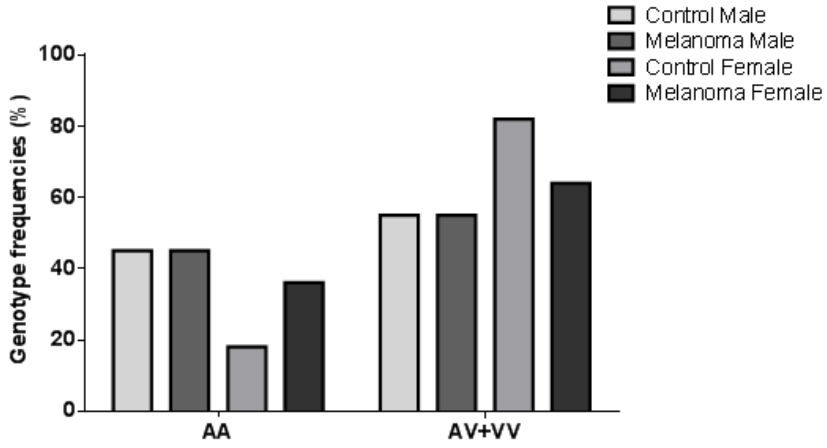
Fig.2 Relative risk of AA genotype and melanoma associated with sex and age. The bars are representing the odds ratio and 95 % confidence interval. The total population of the study was stratified by sex and age (older ≥ 60 years old; younger < 60 years old) to perform the comparisons of genotype frequencies. Women with AA genotype had 2.49-fold increased melanoma risk, while elderly who carried AA genotype were 4-fold more susceptible to melanoma.

Figure 1

A


Control
Melanoma
Genotype frequencies (%)
50
40
30
20
10
0
AA
AV
VV

B


Control Male
Melanoma Male
Control Female
Melanoma Female
Genotype frequencies (%)
100
80
60
40
20
0
AA
AV+VV

C

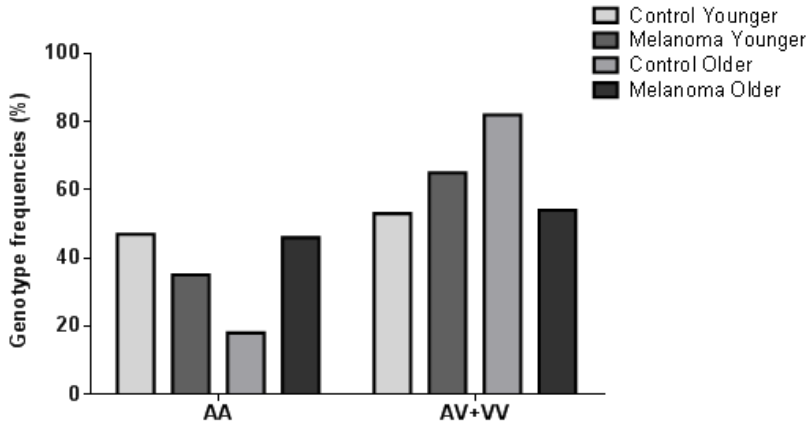

Control Younger
Melanoma Younger
Control Older
Melanoma Older
Genotype frequencies (%)
100
80
60
40
20
0
AA
AV+VV

