SARA SANTOS BERNARDES REAL PRADO

ESTRESSE OXIDATIVO E BIOMARCADORES DO METABOLISMO DO FERRO EM PACIENTES COM MELANOMA CUTÂNEO E MELANOMA RECIDIVADO

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Orientadora: Profa. Dra. Alessandra Lourenço Cecchini Armani

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Londrina, 07 de março de 2014.

Dedico este trabalho a minha família, professores e a todos os pacientes portadores de melanoma que contribuiram com esta pesquisa.

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“A maior recompensa pelo nosso trabalho não é o que nos pagam por ele, mas aquilo em que ele nos transforma.” *John Ruskin*

REAL-PRADO, Sara Santos Bernardes Real. **Estresse oxidativo e biomarcadores do metabolismo do ferro em pacientes com melanoma cutâneo e melanoma recidivado.** 2014. 144f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

Uma das correlações mais aceitas no prognóstico do melanoma cutâneo é a medida da espessura de Breslow, onde tumores mais espessos se relacionam fortemente com o risco de disseminação da doença e morte. O estresse oxidativo sistêmico em pacientes com câncer é reportado na literatura, porém seu papel no prognóstico de doenças malignas ainda é controverso. As ferritinas de cadeia pesada (FCP) e leve (FCL) são proteínas envolvidas no metabolismo do ferro, relacionadas a um pior prognóstico no câncer. O fator de crescimento tumoral beta-1 (TGF-β1) está intimamente relacionado com a iniciação, progressão e metástase tumoral, sendo os radicais livres capazes de regular sua atividade. A relação entre o estresse oxidativo e moléculas relacionadas, como as envolvidas no metabolismo do ferro e na resposta inflamatória, ainda não é bem caracterizado no melanoma. O objetivo deste trabalho foi caracterizar esses parâmetros em pacientes em diferentes estadiamentos dessa doença. A medida de espessura de Breslow e a presença ou não de recidivas foram utilizadas como parâmetros na divisão dos grupos. Um total de 83 pacientes foram recrutados no Hospital do Câncer de Londrina entre abril de 2011 e novembro de 2012. O grupo controle compreendeu 65 indivíduos saudáveis. O sangue venoso foi coletado e usado nas análises sistêmicas. Também foram selecionados aleatoriamente 70 blocos parafinizados de tumores de melanoma analisados no Departamento de Patologia do Hospital do Câncer de Londrina, a fim de estudar o estresse oxidativo e moléculas relacionadas no tecido tumoral por imuno-histoquímica. Quanto maior a espessura de Breslow e agressividade da doença (recidiva), mais evidentes foram as modificações oxidativas e inflamatórias sistêmicas, caracterizadas principalmente pelo aumento dos níveis de malondialdeído (MDA) e proteína C reativa (PCR). Além disso, tumores cutâneos com maior espessura de Breslow apresentaram maior concentração do produto de oxidação de proteínas 3-nitrotirosina nas análises de imunohistoquímica de células de melanoma e no tecido peritumoral quando comparados a tumores menores. Pacientes com recidiva e alto risco de recidiva, apresentaram aumento de FCP sistêmica, enquanto todos os pacientes com melanoma apresentaram aumento da relação FCP:FCL quando comparados a indivíduos saudáveis. O aumento dos níveis de FCP foi acompanhado do aumento do estresse oxidativo sistêmico, avaliado pela lipoperoxidação de hemácias. Células de melanoma apresentam aumento na marcação de FCP por imunohistoquímica porém em níveis iguais entre as diferentes classificações do tumor. A p53, proteína envolvida na proliferação celular, apresentou-se aumentada em tumores metastáticos quando comparados a tumores cutâneos com menores espessuras de Breslow. Pacientes com recidiva apresentaram baixos níveis sistêmicos de TGF-β1, acompanhados de altos níveis de MDA, lipoperoxidação de hemácias e de 3-nitrotirosina. Os resultados mostram o estresse oxidativo e moléculas relacionadas possuem perfis característicos nos diferentes estadiamentos do melanoma, acrescentando uma nova perspectiva sobre o estado redox e seu possível envolvimento com a evolução da doença.

Palavras-chave: Melanoma. Estresse oxidativo. Câncer. Recidiva.

PRADO, Sara Santos Bernardes. **Oxidative stress and iron metabolism biomarkers in patients with cutaneous melanoma and with melanoma recurrence.** 2014. 144p. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2014.

ABSTRACT

Surgical removal of cutaneous melanoma can cure most of the patients, however, the risk of recurrence remains for years. One of the most accepted correlation in cutaneous melanoma prognosis is Breslow thickness, where high tumor thickness values at diagnosis is strongly related to melanoma spread and high morbidity. Oxidative stress in patients with cancer is reported in literature, but their role in prognosis is still controversial. The ferritin heavy (FHC) and light chains (FLC) are proteins involved in iron metabolism, related to a poor prognosis in cancer. The inflammatory mediator transforming growth factor beta-1 (TGF-β1) is closely linked with initiation, progression and tumor metastasis, and free radicals are capable of regulating their activation. The relationship between oxidative stress and related molecules such as those involved in the iron metabolism and inflammatory response is still not well characterized in melanoma. The objective of this study was to characterize these parameters in patients in different stages of this disease. Breslow thickness and the presence or absence of recurrence was used as parameters for group division. A total of 83 patients were recruited at the Cancer Hospital of Londrina between April 2011 and November 2012. The control group comprised 65 healthy volunteers. Venous blood was collected and used in systemic analyzes. Moreover, 70 blocks of paraffin-embedded melanoma from patients treated between 2004 to 2012 were randomly selectedand analyzed at the Department of Pathology, Cancer Hospital of Londrina, in order to study oxidative stress and related molecules in tumor tissue by immunohistochemistry. The higher the Breslow thickness and aggressiveness of the disease (recurrence) the more evident were the systemic inflammatory and oxidative modifications, mainly evidenced by increased levels of malondialdehyde (MDA) and C-reactive protein (CRP). Furthermore, skin tumors with higher Breslow thickness showed increased labeling of the protein oxidation product 3-nitrotyrosine in melanoma cells and peritumoral tissue compared to smaller tumors. Recurrence and high risk recurrence patients showed increased FHC levels, while all patients with melanoma had increased FHC:FLC ratio when compared to healthy subjects. The increased levels of FHC were followed by the increased systemic oxidative stress, measured by red blood cells lipid peroxidation. Melanoma cells showed FHC expression, but the levels did not differ between tumor stages. The p53, a protein involved in cell proliferation, had increased labeling in metastatic tissue compared with thin Breslow thickness cutaneous tumor. Recurrence patients had low TGF-β1 systemic levels, accompanied by high levels of MDA, red blood cells lipid peroxidation and 3-nitrotyrosine in tumoral tissue. The results show that oxidative stress and related molecules have characteristic profiles in different melanoma stages, adding a new perspective on the redox state and their possible involvement in disease progression.

Keywords: Melanoma. Oxidative stress. Neoplasm. Neoplasm recurrence.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Fe^{+2}	Íon ferroso
Fe^{+3}	Íon férrico
3-NT	3-nitrotirosina
8-OHdG	8-hidroxideoxiguanosina
H_2O_2	Peróxido de hidrogênio
HOCl	Ácido hipocloroso
NO	Óxido Nítrico
$^1O_2$	Oxigênio singlet
$O_2^{\bullet-}$	Superóxido
^-OH	Hidróxido
$^\bullet OH$	Hidroxil
$ONOO^-$	Peroxinitrito
AKT	*Protein Kinase B* – Proteína quinase B
AOPP	*Advanced Oxidation Protein Products*- Produtos de oxidação avançada de proteínas
CDK	*Cyclin-Dependent Kinase* – Quinase dependente de ciclina
ERK	*Extracellular Signal-Regulated Kinase* – Quinase regulada por sinal extracelular
ERO	Espécies Reativas de Oxigênio
ERN	Espécies Reativas de Nitrogênio
FDA	*Food and Drugs Administration* – Administração de alimentos e medicamentos
FLIP	*FLICE-like Inhibitory Protein* – Proteína inibitória homóloga a FLICE
GSH	Glutationa reduzida
IκB-α	*Inhibitor kappa B alpha* – Inibidor kappa B alfa
IAP	*Inhibitor of Apoptosis* – Inibidor de apoptose
IAP-1	Inibidor da Ativação do Plasminogênio-1
IKK	*IκB Kinase* – Quinase IκB
IL-1β	Interleucina- 1 beta
INCA	Instituto Nacional do Câncer

JNK	*c-Jun N-terminal Kinase* – Quinase c-Jun N-terminal
LDH	Lactato desidrogense
LPS	Lipopolissacarídeo
MAPK	*Mitogen-activated protein kinases* – Proteínas quinase ativadas por mitógenos
MDA	Malondialdeído
NADH	Nicotinamida-Adenina-Dinucleotídio
NF-κB	*Nuclear Factor kappa B* – Fator nuclear kappa B
PCR	Proteína C reativa
PGC1-α	*Peroxisome Proliferator-Activated Receptor-alpha* – Receptor ativado por proliferadores de peroxissoma-alfa
PI3K	*Phosphatidylinositol-3 kinase* – Fosfatidilinositol 3-quinase
PTEN	*Phosphatase and Tensin homolog* – Homológo da fosfatase e tensina
PUFA	*Polyunsaturated Fatty Acids* – Ácidos graxos poli-insaturados
SMAD	*sma and MAD related proteins* – Proteínas homólogas de Sma e MAD
SOD	Superóxido dismutase
TAK-1	*TGF-beta Activated Kinase 1* – Quinase ativadora de TGF-beta
TGF- β	*Transforming growth factor beta* – Fator transformador de crescimento beta
TNF-α	*Tumor necrosis factor alpha* – Fator de necrose tumoral
UV	Ultravioleta

SUMÁRIO

1 INTRODUÇÃO

1.1 MELANOMA

O melanoma cutâneo é uma neoplasia maligna com origem nos melanócitos, células originadas da crista neural e localizadas na camada basal da pele. Em reposta à radiação ultravioleta (UV), os queratinócitos da epiderme secretam fatores que regulam a sobrevivência, proliferação e motilidade dos melanócitos, estimulando a síntese de melanina (GRAY-SCHOPFER; WELLBROCK; MARAIS, 2007). A melanina atua como uma barreira, diminuindo a absorção da radiação UV, além de definir a cor da pele, olhos e cabelos (TADOKORO et al., 2005). Mutações em genes críticos que regulam o crescimento celular, a produção autócrina de fatores de crescimento e a perda de receptores de adesão contribuem na interrupção da sinalização intracelular dos melanócitos, permitindo-lhes escapar de sua regulação controlada pelos queratinócitos (HAASS; SMALLEY; HERLYN, 2004).