Figure 2

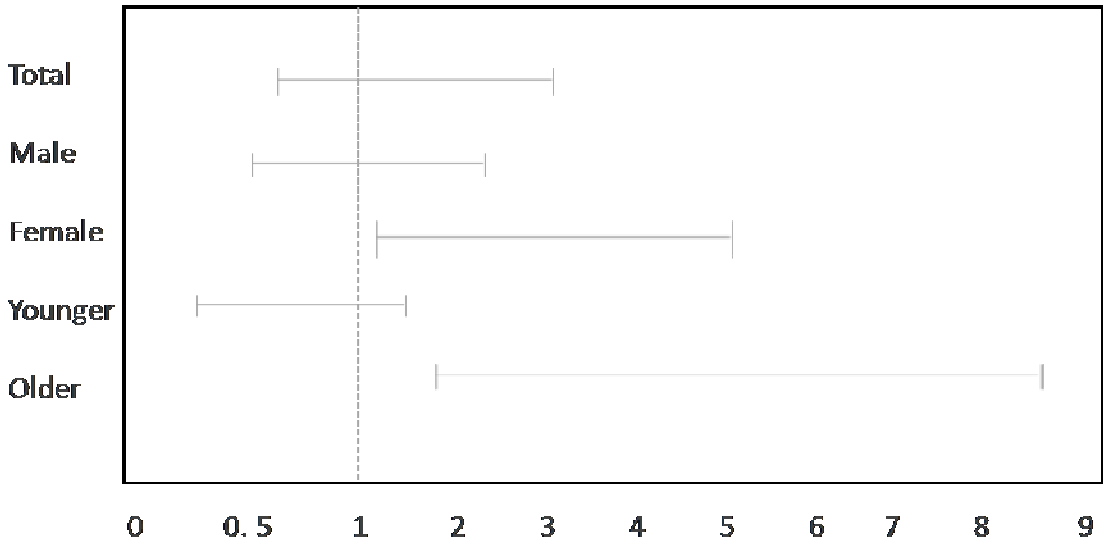


Table 1

Characteristics	*N*	%
Tumor Site		
Head/neck	13	21
Trunk	32	51.6
Upper limb	10	16.1
Lower limb	7	11.3
Histological subtype		
Superficial spreading	47	75.8
Lentigo maligna	7	11.3
Nodular	5	8.1
Acral lentiginous	2	3.2
Desmoplasic	1	1.6
Clark's level		
I	13	21
II	10	16.1
III	14	22.6
IV	22	35.5
V	3	4.8
Breslow thickness		
T*is*	13	21
≤ 1 mm	25	40.3
1.01 - 2 mm	10	16.1
2.01 - 4 mm	10	16.1
> 4 mm	4	6.5
Presence of ulceration		
Yes	7	11.3
No	55	88.7
Mitotic rate		
Mean ± SD	1.94 ± 2.47	
Metastasis		
Yes	16	25.8
No	46	74.2

T*is* = tumor *in situ*; SD = standard deviation

Table 2

Variables		Cases	Controls	*P*
Age (years, mean ± SD)		57.55 ± 11.98	56.17 ± 15.32	0.49
Sex (*n*)				0.55
	Male	29	237	
	Female	33	263	
Genotype *n* (%)				*0.04
	AA	25 (40.3)	156 (31.2)	
	VV	17 (27.4)	110 (22)	
	AV	20 (32.3)	234 (46.8)	

SD = standard deviation; Statistical test: age comparison between groups was analyzed with Student's *t* test; other variables were analyzed with Chi-square test; Statistical significance was considered when $P < 0.05$.

4. CONSIDERAÇÕES FINAIS

O presente estudo faz parte de um projeto maior intitulado "**Avaliação de Marcadores Prognósticos no Melanoma",** aprovado pelos Comitês de Ética em Pesquisa (CEP) da UFCSPA (Parecer N° 465.145) e da ISCMPA (Parecer do projeto original Nº 436.339; Parecer da emenda Nº 843.196). Além da presente tese de doutorado, também está sendo desenvolvida uma dissertação de mestrado como parte integrante deste projeto.

O Núcleo de Melanoma do Hospital Santa Rita da ISCMPA é um centro de referência no tratamento de pacientes com melanoma e coordenado pelo Dr. Felice Riccardi, colaborador deste projeto. A parceria deste serviço com os pesquisadores pertencentes ao Grupo de Pesquisa de Marcadores Tumorais de Diagnóstico e Prognóstico da UFCSPA teve início em 2013. Um objetivo comum aos dois grupos é o desenvolvimento de biomarcadores de prognóstico para o melanoma capaz de serem incorporados na prática clínica visando melhor classificar os pacientes com alto e baixo risco de doença metastática.

O desenvolvimento de marcadores de prognóstico é extremamente importante para o manejo desta doença. O biomarcador ideal deve ser sensível, específico, confiável, de rápida análise, de baixo custo e deve "agregar valor", em termos prognósticos ou terapêuticos, às ferramentas atuais de avaliação.

A vantagem da triagem precoce de pacientes com alto risco é a oportunidade de identificar aqueles que necessitam de um acompanhamento clínico intensificado ou mesmo tratamentos adjuvantes, enquanto que, por outro lado, minimizaria intervenções desnecessárias em pacientes de baixo risco.

Na busca por artigos para compor a revisão sistemática de marcadores genéticos e epigenéticos no melanoma (artigo I da tese), ficou claro que embora tenha havido recente progresso no entendimento dos mecanismos genéticos por trás do melanoma, poucos biomarcadores provaram serem clinicamente significantes e, por isto, nenhum deles foi incorporado à rotina clínica até hoje. Muitos pesquisadores da área vêm apostando na abordagem de múltiplos marcadores para acessar o desfecho dos pacientes portadores de melanoma, principalmente aqueles que avaliam a expressão de microRNAs no sangue e tecido, por se tratarem de amostras facilmente obtidas de materiais biológicos escassos e/ou mal-preservados.

Devido a um maior conhecimento da doença por parte da população, nos últimos anos há uma tendência de diagnóstico precoce da doença. Na experiência obtida durante a condução deste estudo, foi notória a identificação de um grande número de pacientes com lesões finas ou *in situ*, usualmente curáveis cirurgicamente. No entanto, houve um subgrupo de pacientes com melanomas igualmente finos que vieram a óbito após o desenvolvimento de metástases sistêmicas, salientando o caráter heterogêneo desta neoplasia que não pode ser acessado em sua totalidade pelo sistema de classificação da AJCC. Portanto, acreditamos que este fato justifique o desenvolvimento de novos marcadores de prognóstico.

Biomarcadores sanguíneos são atrativos por se tratarem de métodos fáceis de obtenção de amostras e minimamente invasivos comparados com biópsias de tecido, por exemplo. Por esta razão, optamos por avaliar o potencial da quantificação de *cfDNA* no prognóstico do melanoma.

Em uma análise preliminar apresentada em forma de resumo e pôster no Congresso Brasileiro de Melanoma (2015), o trabalho intitulado “Potencial marcador plasmático de *screening* em pacientes com melanoma” foi nomeado como um dos destaques do evento e escolhido para apresentação oral, a qual foi realizada pelo Dr. Felice Riccardi. Nosso estudo foi reconhecido como segundo melhor trabalho do evento, além de recebermos premiação financeira.

Com a inclusão de novos casos, além da incorporação da análise para avaliar o status inflamatório dos pacientes, escrevemos o artigo II da tese que foi submetido para publicação na revista científica *Biomarkers*. Como principais resultados observados, destacamos: (1) níveis de *cfDNA* significativamente aumentado nos pacientes com melanoma em relação aos indivíduos saudáveis; (2) níveis aumentados de citocinas pró-inflamatórias e reduzidos da citocina anti-inflamatória nos casos quando comparado aos controles, corroborando o potencial do *cfDNA* como marcador inflamatório neste câncer; e (3) demonstramos o impacto do tratamento cirúrgico na redução dos níveis de *cfDNA*. Consideramos nossos achados cientificamente relevantes por este ser o único estudo na literatura a quantificar este marcador com amostras pré e pós-cirúrgicas em pacientes com melanoma.

Seguindo a linha de marcadores relacionados à inflamação no câncer, avaliamos a associação do melanoma com o polimorfismo da enzima

antioxidante superóxido dismutase dependente de manganês (MnSOD). O que encontramos, também de forma inédita na literatura, foi que o genótipo AA (Ala/Ala) do polimorfismo Ala16Val-SOD2, está associado com maior risco de melanoma. Entretanto, está associação é influenciada pelo sexo e idade. Sendo assim, observamos que mulheres e idosos portadores do genótipo AA estão mais susceptíveis ao câncer. A partir destes resultamos, foi desenvolvido um artigo científico encaminhado para a revista *Pathology and Oncology Research*.