Apesar de raro – cerca de 4% dos tipos de cânceres cutâneos - o melanoma é a mais grave neoplasia maligna de pele, possuindo altas taxas de mortalidade devido sua característica altamente invasiva e agressiva (CAINI et al., 2009). Segundo estimativas da Organização Mundial da Saúde, anualmente podem ocorrer cerca de 132 mil novos casos de melanoma cutâneo no mundo. No Brasil, estima-se 7.990 novos casos (3.910 em homens e 4.080 em mulheres) no ano de 2014, sendo a maior parte na região sul do país (INCA, 2014). Quando diagnosticado precocemente, o melanoma é curável através de cirurgia, e 80% dos pacientes apresentam-se livres de recidiva até 10 anos após a remoção do tumor cutâneo primário. Quando a doença encontra-se disseminada para os nódulos linfáticos ou órgãos distantes, torna-se refratária a terapias comuns, e, portanto, incurável (DAS THAKUR; STUART, 2013). A gênese do melanoma parece estar mais relacionada a fatores genéticos do que ambientais, embora alguns estudos mostrem que exposições solares intermitentes intensas aumentam em até 40% a probabilidade de desenvolvimento da doença (MILLER; MIHM, 2006). Esse fato explica a maior frequência da localização do melanoma em regiões geralmente expostas ao sol em

momentos de recreação e lazer, como costas, tórax, pernas e abdômen (GILCHREST et al., 1999).

Podem ser considerados fatores de risco para o desenvolvimento do melanoma o fenótipo da pele, sendo a pele clara mais susceptível por produzir menos pigmento melanina, a presença de nevos melanocíticos em grande quantidade e fatores genéticos-familiares (PAEK et al., 2008). Mutações no gene CDKN2A, que sinaliza a produção de proteínas inibidoras do ciclo celular, e dos genes BRAF e N-RAS, envolvidos na via de sinalização MAPK (Do inglês *Mitogen-Activated proteins kinases*), são as alterações genéticas mais frequentes em pacientes com melanoma, sendo a primeira relacionada ao melanoma familiar (MILLER; MIHM, 2006; PAEK et al., 2008).

No melanoma, devido a mutações no gene N-RAS da família RAS, a ativação do receptor tirosina quinase resulta em aumentos aberrantes da sinalização MAPK/ERK (Do inglês *Mitogen-Activated protein kinases/ Extracellular Signal-Regulated Kinases*) e PI3K/AKT (Do inglês *Phosphatidylinositol-3 kinase/ Protein Kinase B*), vias que promovem a proliferação e sobrevivência celulares. As mutações ativadoras em BRAF, que codificam uma serina/treonina quinase que regula positivamente o RAS, são observadas em 60 a 70% dos melanomas, enquanto as mutações ativadoras em N-RAS (que regula positivamente o BRAF) ocorrem em 10-15% dos demais tumores. PTEN (Do inglês *Phosphatase and Tensin homolog*), um supressor tumoral que atua na regulação negativa da sinalização de PI3K/AKT é epigeneticamente silenciado em 20% dos melanomas (CURTIN et al., 2006; LAZAR; MURPHY, 2010). O gene CDKN2A codifica três proteínas supressoras tumorais, p15/INK4b, p16/INK4a e p14/ARF, sendo a perda do lócus que codifica p16/INK4a claramente envolvida no melanoma humano. A proteína p16/INK4a, também conhecida como proteína p16, é uma proteína supressora tumoral da família do retinoblastoma, que atua através da inibição da quinases dependentes de ciclina 4 e 6 (CDK4/ CDK6, do inglês *Cyclin-Dependent Kinase*). Uma proteína p16 mutante geralmente leva à perda do controle do ciclo celular (LAZAR; MURPHY, 2010). As vias de sinalização envolvidas com as mutações citadas acima estão representadas na Figura 1.

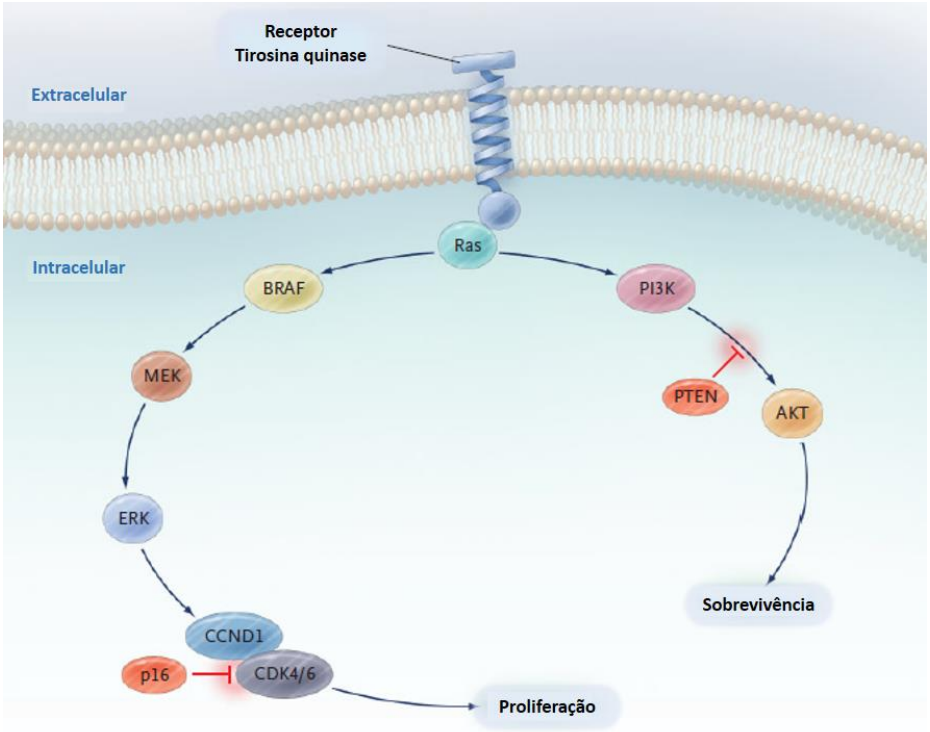


Figura 1. Vias de sinalização da MAPK/ERK e PI3K/AKT. A sinalização do receptor Tirosina quinase promove proliferação celular através da via MAPK/ERK (Do inglês *Mitogen-Activated protein kinases/ Extracellular Signal-Regulated Kinases*, ramificação esquerda) e sobrevivência celular através da via PI3K/AKT (Do inglês *Phosphatidylinositol-3 kinase/ Protein Kinase B*, ramificação direita). No melanoma, mutações ativadoras no gene N-RAS e BRAF aumentam a sinalização MAPK/ERK, bem como mutações que modificam estruturalmente a proteína p16. A via da PI3K/AKT também encontra-se ativada no melanoma, sendo uma das causas o desenvolvimento de mutações que levam à alterações estruturais da proteína PTEN (Do inglês *Phosphatase and Tensin homolog*), importante inibidora dessa via. Adaptado de Curtin et al., 2006.

O melanoma pode ser classificado em extensivo superficial, nodular, acral lentiginoso, lentigo maligno e lentigo maligno melanoma. O melanoma extensivo superficial é o tipo mais frequente em caucasianos, apresentando crescimento lento e melhor prognóstico, enquanto o melanoma nodular apresenta crescimento rápido e pior prognóstico. O melanoma acral lentiginoso acomete as palmas das mãos, sola dos pés e o leito ungueal, e corresponde a 2 – 5% dos casos de melanoma em indivíduos caucasianos, porém é o tipo histológico mais comum entre indivíduos com a pele pigmentada, como afro-descendentes, hispânicos e asiáticos (KAY; FUJIWARA, 2013;

PAEK et al., 2008). Pacientes com esse diagnóstico apresentam pior prognóstico, devido o melanoma acral lentiginoso apresentar biologia diferente dos demais tipos, e também porque o diagnóstico desses pacientes costuma ser tardio, uma vez que esses tumores acometem regiões pouco visíveis (BELLO et al, 2013). Já o lentigo maligno (melanoma *in situ*) é mais comum em indivíduos com mais de 70 anos, possui crescimento lento, que pode durar anos, e ocorre frequentemente em regiões expostas ao sol, como a face. O termo lentigo maligno melanoma é usado quando o lentigo maligno assume caráter invasivo.

O melanoma pode apresentar dois padrões de crescimento, o radial (crescimento horizontal) ou vertical (crescimento invasivo em profundidade). Os melanomas com padrão de crescimento radial apresentam melhor prognóstico comparado ao padrão vertical. O tipo extensivo superficial apresenta crescimento radial na maioria dos casos, enquanto os tipos nodular, acral-lentiginoso e lentigo-maligno frequentemente apresentam crescimento vertical (THÖRN et al., 1994). As características histopatológicas dos dois padrões de crescimento e do aspecto macroscópico do melanoma cutâneo estão ilustradas na Figura 2.

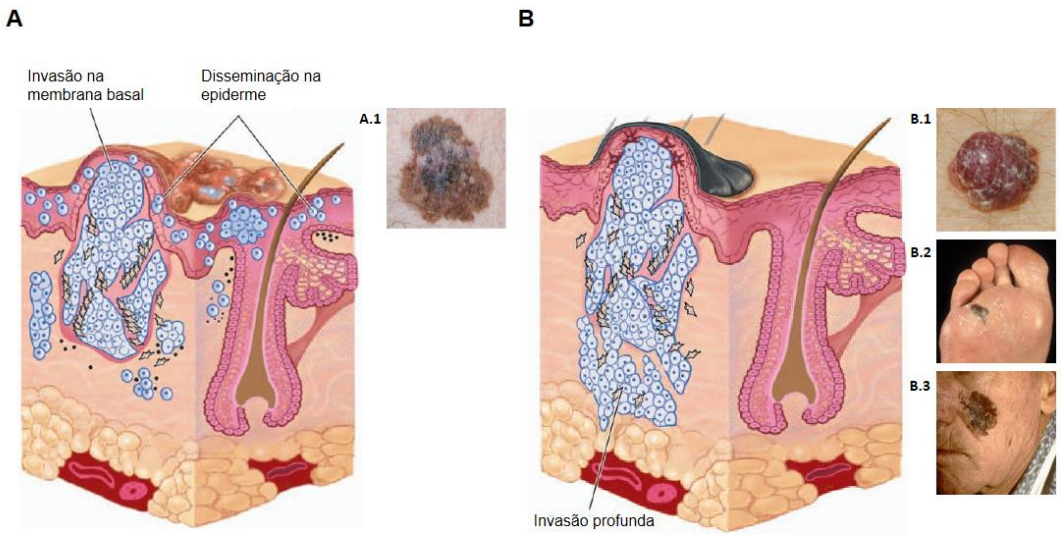


Figura 2. Padrões de crescimento e características macroscópicas do melanoma cutâneo. O melanoma extensivo superficial apresenta padrão de crescimento radial, enquanto os tipos nodular, acral lentiginoso e lentigo maligno melanoma apresentam comumente padrão de crescimento

vertical. A) Crescimento radial, com invasão de células tumorais na epiderme; A.1) Características macroscópicas do melanoma extensivo superficial; B) Crescimento vertical, com invasão profunda da derme; B.1) Características macroscópicas do melanoma nodular; B.2) Características macroscópicas do melanoma acral-lentiginoso; B.3) Características macroscópicas do lentigo maligno melanoma.

A classificação clínico-histopatológica do melanoma cutâneo pode ser realizada através dos níveis de Clark e/ou medida de espessura de Breslow, sendo que atualmente a última tem sido mais utilizada, por apresentar maior correlação com o prognóstico do paciente (BICHAKJIAN et al., 2011; SOONG et al., 1998). O nível de Clark mede o grau de invasão, de I a V, da epiderme (Nível I, tumor in *situ*) à hipoderme (Nível V). A medida de espessura de Breslow mede a profundidade em milímetros. O melanoma é classificado em 5 estágios: Estágio 0 ou melanoma *in situ*; Estágio I, onde a espessura de Breslow do tumor não ultrapassa 2 mm; Estágio II, caracterizado principalmente pela presença de ulceração em tumores com espessura de Breslow maior que 1 mm; Estágio III, onde o tumor encontra-se disseminado nos linfonodos ou na pele e tecido subcutâneo, com até 2 cm de distância do tumor primário, também chamado de melanoma regional; e Estágio IV, quando a doença encontra-se disseminada para órgãos distantes, também chamada de melanoma altamente disseminado (BALCH et al., 2009).

Os estágios I e II (melanoma cutâneo ou localizado) são subdivididos, e as principais características de cada uma delas estão representadas na Figura 3. Além das características já citados, o comportamento biológico dos tumores de melanoma dependem de fatores como ulceração, índice mitótico e infiltrado linfocitário (BALCH et al., 2009).

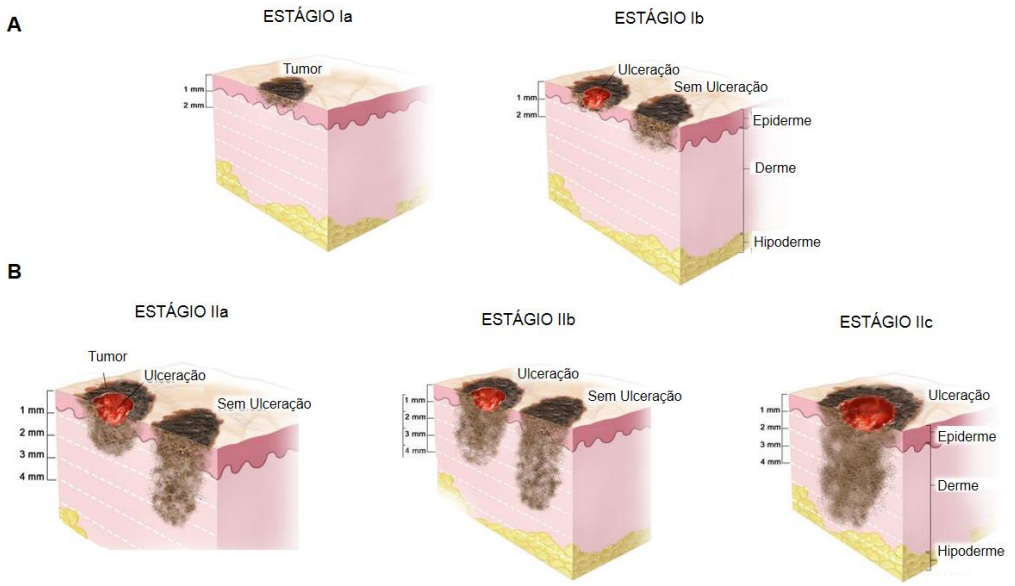


Figura 3. Subtipos de melanoma cutâneo (Estágio I e Estágio II). A) Estágio I: No estágio Ia, o tumor é menor do que um milímetro de espessura e sem ulceração. No estágio Ib, o tumor é ≤ 1 milímetro de espessura, com ulceração, ou entre 1 e 2 milímetros de espessura, sem ulceração; B) No estágio IIa, o tumor tem entre 1 e 2 milímetros de espessura, com ulceração, ou entre 2 e 4 milímetros de espessura, sem ulceração. No estágio IIb, o tumor tem entre 2 e 4 milímetros de espessura, com ulceração, ou é maior de 4 milímetros de espessura, sem ulceração. No estágio IIc, o tumor é superior a 4 milímetros de espessura, com ulceração. Adaptado de National Cancer Institute at the National Institutes of Health, 2008.

No melanoma, mesmo com a ressecção cirúrgica do tumor, o risco de recidiva permanece por anos, fazendo com que o acompanhamento clínico seja uma ferramenta importante em seu tratamento (HOHNHEISER et al., 2011). O uso de biomarcadores sistêmicos de progressão tumoral ou de recidiva da doença ainda é controverso, embora alguns testes bioquímicos como lactato desidrogenase (LDH), proteína-C reativa (PCR) e a proteína S-100β tenham sido utilizadas na prática clínica (BOUWHUIS et al., 2011; MARTENSON et al., 2001; KLUGER et al., 2011). Embora inespecífica, a LDH plasmática pode indicar a taxa de crescimento e volume tumoral, constituindo uma importante ferramenta no prognóstico da doença metastática (ALMEIDA et al., 2007). A PCR é uma proteína inflamatória de fase aguda, inespecífica, porém encontra-se aumentada em pacientes com melanoma em todos os estágios da

doença (FINDEISEN et al., 2009). A proteína S-100β é normalmente expressa em células derivadas da crista neural, como os melanócitos. Células de melanoma podem produzir S-100β de forma aberrante, tornando os níveis séricos dessa proteína detectáveis em pacientes portadores dessa neoplasia (MARTENSON et al., 2001).

1.1.1 Melanoma Metastático

As células metastáticas do melanoma são geneticamente heterogêneas, e os pacientes com doença disseminada apresentam menos de 10% de sobrevida em um período de 5 anos (FRANCKEN et. al. 2008; JACOBS; LANGE; BALCH, 2008). A cirurgia, a quimioterapia e a imunoterapia são oferecidas a pacientes com metástase e são essencialmente paliativas, com baixa previsão de benefício para o paciente (BALCH et. al. 2001; DAS TAKHUR et al., 2013).

Na doença regional, o número de linfonodos acometidos é o fator prognóstico mais importante. Para os pacientes com doença disseminada para órgãos distantes, as únicas variáveis de significado prognóstico são o número de metástases locais, ressecabilidade cirúrgica, duração da remissão e localização das metástases. As metástases sistêmicas implicam um ruim prognóstico, com sobrevida média de 6 a 9 meses após o reconhecimento da disseminação metastática. Apesar dos avanços na quimioterapia, inclusive na imunoterapia, o sucesso no tratamento medicamentoso da doença disseminada permanece com limitações, e o prognóstico da doença metastática é reservado (ASCIERTO et al., 2013; DAS TAKHUR et al., 2013).