5. ANEXOS

5.1 Parecer do Comitê de Ética em Pesquisa da UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de Marcadores Prognósticos no Melanoma
Pesquisador: Claudia Giuliano Bica
Área Temática:
Versão: 3
CAAE: 14404513.4.0000.5335
Instituição Proponente: Irmandade da Santa Casa de Misericordia de Porto Alegre - ISCMPA
Patrocinador Principal: Universidade Federal de Ciências da Saúde de Porto Alegre

DADOS DO PARECER

Número do Parecer: 465.145
Data da Relatoria: 21/11/2013

Apresentação do Projeto:
Trata-se de um projeto de doutorado da aluna Munique Pereira Mendonça, orientado pela Profa. Cláudia G. Bica. Neste estudo, pacientes com diagnóstico de melanoma, provenientes do Núcleo de Melanoma do Hospital Santa Rita, serão seguidos semestralmente durante dois anos e avaliados quanto a identificaçãp de potenciais biomarcadores que possam influenciar no prognóstico do mesmo. A presença de biomarcadores será comparada com populção controle sem melanoma.
O referido projeto será realizado no Complexo da Santa Casa onde já recebeu aprovação do CEP. A Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) será centro coparticipante uma vez que algumas análises moleculares e bioquímicas serão realizadas no Laboratório de Patologia da Pós-Graduação da UFCSPA.

Objetivo da Pesquisa:
O objetivo geral do projeto é avaliar o potencial de marcadores moleculares no prognóstico do melanoma maligno cutâneo.
Os objetivo secundário do estudo são: I. Comparar os parâmetros inflamatórios/oxidativos e a quantidade de cell-free DNA entre indivíduos hígidos e portadores de melanoma. II. Quantificar os níveis de cell-free DNA (cfDNA) de pacientes com melanoma; III. Estabelecer o perfil inflamatório/oxidativo dos pacientes portadores da doença; IV. Identificar a expressão de moléculas de adesão nas peças cirúrgicas.

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Avaliação dos Riscos e Benefícios:

o projeto não oferece maiores riscos aos pacientes, uma vez que serão aproveitados a sobra de sangue dos exames de rotina realizados no Hospital Santa Rita da ISCMPA para o estudo. Exames esses feitos assistencialmente, rotineiramente.

Embora sem benefícios diretos poderá auxiliar na identificação de fatores que possam auxiliar prognóstico da doença.

Comentários e Considerações sobre a Pesquisa:

O projeto é relevante e exequível. O mesmo já foi aprovado pelo CEP da Santa Casa. A Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) será centro coparticipante uma vez que algumas análises moleculares e bioquímicas serão realizadas no Laboratório de Patologia da Pós-Graduação da UFCSPA.

Considerações sobre os Termos de apresentação obrigatória:

Documentos regulatórios estão adequados. Possui carta de concordância das instituições.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

O projeto está dentro dos padrões e julgo que o mesmo possa ser aprovado.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Data de início 23/10/13

Data de término 06/03/17

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Continuação do Parecer: 465.145

PORTO ALEGRE, 22 de Novembro de 2013

Assinador por:
José Geraldo Vernet Taborda
(Coordenador)

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Bairro: **CEP:** 90.050-170
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Telefone: (513)303 -8804 **E-mail:** cep@ufcspa.edu.br

5.2 Parecer do Comitê de Ética em Pesquisa da ISCMPA versão 3

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de Marcadores Prognósticos no Melanoma
Pesquisador: Claudia Giuliano Bica
Área Temática:
Versão: 3
CAAE: 14404513.4.0000.5335
Instituição Proponente: Irmandade da Santa Casa de Misericordia de Porto Alegre - ISCMPA
Patrocinador Principal: Universidade Federal de Ciências da Saúde de Porto Alegre

DADOS DO PARECER

Número do Parecer: 436.339
Data da Relatoria: 25/09/2013

Apresentação do Projeto:
Projeto de pós-graduação em nível de doutorado de MUNIQUE PEREIRA MENDONÇA. ORIENTADORA: DRA. CLÁUDIA G. BICA. Estudo Prospectivo de Coorte com pacientes portadores de melanoma maligno cutâneo sem metástases do Núcleo de Melanoma do Hospital Santa Rita. Os pacientes serão acompanhados semestralmente, totalizando 4 amostragens de cada paciente.

Objetivo da Pesquisa:
Objetivo Primário: Avaliar o potencial de marcadores moleculares no prognóstico do melanoma maligno cutâneo.
Objetivo Secundário: I. Comparar os parâmetros inflamatórios/oxidativos e a quantidade de cell-free DNA entre indivíduos hígidos e portadores de melanoma. II. Quantificar os níveis de cell-free DNA (cfDNA) de pacientes com melanoma; III. Estabelecer o perfil inflamatório/oxidativo dos pacientes portadores da doença; IV. Identificar a expressão de moléculas de adesão nas peças cirúrgicas.