O melanoma também pode progredir rapidamente de uma forma branda para uma doença metastática agressiva com alta mortalidade e pobre resposta às terapias, não sendo os mecanismos de progressão e resistência aos agentes terapêuticos bem elucidados (JEFFS et al., 2009). A base mais provável para a resistência às drogas no melanoma é a inibição da apoptose, embora outros mecanismos, incluindo alterações no transporte e desintoxicação de drogas e perda do reparo de DNA também desempenhem um papel importante (GROSSMAN; ALTIERI, 2001).

Células de melanoma apresentam baixos índices de apoptose espontânea *in vivo* em comparação com outros tipos de células tumorais, e são relativamente resistentes à apoptose induzida por agentes quimioterápicos. Dois membros das famílias de proteínas inibidoras da apoptose IAP (Do inglês *Inhibitor of Apoptosis*) e FLIP (Do inglês *FLICE-like Inhibitory Protein*) têm sido associados com a progressão do melanoma, sendo detectáveis no início da disfunção melanocítica (nevos melanocíticos) e em maior quantidade em melanomas metastáticos altamente invasivos (SOENGAS; LOWE, 2003).

Mutações no gene p53 estão envolvidas em falhas nos mecanismos de apoptose e no aumento da proliferação celular, sendo comuns em aproximadamente metade das neoplasias humanas (BRASILEIRO-FILHO; PEREIRA; GUIMARÃES, 2013). No melanoma, mutações nesse gene estão relacionadas com a transformação maligna do nevo, embora a frequência da mutação da p53 em tumores de melanoma sejam divergentes (HOUBEN et al., 2011; SOENGAS; LOWE, 2003). O gene p53, o "guardião do genoma", é um fator de transcrição que pode se ligar a regiões promotoras de diferentes genes, sendo capaz de ativar ou suprimir a expressão gênica (HOUBEN et al., 2011). Quando as células sofrem lesão e erros na replicação do DNA, a p53 é fosforilada e atua aumentando proteínas inibidoras do ciclo celular, a fim de que o DNA seja reparado antes da divisão celular, ou induzindo apoptose caso o reparo não seja possível (BRASILEIRO-FILHO; PEREIRA; GUIMARÃES, 2013). Recentemente foi mostrado por Avery-Kiejda e colaboradores (2011) que em tumores de melanoma metastático humanos e em linhagens celulares de melanoma cultivadas *in vitro*, o p53 apresenta menor capacidade de regulação sobre genes alvo envolvidos no controle do ciclo celular e apoptose. As alterações nos genes alvo da p53 observados nesse estudo apontam para uma falência potencial dessas células em responder adequadamente a estímulos indutores de apoptose, como os sinalizados pela quimioterapia.

O principal mecanismo indutor de apoptose induzido pela quimioterapia é a produção de radicais livres no tecido tumoral. Essas moléculas são capazes de causar dano mitocondrial, e também ativar a via de sinalização JNK (Do inglês *c-Jun N-terminal Kinase*), induzindo a apoptose via ação de caspases (SIMON; HAJ-YEHIA; LEVI-SCHAFFER, 2000).

1.2 Radicais Livres e Estresse Oxidativo

Radicais livres podem ser definidos como fragmentos moleculares com capacidade oxidante, contendo um ou mais elétrons desemparelhados em seu orbital atômico ou molecular, o que lhes confere alta reatividade (HALLIWELL; GUTTERIDGE, 2007). Basicamente, os radicais livres são constituídos de espécies reativas de oxigênio (ERO) e de espécies reativas de nitrogênio (ERN). Flutuações dos níveis de radicais livres possuem importante função regulatória. Quando os níveis se encontram altos e/ou sustentados, os radicais livres podem causar diversos danos ao DNA, lipídeos e proteínas, levando a uma condição chamada de estresse oxidativo, definida como a ruptura da sinalização redox em uma situação controle, levando a um desequilíbrio entre os níveis de radicais livres e substâncias antioxidantes (JONES, 2006; HALLIWELL; GUTTERIDGE, 2007).

As células são expostas a uma variedade de ERO e ERN derivadas de fontes endógenas e exógenas. Como exemplo de fontes exógenas, pode-se citar a radiação ionizante, a radiação ultravioleta, xenobióticos, solventes orgânicos, componentes da fumaça do cigarro e outros poluentes atmosféricos (KOHEN; NISKA, 2002). Os mecanismos endógenos de geração de radicais livres ocorrem nas mitocôndrias, membranas celulares e no citoplasma, sendo a mitocôndria, por meio da cadeia transportadora de elétrons, a principal fonte (FINKEL; HOLBROOK, 2000; RAHA; ROBINSON, 2000). As principais fontes exógenas e endógenas de radicais livres estão representadas na figura 4.

Aproximadamente 95% do oxigênio consumido pelos organismos aeróbicos é reduzido a água pela enzima citocromo c oxidase na reação terminal da cadeia respiratória mitocondrial. No entanto, uma pequena parte das moléculas de oxigênio (1-2%) é convertida em radical ânion superóxido ($O_2^{\bullet -}$) por outros componentes da cadeia respiratória, principalmente o Complexo I – NADH desidrogenase e o complexo III – ubisemiquinona (UQ10), sendo o último uma fonte constante de geração de $O_2^{\bullet -}$ (RAHA; ROBINSON, 2000; TURRENS, 1997). O aumento do $O_2^{\bullet -}$ é acompanhado do aumento de enzimas que participam de sua degradação e metabolismo, como a superóxido dismutase, catalase e glutationa peroxidase (FINKEL;

HOLBROOK, 2000). A enzima superóxido dismutase converte o $O_2^{\bullet-}$ em H_2O_2, que por sua vez é degradado em água pelas enzimas catalase e glutationa peroxidase. O H_2O_2 não possui elétrons desemparelhados na sua última camada de valência e, portanto, não é considerado um radical livre, embora apresente alto potencial reativo. Diferente dos radicais livres, o H_2O_2 tem vida longa e é capaz de atravessar as membranas celulares, apresentando-se potencialmente tóxico para as células. Esta toxicidade pode ser aumentada em dez mil vezes pela presença de ferro. Por participar da reação de geração de $^{\bullet}OH$, o H_2O_2 tem ação deletéria potencial, uma vez que esse é um dos radicais livres mais reativos (BARBOSA et al., 2010).

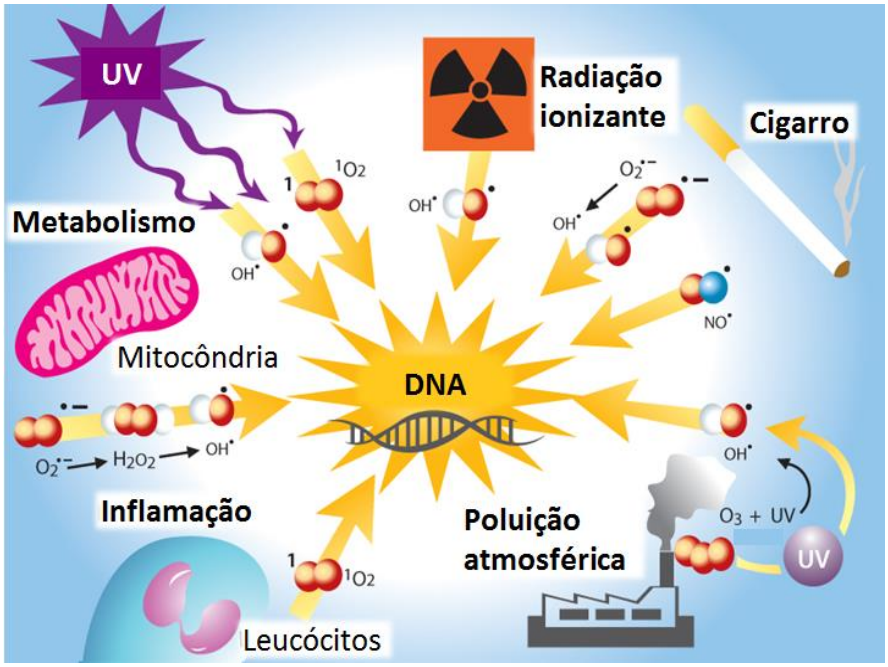


Figura 4. Principais fontes endógenas e exógenas de radicais livres. Os mecanismos endógenos de geração de radicais livres incluem o metabolismo mitocondrial, o metabolismo de xenobióticos e a resposta inflamatória. Como exemplo de fontes exógenas, pode-se citar a radiação ionizante, a radiação ultravioleta, componentes da fumaça do cigarro e outros poluentes atmosféricos. Adaptado de Kohen e Niska, 2002.