Avaliação dos Riscos e Benefícios:
Riscos: Segundo os autores, o projeto não oferece maiores riscos aos pacientes, uma vez que serão aproveitados a sobra de sangue dos exames de rotina realizados no Hospital Santa Rita da ISCMPA para o estudo. Do mesmo modo, serão utilizadas peças cirúrgicas dos pacientes, as quais

serão devolvidas ao Laboratório de Patologia da Santa Casa assim que o estudo for concluído. NO TCLE foi descrito que as informações contidas no prontuário serão de acesso da equipe do estudo, e que a equipe garante a realização de todas as medidas cabíveis para que os dados sejam mantidos em sigilo absoluto. Benefícios: Os pacientes não terão nenhum benefício direto com esta pesquisa, entretanto os dados científicos gerados poderão contribuir para o maior conhecimento desta importante neoplasia.

Comentários e Considerações sobre a Pesquisa:

Estudo prospectivo de coorte.

Tamanho da Amostra: 170 (85 casos e 85 controles), orçamento R$ 10.595,00, apoio financeiro Universidade Federal de Ciências da Saúde de Porto Alegre. Local de realização do projeto: Hospital Santa Rita (Núcleo de melanoma e Laboratório de Patologia) e Laboratório Central e Análises Clínicas - Irmandade da Santa Casa de Misericórdia de Porto Alegre; Laboratório de Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) e Laboratório de Biogenômica da Universidade Federal de Santa Maria (UFSM).

Quem pagará as despesas: Verbas dos pesquisadores, agência de fomento ao qual o projeto será submetido e apoio da Universidade.

Critérios de Inclusão: Casos: serão incluídos à pesquisa pacientes portadores de melanoma maligno cutâneo sem metástases e maiores de idade, após a assinatura de Termo de Consentimento Livre e Esclarecido (TCLE).

Controles: serão incluídos indivíduos hígidos, pareados aos casos por sexo e idade e cor da pele, oriundos da comunidade universitária da UFCSPA ou da UFSM (Universidade Federal de Santa Maria), onde a colaboradora do projeto atua em cooperação, após a assinatura Termo de Consentimento Livre e Esclarecido (TCLE).

Critério de Exclusão: Ficarão excluídos deste estudo pacientes com melanoma metastático, menores de idade e que não concordarem em assinar o TCLE.

Considerações sobre os Termos de apresentação obrigatória:

Foi apresentada declaração de isenção de ônus à instituição. Declaração de confidencialidade do sujeito no estudo e declaração de uso de dados e materiais.

TCLE redigido de forma clara, com 3 páginas.

Recomendações:

Solicita-se e aguarda-se modificação no TCLE a respeito de comparação com estudos futuros,

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

onde deve ser colocado a seguinte consideração, na solicitação do paciente de autorizar ou não: "desde que os futuros projetos, sejam aprovados pelo CEP."

Conclusões ou Pendências e Lista de Inadequações:

Pendências anteriormente solicitadas foram atendidas.

Aguarda-se inclusão de frase no TCLE, conforme descrito nas "recomendações" que são necessárias novas aprovações do CEP em projetos futuros.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Após reavaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer.

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA. Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 - Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

Endereço: R. Profº Annes Dias,285 Hosp.Dom Vicente Scherer
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IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Continuação do Parecer: 436.339

PORTO ALEGRE, 25 de Outubro de 2013

Assinador por:
Claudio Teloken
(Coordenador)

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5.3 Parecer do Comitê de Ética em Pesquisa da ISCMPA versão 4

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de Marcadores Prognósticos no Melanoma
Pesquisador: Claudia Giuliano Bica
Área Temática:
Versão: 4
CAAE: 14404513.4.0000.5335
Instituição Proponente: Irmandade da Santa Casa de Misericordia de Porto Alegre - ISCMPA
Patrocinador Principal: Universidade Federal de Ciências da Saúde de Porto Alegre

DADOS DO PARECER

Número do Parecer: 843.196
Data da Relatoria: 25/09/2014

Apresentação do Projeto:
Solicitação de Emenda, referente:
-Projeto modificado 20/08/2014: critérios de inclusão para pacientes.
-Termo de Consentimento Livre e Esclarecido, modificado - Versão 3.0 datada de 23/09/2013.
_Inclusão de Pesquisador: Luciana Schuch.