O excesso de $O_2^{\bullet-}$ provoca a liberação de ferro de moléculas que possuem sítios ferro-sulfúricos, como a aconitase, a succinato desidrogenase e a NADH-ubiquinona oxiredutase, e também da molécula de armazenamento de ferro

ferritina (HALLIWELL & GUTTERIDGE, 2007; RAHA & ROBINSON, 2000). O ferro liberado encontra-se na forma reduzida (Fe^{2+}), sendo capaz de participar da conversão do H_2O_2 em radical hidroxil ($^{\bullet}OH$), através da reação de Fenton. A reação de Haber-Weiss também apresenta um papel importante na geração de $^{\bullet}OH$, atuando como fonte de Fe^{2+}. Outros metais de transição podem participar da geração de $^{\bullet}OH$, tendo o Cu^{2+} importante participação em sistemas biológicos. A reação de geração de $^{\bullet}OH$ através da reação de Haber-Weiss e Fenton estão descritas abaixo:

Reação de Haber-Weiss: $\mathbf{Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2}$

Reação de Fenton: $\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\bullet}OH}$

Na resposta inflamatória, os radicais livres são produzidos principalmente durante a fagocitose, no *burst* oxidativo. A produção de ERO é uma característica marcante da fagocitose dos neutrófilos. Esta produção é muito superior à observada em macrófagos. Nesse último, a explosão respiratória é dependente de enzimas da família NADPH oxidase (do inglês *Nicotinamide Adenine Dinucleotide Phosphate Oxidases*) internalizada da membrana plasmática durante a formação do fagossomo. Já em neutrófilos, ocorre um recrutamento adicional de NADPH oxidases para o fagossomo. Ainda na fagocitose de neutrófilos, a ação catalítica da mieloperoxidase transforma o superóxido em espécies reativas ainda mais tóxicas, como o HOCl (ácido hipocloroso) (NORDENFELT; TAPPER, 2011). O ácido hipocloroso e outros produtos oxidantes clorados derivados do *burst* oxidativo reagem com proteínas, formando Produtos de Oxidação Avançada de Proteínas (AOPP, do inglês *Advanced Oxidation Protein Products*) (KORKMAZ et al. 2013). A família NADPH também faz parte de um grupo de proteínas transmembrana com função de transferir elétrons através das membranas celulares. Geralmente, o aceptor de elétrons nessa reação é o oxigênio e em decorrência desse processo, gera-se o radical $^{\bullet}OH$ (HALLIWELL; GUTTERIDGE, 2007; SIES, 1997). Outra importante fonte endógena de geração de radicais livres decorre da ação de enzimas da família do citocromo P450, que atuam juntamente com enzimas NADPH na metabolização de xenobióticos.

Os ácidos graxos poli-insaturados contidos nas membranas celulares fazem com que essas sejam potentes geradoras dos radicais livres alcoxila e peroxila, por meio do dano oxidativo a lipídeos, chamado lipoperoxidação (BARBOSA et al., 2010; HALLIWELL; GUTTERIDGE, 2007). O radical $^{\bullet}OH$, por meio da retirada de um átomo de hidrogênio dos ácidos graxos poli-insaturados da membrana celular, desempenha importante papel na lipoperoxidação, sendo considerado o principal iniciador desse processo, onde o Fe^{2+} também desempenha papel importante (BARBOSA et al., 2010; HALLIWELL; GUTTERIDGE, 2007). Um dos produtos finais da lipoperoxidação é o malondialdeído (MDA), bastante utilizado na avaliação do estresse oxidativo *in vitro* e *in vivo* (HALLIWELL; GUTERIDGE, 2007). A principal fonte de geração de MDA é a oxidação de ácidos graxos poli-insaturados (PUFA, do inglês *Polyunsaturated Fatty Acids*), e uma vez que o estado pró-inflamatório aumenta a disponibilidade de PUFA, um aumento de MDA é esperado em doenças inflamatórias, como o câncer (BARTSCH; NAIR, 2004).

Além da capacidade do $O_2^{\bullet -}$ em participar de reações de geração de $^{\bullet}OH$, ele pode ainda, por meio da reação com o radical livre óxido nítrico (NO), gerar peroxinitrito ($ONOO^-$), também potencialmente reativo. A reação do $ONOO^-$ com o aminoácido tirosina de proteínas forma a 3-nitrotirosina (3-NT), um importante produto de modificação oxidativa de proteínas, podendo afetar a atividade de enzimas envolvidas na metabolização de xenobióticos, sinalização celular e enzimas com potencial antioxidante, como a superóxido dismutase e a ceruloplasmina (HALLIWELL; GUTTERIDGE, 2007).

O dano oxidativo ao DNA pode desencadear morte celular ou mutações que propiciam o desenvolvimento de células cancerígenas. O principal produto do dano oxidativo ao DNA é a 8-hidroxideoxiguanosina (8-OHdG), formada pela oxidação da base nitrogenada guanina. A ligação do $OH^{\bullet}$ com a guanina leva a formação de um aduto de DNA, e a abstração subsequente de mais um elétron forma a 8-OHdG. A 8-OHdG sofre tautomerismo ceto-enólico, que favorece o produto na sua forma oxidada, 8-oxo-7,8-diidro-deoxiguanosina (8-oxo-dG). Na literatura, a 8-OHdG e 8-oxo-dG são consideradas sinônimos (VALAVANIDIS; VIACHOGIANNI; FIOTAKIS, 2009). O potencial mutagênico da 8-OHdG é suportado pela perda de especificidade no

emparelhamento de bases, na leitura errada de pirimidinas adjacentes, ou na inserção de adenina na fita oposta a lesão. Além disso, a 8-OHdG é um potente agente pró-oxidante, uma vez que é capaz de gerar radicais $^{\bullet}OH$, $ONOO^-$ e $^1O_2$ (oxigênio singlet) (ICHIHASHI et al., 2003). Outras bases de DNA também podem reagir com $^{\bullet}OH$ de uma maneira semelhante, porém a oxidação da guanosina é a lesão de DNA mais comum, sendo um biomarcador potencial da carcinogênese devido sua ação pró-mutagênica (VALAVANIDIS; VIACHOGIANNI; FIOTAKIS, 2009).

Em condições fisiológicas, a maior parte dos radicais livres é neutralizada por substâncias antioxidantes, garantindo a homeostasia. Segundo Halliwelll e Gutteridge (2007), antioxidante é qualquer substância que atrasa, previne ou remove o dano oxidativo a uma molécula alvo. Esta definição inclui compostos de natureza enzimática e não enzimática, como as enzimas superóxido dismutase, catalase e do sistema glutationa (glutationa peroxidase e glutationa redutase); substâncias queladoras de metais, que previnem o desencadeamento de reações oxidativas dependentes de ferro e cobre; e compostos fenólicos que atuam como removedores de radicais livres. Os principais sistemas geradores de radicais livres e sistemas antioxidantes *in vivo* estão representados na figura 5.

Em nível celular, as ERO podem, direta ou indiretamente, modular as funções de muitas enzimas e fatores de transcrição através de uma infinidade de cascatas de sinalização celular e levar ao desenvolvimento de diferentes tipos de doenças. A ação das ERO sobre a sinalização celular pode ser positiva ou negativa. Essas sinalizações resultam em mudanças na expressão gênica, que influenciam a capacidade de sobrevivência ou morte da célula. A magnitude e a duração do estresse, bem como o tipo de células envolvidas são fatores importantes na determinação de quais caminhos são ativados, bem como no resultado celular final (HALLIWELL, 2007; MARTINDALE; HOOLBROK, 2002).

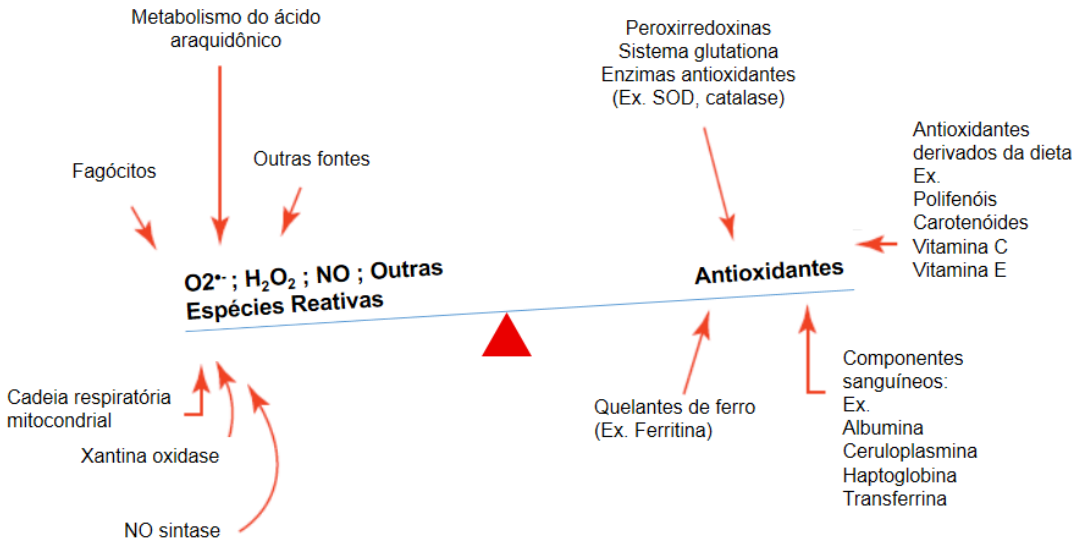


Figura 5. Balanço oxidativo *in vivo*. Os radicais livres podem ser gerados a partir de processos inflamatórios, através do *burst* respiratório de fagócitos; durante o metabolismo de produtos derivados do ácido araquidônico; durante a redução do oxigênio na cadeia respiratória mitocondrial; e pelas enzimas xantina oxidase e óxido nítrico sintase. São consideradas substâncias antioxidantes as quelantes de íons ferro; algumas proteínas plasmáticas; enzimas antioxidantes; substâncias de baixo peso molecular, como a glutationa reduzida; e substâncias derivadas da dieta. Quando o equilíbrio entre radicais livres e substâncias antioxidantes é perdido, ocorre o estresse oxidativo. NO: Óxido Nítrico; SOD: Superóxido dismutase. Adaptado de Halliwell, 2011.

O estresse oxidativo é amplamente reportado em pacientes com diferentes tipos de câncer, tanto em nível tumoral quanto sistêmico (GADJEVA et aL, 2008; GERBER et al, 1997; GUPTA; BHATT; MISRA, 2009; MANTOVANI et al, 2002; PANIS et al, 2012a; 2012b; SAINTOT et al, 2002). Em geral, o papel dos radicais livres na progressão do câncer é pouco compreendido. Apesar do estresse oxidativo sistêmico ser considerado um bom indicador prognóstico em pacientes com câncer (GERBER et al, 1997; SAINTOT et al, 1996; 2002; SALZMAN et al, 2010), alguns autores afirmam que um estado pró-oxidativo, como por exemplo altos níveis de MDA e presença de polimorfismos em genes antioxidantes, atua como um fator protetor contra recidivas da

doença (AMBROSONE et al, 2005; GAGO-DOMINGUEZ et al, 2007; MARTIN et al, 2006).

1.2.1 Estresse Oxidativo no Melanoma

Durante a melanogênese o controle antioxidante intrínseco é perdido e ocorre a ativação inapropriada de fatores de transcrição sensíveis ao *status* redox, contribuindo para um fenótipo antiapoptótico, e possivelmente contribuindo para a determinação da resistência das células de melanoma aos agentes quimioterápicos (MEYSKENS et al., 2001).

O estresse oxidativo está altamente associado a transformação da célula sadia em célula cancerosa, além de participar da proliferação de células malignas. Melanócitos normais de pacientes com melanoma, *in vitro*, apresentam modificações das enzimas antioxidantes SOD e catalase e no conteúdo da vitamina E que propiciam o aumento do estresse oxidativo, e provavelmente o processo de malignização (GRAMMATICO et al., 1998; PICARDO et al., 1996). Melanomas primários e metastáticos apresentam diferentes mecanismos de evasão do estresse oxidativo. Ao contrário dos melanócitos, as células de melanoma são equipadas com uma alta capacidade antioxidante, e podem usar essa habilidade para gerar radicais livres para as células vizinhas ao mesmo tempo em que não são atingidas, favorecendo sua proliferação e invasão (MEYSKENS et al., 2001; SANDER et al., 2003; WITTGEN; VAN KEMPEN, 2007).

Gadjeva, Dimov e Georgieva (2008) mostraram que pacientes com melanoma não tratado demonstraram aumento do estresse oxidativo sistêmico e um comprometimento do sistema antioxidante. Assim, alterações redox em pacientes e em células de melanoma podem ser um dos fatores responsáveis pela sua fraca resposta ao tratamento com quimioterápicos e imunoterápicos, uma vez que a maioria dos quimioterápicos atuam aumentando os níveis de ERO nessas células como indutor de apoptose (ZHELEVA; GADJEVA, 2001). Nos últimos anos foi aprovado o tratamento do melanoma com os anticorpos monoclonais ipilimumabe e vemurafenibe pela FDA (Do

inglês *Food and Drugs Administration*). O vemurafenibe possui um rápido benefício clínico no tratamento de pacientes com mutação no gene BRAF no estadiamento IV da doença, embora haja relatos na literatura do desenvolvimento de resistência ao tratamento após 6 meses de terapia (ASCIERTO et al., 2013; DAS TAKHUR et al., 2013). Um trabalho recente mostra que a resistência ao vemurafenibe pode estar relacionada a produção de ERO pela mitocôndria, com alterações nos níveis do peptídeo antioxidante glutationa reduzida (GSH) e de enzimas antioxidantes intracelulares (KLUZZA et al., 2013).

Células de melanoma, cultivadas *in vitro* ou implantadas em animais, apresentam altos níveis do co-ativador do receptor ativado por proliferadores de peroxissoma gama alfa (PGC1-alfa). Esta molécula está envolvida na biogênese mitocondrial e na desintoxicação de ERO, atuando tanto no aumento da respiração mitocondrial - com consequente geração de ERO - quanto na expressão de numerosas enzimas antioxidantes. Isso confere uma seletiva vantagem para as células de melanoma, uma vez que proporciona uma forte proteção contra danos oxidativos e morte induzida por ERO (VAZQUEZ et al., 2013). Outro mecanismo de defesa contra o ERO das células de melanoma é o aumento intracelular da ferritina.

1.3 FERRITINA E CÂNCER

A ferritina é uma proteína de fase aguda com expressão aumentada em estados inflamatórios, infecciosos e no câncer. Níveis aumentados de ferritina no tecido tumoral têm sido reportados em diferentes tipos de neoplasias, como câncer de cólon, mama e seminoma (STEEGMANN-OLMEDILLAS, 2011). A ferritina é uma proteína intracelular ligadora de íons ferro, constituída dos subtipos cadeia leve e cadeia pesada. Devido sua capacidade de ligar íons Fe^{+2} e diminuir sua disponibilidade intracelular, ela exerce papel antioxidante. A cadeia pesada apresenta atividade ferroxidase importante, e consequentemente, é capaz de inibir a apoptose mediada por ERO (AROSIO; INGRASSIA; CAVADINI, 2009; PHAM et al., 2004). As funções gerais das ferritinas de cadeia leve e pesada estão representadas na figura 6.

Recentemente, Liu e colaboradores (2011) mostraram que o silenciamento do gene da ferritina de cadeia pesada em células de glioma e câncer de mama aumentou a vulnerabilidade dessas à quimioterapia, tanto *in vitro* quanto *in vivo*, através ativação da caspase-3. Em humanos, a ferritina de cadeia pesada sérica induz a proliferação de linfócitos T regulatórios, levando a um estado de imunossupressão em pacientes com melanoma, o que favoreceria o tumor e promoveria a progressão da doença (GRAY, AROSIO; HERSEY, 2003). Metástases de melanoma apresentam alta expressão de ferritina de cadeia leve em relação aos respectivos tumores primários, mostrando um importante papel dessa molécula na proliferação e invasão do melanoma (BALDI et al., 2005).

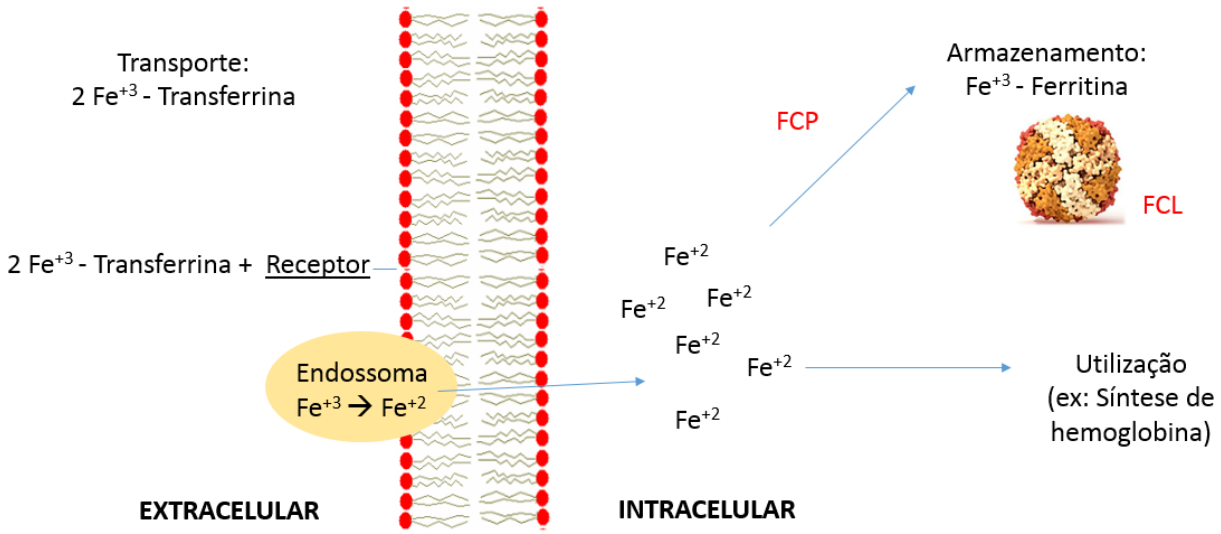


Figura 6. Papel das ferritinas de cadeia leve e pesada no metabolismo intracelular do ferro. No plasma, o ferro é transportado pela transferrina na forma Fe^{+3}. Ao se ligar ao seu receptor específico na membrana das células, o Fe^{+3} é liberado da transferrina e internalizado através do endossoma, sendo convertido em Fe^{+2} dentro dessa vesícula. No meio intracelular, o Fe^{+2} pode ser utilizado, como, por exemplo, na síntese do grupamento heme, ou pode ser oxidado a Fe^{+3} e armazenado pela FCL (Ferritina de Cadeia Leve). A FCP (Ferritina de Cadeia Pesada) possui atividade ferroxidase e também atua na etapa de conversão do Fe^{+2} em Fe^{+3} para incorporação na FCL.