Objetivo da Pesquisa:
Já descrito no parecer anterior.

Avaliação dos Riscos e Benefícios:
Adequados e já apresentados.

Comentários e Considerações sobre a Pesquisa:
Solicitação de Emenda referente ao critérios de Inclusão, TCLE modificado e inclusão de pesquisadora, todos apresentados em anexo e adequados.

Considerações sobre os Termos de apresentação obrigatória:
Adequados.

Recomendações:
Não se aplica.

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Continuação do Parecer: 843.196

Conclusões ou Pendências e Lista de Inadequações:

Solicitamos no TCLE, modificado - Versão 3.0 datada de 23/09/13, retirar onde cita a resolução 196/96, trocar para Resolução nº.: 466/12 vigente!.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Após reavaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer.

-Projeto modificado 20/08/2014: critérios de inclusão para pacientes.
-Termo de Consentimento Livre e Esclarecido, modificado - Versão 3.0 datada de 23/09/2013.
_Inclusão de Pesquisadora: Luciana Schuch.

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA. Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 – Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

Endereço: R. Profº Annes Dias,285 Hosp.Dom Vicente Scherer
Bairro: 6º andar - Centro **CEP:** 90.020-090
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Continuação do Parecer: 843.196

PORTO ALEGRE, 23 de Outubro de 2014

Assinado por:
Claudio Teloken
(Coordenador)

Endereço: R. Profº Annes Dias,285 Hosp.Dom Vicente Scherer
Bairro: 6º andar - Centro **CEP:** 90.020-090
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

5.4 Comprovante de submissão de artigo à revista Pathology & Oncology Research

Gmail **Munique Mendonça <muniquepm@gmail.com>**

[Fwd: PORE-D-17-00535 - Submission Confirmation]

claudia@ufcspa.edu.br <claudia@ufcspa.edu.br> 6 de novembro de 2017 07:45
Para: muniquepm@gmail.com

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Assunto: PORE-D-17-00535 - Submission Confirmation
De: "PORE" <em@editorialmanager.com>
Data: Seg, Novembro 6, 2017 7:45 pm
Para: "Claudia Giuliano Bica" <claudia@ufcspa.edu.br>

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5.5 Normas da revista Pathology & Oncology Research

Pathology & Oncology Research

Official Journal of the Arányi Lajos Foundation

Editors-in-Chief: L. **Kopper**; J. **Tímár**
ISSN: 1219-4956 (print version)
ISSN: 1532-2807 (electronic version)
Journal no. 12253

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Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

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- Book chapter

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- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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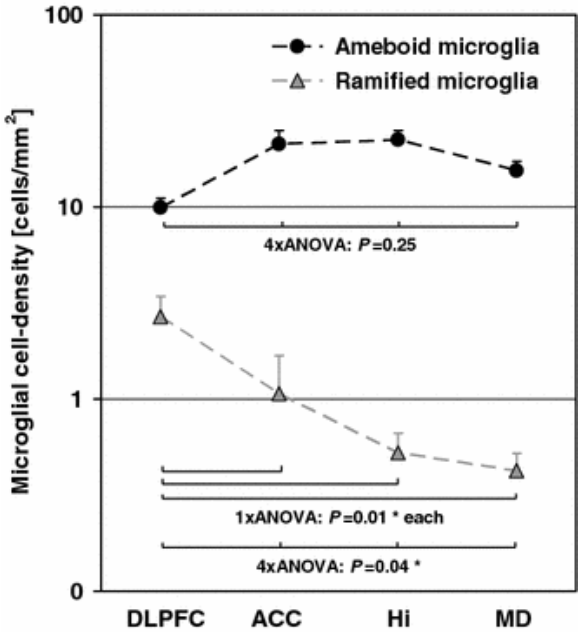