1.4 Fator transformador de Crescimento beta e Câncer

O fator transformador de crescimento beta (TGF-beta, do inglês *Transforming Growth Factor Beta*) é um ligante secretado por diferentes tipo celulares, intimamente relacionado à iniciação, progressão tumoral e metástase (LEBRUN, 2012). Existem 3 isotipos de TGF-beta, sendo o TGF-beta 1 mais abundante, e utilizado na literatura como sinônimo de TGF-beta. A ação do TGF-beta se dá através de sua ligação a um complexo de receptores composto por receptor de TGF-beta tipo I (TβRI), tipo II (TβRII) e tipo III (TβRIII). O complexo de receptores ativados pela ligação ao TGF-beta interage com um grupo de proteínas controladas por fosforilação, as SMAD 2 e SMAD 3 (Do inglês *Sma and Mad related proteins*). As SMAD 2 e 3 combinam-se com a SMAD 4, que é translocada até o núcleo, onde se liga à sequências específicas dos genes alvo, regulando a transcrição gênica. As ações do TGF-beta são antagonizadas pela SMAD 7, que atua interagindo com o TβRI, impedindo a ativação das SMAD 3 e 4 (LEBRUN, 2012).

As isoformas do TGF-beta são sintetizadas como um complexo latente, onde o TGF-beta deve ser efetivamente liberado para exercer sua atividade biológica. Embora muitos estudos foquem na regulação da transcrição gênica do TGF-beta, a latência parece ser uma etapa crítica no controle da sua atividade, e um aumento da sua expressão nem sempre está relacionada à sua atividade biológica (GLEIZES et al., 1997). A ativação do TGF-beta latente é dependente de diversos estímulos, como atividade de enzimas proteolíticas, calor e ERO (LEBRUN, 2012). O sistema antioxidante tem sido descrito como um fator protetor da ativação do TGF-beta em processos patológicos, atuando como um regulador da sua ativação redox (ISLAM et al., 1996; THANNICKAL; FANBURG, 2000; BARCELLOS-HOFF; DIX, 1996).

Experimentalmente, o silenciamento do gene TGF-beta 1 resulta em resposta inflamatória excessiva, e o tratamento com TGF-beta previne o choque séptico induzido por LPS (Lipopolissacarídeo), além de suprimir o *burst* respiratório de macrófagos ativados (LE et al., 2004; LETTERIO; ROBERTS, 1998). O TGF-beta tem seu efeito anti-inflamatório antagonizado pela ativação do fator de transcrição NF-κB (Do inglês *Nuclear Factor kappa B*). A ativação do NF-κB ocorre principalmente por

ação das citocinas pró-inflamatórias TNF-alfa (Do inglês *Tumor Necrosis Factor alpha*) e IL-1 (Interleucina 1) e ERO, e ele é considerado o principal fator de transcrição coordenador da resposta pró-inflamatória (SUN; ANDERSSON, 2002). O NF-κB atua como um regulador negativo da ação do TGF-beta por aumentar os níveis da proteína inibitória SMAD 7 (LE et al., 2004; MONTELEONE et al., 2004). Paradoxalmente, o TGF-beta é capaz de ativar o NF-κB via ativação da proteína TAK-1 (Do inglês *TGF-beta Activated Kinase 1*), levando à degradação da proteína citoplasmática IκB-α (Do inglês *Inhibitor kappa B alpha*), inibitória da NF-κB. Porém, como o NF-κB pode ser ativado por outros estímulos, o aumento da SMAD 7 suprime preferencialmente a sinalização canônica do TGF-beta (FREDLSPERGER et al., 2013). A interação entre essas duas vias encontra-se ilustrada na Figura 7.

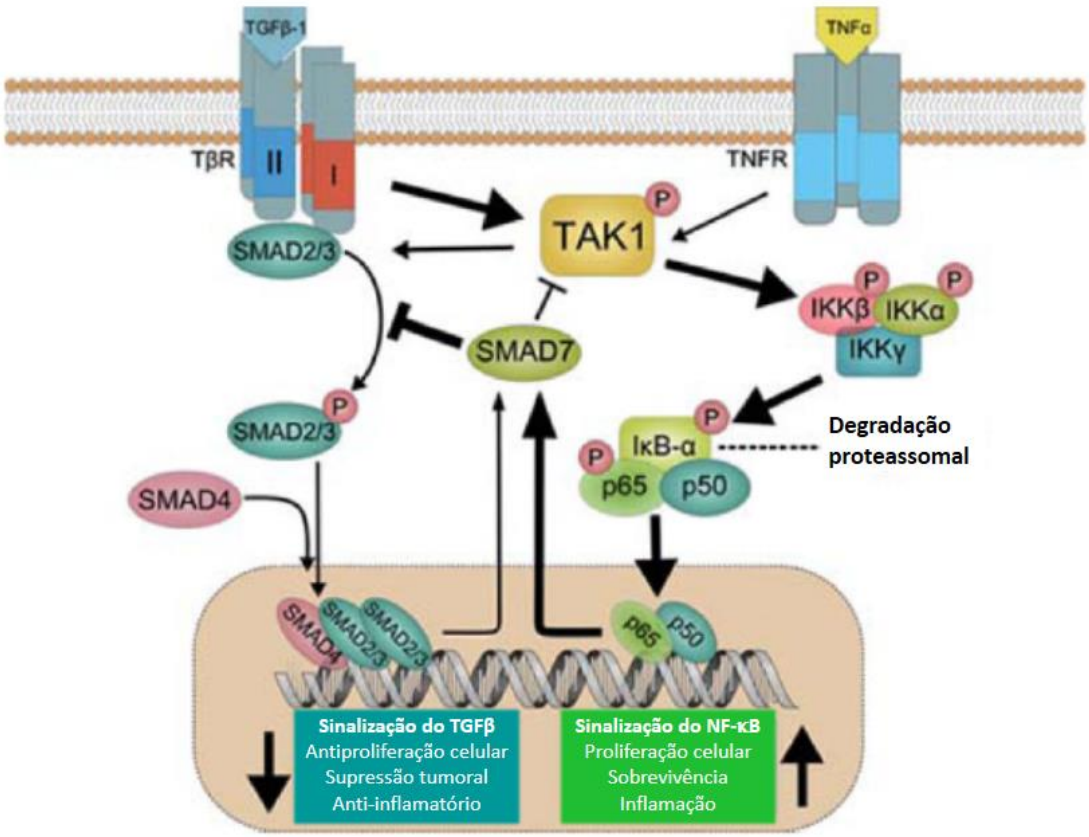


Figura 7. Interação entre as vias de sinalização do TGFβ e NF-κB. O TGFβ (Do inglês *Transforming Growth Factor Beta*) ativa a sinalização do NF-κB (Do inglês *Nuclear Factor kappa B*)

através da regulação das quinases TAK-1 (Do inglês *TGF-beta Activated Kinase 1*) e IKK (Do inglês *IκB Kinase*) levando à fosforilação do IκB-α (Do inglês *Inhibitor kappa B alpha*), translocação nuclear e fosforilação da subunidade p65 do NF-κB. Tanto a sinalização do TGF-β quanto do NF-κB induzem a síntese de SMAD 7, que suprime preferencialmente a via canônica de sinalização do TGF-β e a sinalização não-canônica do NF-κB, comparado à via de ativação canônica do NF-κB pelo TNF-α (Do inglês *Tumor Necrosis Factor Alpha*). Adaptado de Fredlsperger et al., 2013.

No câncer, a ação do TGF-beta é complexa, agindo desde a inibição do crescimento celular até a migração e invasão das células tumorais. O efeito de supressão tumoral sobre células malignas de origem epitelial, endotelial, mielóide e linfóide é bem caracterizado, porém no melanoma, cujo o melanócito possui origem embrionária da crista neural, os efeitos supressores e pró-metastáticos ainda são controversos (LEBRUN, 2012; MOUSTAKAS, 2008; SCHRIEK at al., 2005). Nos melanócitos, o TGF-beta apresenta um forte efeitos antiproliferativo (JAVELAUD; ALEXAKI; MAUVIEL, 2008). Evidências apontam que o TGF-beta pode inibir o crescimento e a migração tumoral de células de melanoma murino *in vivo*, e também de células de melanoma humano *in vitro*, embora o TGF-beta plasmático esteja aumentado em pacientes com melanoma em todos os estágios da doença (HUMBERT; LEBRUN, 2013; KRASAKIS et al., 1998; MALAPONTE et al., 2010; RAMONT et al., 2003).

O TGF-beta, assim como a inflamação e o estresse oxidativo estão relacionados com a promoção e progressão do câncer, porém essa relação é pouco compreendida na evolução do melanoma. Uma vez que o TGF-beta é secretado *in vitro* por leucócitos, é possível assumir que *in vivo* os leucócitos também podem secretar TGF-beta no plasma, embora ainda não exista evidências concretas desse fato (GRAIGER; MOSEDALE; METCALFE, 2000). A presença do TGF-beta, principalmente o TGF-beta 2 no tecido tumoral de melanoma humano, é reportada na literatura, sendo sua expressão ainda maior em tumores metastáticos (SCHIMID; ITIN; RUFLI, 1995; VAN BELLE et al., 1996; MORETI et al., 1999). Tanto o TGF-beta 1 quanto o TGF-beta 2 estão aumentados no plasma de pacientes com melanoma metastático, e os níveis

séricos de TGF-beta 1 está correlacionada com os níveis tumorais dessa citocina (KRASAGAKIS et al., 1999; MALAPONTE et al., 2010).

Recentemente, Humbert e Lebrun (2013) mostraram um papel protetor do TGF-beta na migração e invasão de células de melanoma humano, *in vitro*, por regular positivamente o IAP-1 (Inibidor da Ativação do Plasminogênio-1). Em um modelo de melanoma experimental, o TGF-beta foi capaz de diminuir o processo metastático *in vivo* (RAMONT et al., 2003). Uma evidência de que o TGF-beta pode atuar como protetor no câncer seria um recente trabalho que mostra que pacientes com câncer de mama triplo negativo e com metástases apresentam baixos níveis de TGF-beta circulante, relacionado a um ruim prognóstico (PANIS et al., 2013).