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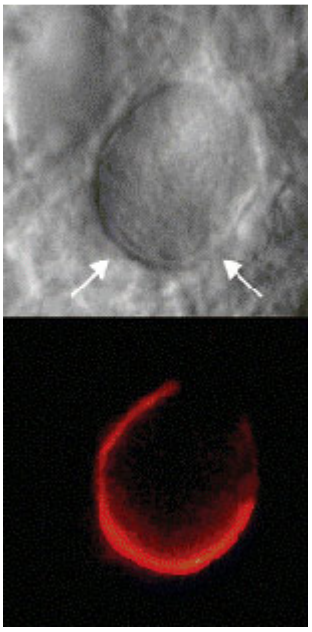

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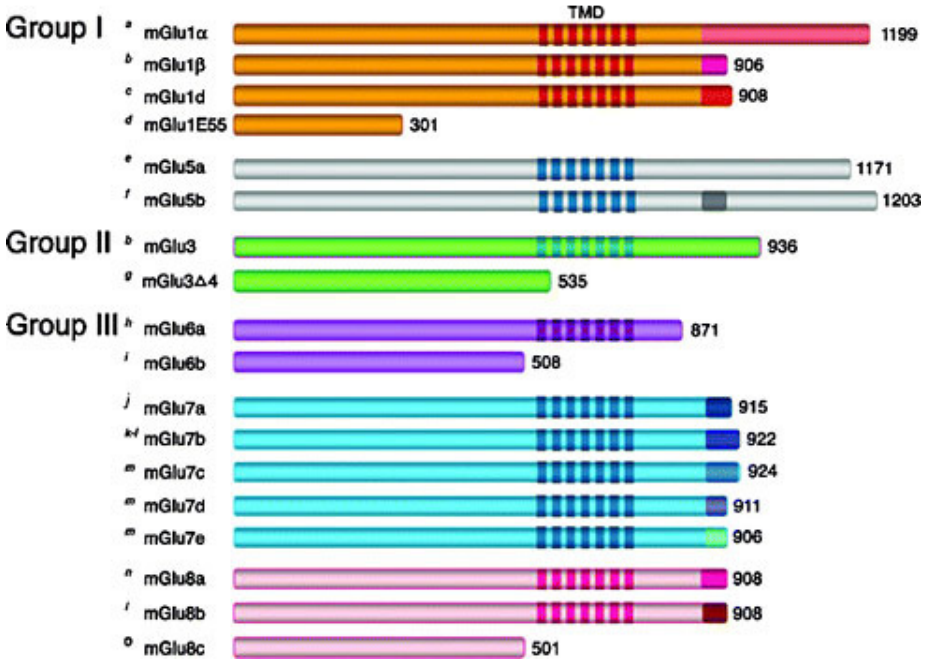

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Munique Mendonça Azevedo <azevedo.munique@gmail.com>

[Fwd: BIOMARKERS – Manuscript ID TBMK-2017-0033]

Claudia@ufcspa.edu.br <claudia@ufscpa.edu.br> 24 de novembro de 2017 10:13
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5.7 Normas da revista Clinical Biochemistry

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Gmail **Munique Mendonça <mAuniquepm@gmail.com>**

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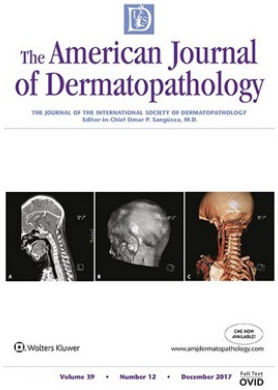

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Software
4. *Epi Info* [computer program]. Version 6. Atlanta, GA: Centers for Disease Control and Prevention; 1994.

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5. Friedman SA. Preeclampsia: a review of the role of prostaglandins. Obstet Gynecol [serial online]. January 1988;71:22-37. Available from: BRS Information Technologies, McLean, VA. Accessed December 15, 1990.

Database
6. CANCERNET-PDQ [database online]. Bethesda, MD: National Cancer Institute; 1996. Updated March 29, 1996.

World Wide Web
7. Gostin LO. Drug use and HIV/AIDS [*JAMA* HIV/AIDS Web site]. June 1, 1996. Available at: http://www.ama-assn.org/special/hiv/ethics. Accessed June 26, 1997.

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