Embora ainda pouco explorados no melanoma, o estresse oxidativo e moléculas relacionadas com o equilíbrio redox, como as envolvidas no metabolismo do ferro e na resposta inflamatória, exercem importante papel na progressão em diversos tipos de câncer. O estudo dessas moléculas pode auxiliar no desenvolvimento de tratamentos eficazes para o melanoma disseminado, até então, incurável.

2 OBJETIVO

Caracterizar biomarcadores oxidativos e relacionados no sangue e no tecido tumoral de pacientes com melanoma cutâneo e melanoma metastático.

2. 1 OBJETIVOS ESPECÍFICOS

A) Artigo 1: Caracterizar o estresse oxidativo sistêmico e tumoral e parâmetros inflamatórios sanguíneos em pacientes com melanoma estágios I e II, com ressecção cirúrgica do tumor entre 6 meses e 5 anos, de acordo com a medida T (do inglês *thickness*, que significa espessura do tumor) estabelecida pelo Comitê Americano de Câncer.

B) Artigo 2: Caracterizar os níveis sistêmicos de ferritina de cadeia pesada (FCP) e de ferritina de cadeia leve (FCL), a lipoperoxidação de hemácias, e a expressão de FCP e p53 no tecido tumoral de pacientes com melanoma cutâneo com baixo risco de recidiva, com alto risco de recidiva, e com doença recidivada.

C) Artigo 3: Caracterizar o estresse oxidativo sistêmico, marcadores plasmáticos de doença avançada e os níveis de TGF-β circulante em pacientes com melanoma sem recidiva em um período de 3 anos, e em pacientes com melanoma recidivado.

3 CONCLUSÕES GERAIS

- Pacientes com melanoma cutâneo apresentam alterações de parâmetros de estresse oxidativo sistêmico quando comparado a indivíduos saudáveis, evidenciado pelo aumento do MDA plasmático, mesmo após a remoção cirúrgica do tumor. Quanto maior a medida de espessura de Breslow do tumor primário, maiores os níveis sistêmicos de MDA e de PCR nesses pacientes. No tecido tumoral de melanoma cutâneo, o aumento da expressão de 3-NT nas células tumorais e no microambiente de tumores com Breslow > 2 mm indica que o melanoma é capaz de induzir estresse oxidativo no tecido saudável ao redor. Essa evidência pode ser considerada uma das causas do maior risco de recidiva da doença em pacientes com tumores > 2 mm, uma vez que o estresse oxidativo está relacionado ao processo de invasão e migração das células tumorais.

- Embora as células tumorais de melanoma expressem FCP, os níveis dessa proteína não são estatisticamente significativos entre os diferentes estágios da doença. A expressão de p53 encontra-se aumentada em tumores metastáticos quando comparados à tumores com Breslow < 2 mm. Os níveis sistêmicos de FCP apresentou-se aumentado somente nos grupos com alto risco de recidiva e com recidiva quando comparados ao grupo controle, embora todos os pacientes com melanoma terem apresentado aumento na relação FCP:FCL em relação ao grupo controle.

- Pacientes com doença recidivada apresentam menores níveis de TGF-β circulantes quando comparado a indivíduos saudáveis, correlacionado à maiores níveis de MDA e AOPP, indicando um possível papel pró-inflamatório dessa citocina quando presente em baixas quantidades.

- Os resultados encontrados mostram a participação do estresse oxidativo em diferentes estágios do melanoma, bem como a interação com outras biomoléculas envolvidas na resposta inflamatória e bioquímica, como a PCR, ferritinas de cadeia pesada e leve e TGF-β. Quanto maior a espessura de Breslow e

agressividade da doença (alto risco de recidiva e doença recidivada), mais evidentes são as alterações nesses pacientes.

- Os dados obtidos acrescentam uma nova perspectiva sobre o estado redox em pacientes com melanoma e seu possível envolvimento com a evolução da doença, que podem auxiliar no desenvolvimento de estratégias clínicas para a detecção precoce do melanoma avançado.

4 REFERÊNCIAS

ALMEIDA, J.R.C. Marcadores Tumorais: Revisão de Literatura. **Revista Brasileira de Cancerologia**, Rio de Janeiro, v. 53, p. 305-316, 2007.

AMBROSONE, C.B. et al. Polymorphisms in genes related to oxidative stress (MPO, MnSOD, CAT) and survival after treatment for breast cancer. **Cancer Research**, Philadelphia, v.65, p.1105-1111, 2005.

AROSIO, P.; INGRASSIA, R.; CAVADINI, P. Ferritins: A family of molecules for iron storage, antioxidation and more. **Biochimica et Biophysica Acta**, Amsterdam, v.1790, p. 589-599, 2009.

ASCIERTO, P.A. et al. Biomarkers for immunostimulatory monoclonal antibodies in combination strategies for melanoma and other tumor types. **Clinical Cancer Research**, Philadelphia, v.19, p.1009-1020, 2013.

AVERY-KIEJDA, K.A. et al. p53 in human melanoma fails to regulate target genes associated with apoptosis and the cell cycle and may contribute to proliferation. **BMC Cancer**, London, v.11, p.203, 2011.

BALCH, C.M. et al. Final version of 2009 AJCC melanoma staging and classification. **Journal of Clinical Oncology**, Alexandria, v. 27, p. 6199-6206, 2009.

BALCH, C.M.. et al. Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. **Journal of Clinical Oncology**, Alexandria ,v.19, p. 3622-3634, 2001.

BALDI, A. et al. Ferritin contributes to melanoma progression by modulating cell growth and sensitivity to oxidative stress. **Clinical Cancer Research**, Philadelphia, v.11, p. 3175–3183, 2005.

BARBOSA, K.B.F. et al. Estresse oxidativo: conceito, implicações e fatores modulatórios. **Revista de Nutrição**, Campinas, v. 23, p. 629-643, 2010.

BARCELLOS-HOFF, M.H.; DIX, T.A. Redox-mediated activation of latent transforming growth factor-beta 1. **Molecular Endocrinology**, Baltimore, v.10, p.1077-1083, 1996.

BARTSCH, H.; NAIR, J. Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis. **Cancer Detection and Prevention,** Amsterdam, v. 28, p. 385-391, 2004.

BELLO, D.M. et al. Prognosis of acral melanoma: a series of 281 patients. **Annals of Surgical Oncology**, Dordrecht, v. 20, p. 3618-3625, 2013.

BICHAKJIAN, C.K. et al. Guidelines of care for the management of primary cutaneous melanoma. American Academy of Dermatology. Journal of the **American Academy of Dermatology**, Schaumburg, v. 65, p. 1032-1047, 2011.

BOUWHUIS, M.G. et al. Changes of ferritin and CRP levels in melanoma patients treated with adjuvant interferon-α (EORTC 18 952) and prognostic value on treatment outcome. **Melanoma Research**, Pittsburgh, v.21, p. 344-351, 2011.

BRASILEIRO-FILHO, G.; PEREIRA, F. E. L.; GUIMARÃES, R. C. Alterações da Proliferação e Diferenciação Celulares. In: BRASILEIRo-FILHO, G. (ed). **Bogliolo - Patologia Geral**. 5 ed. Rio de Janeiro:Guanabara Koogan, 2013. pp. 233-284.

CAINI, S. et al. Meta-analysis of risk factors for cutaneous melanoma according to anatomical site and clinico-pathological variant. **European Journal of Cancer, Oxford**, v. 45, p. 3054-3063, 2009.

CURTIN, J.A. et al. Distinct sets of genetic alterations in melanoma. **The New England Journal of Medicine**, Boston, v. 353,p. 2135-2147, 2005.

DAS THAKUR, M.; STUART, D.D. The evolution of melanoma resistance reveals therapeutic opportunities. **Cancer Research**, Philadelphia, v. 73, p. 6106-6110, 2013.

FINDEISEN, P. et al. Serum amyloid A as a prognostic marker in melanoma identified by proteomic profiling. **Journal of Clinical Oncology**, Alexandria, v.27, p. 2199 – 2208, 2009.

FINKEL, T.; HOLBROOK, N.J. Oxidants, oxidative stress and the biology of ageing. **Nature**, London, v. 408, p. 239-247, 2000.

FRANCKEN, A.B. et al. Prognosis and determinants of outcome following locoregional or distant recurrence in patients with cutaneous melanoma. **Annals of Surgical Oncology**, Dordrecht, v.15, p.1476-1484, 2008.

FREUDLSPERGER et al. TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. **Oncogene**, Hampshire, v. 21, p.1549-1559, 2013.

GADJEVA, V.; DIMOV, A.; GEORGIEVA, N. Influence of therapy on the antioxidant status in patients with melanoma. **The Journal of Clinical Pharmacology**, Oxford, v.33, p. 179-185, 2008.

GAGO-DOMINGUEZ M, JIANG X, CASTELAO JE. Lipid peroxidation, oxidative stress genes and dietary factors in breast cancer protection: a hypothesis. **Breast Cancer Research**, London, v.9, p. 201, 2007.

GERBER, M. et al. Oxidant-antioxidant status alterations in cancer patients: relationship to tumor progression. **The Journal of Nutrition**, Rockville, v.126, p.1201S-127S, 1996.

GERBER, M. et al. Tumor progression and oxidant-antioxidant status. **Cancer Letters**, Amsterdam, v.114, p. 211-214, 1997.

GILCHREST, B.A. et al. The pathogenesis of melanoma induced by ultraviolet radiation. **The New England Journal of Medicine**, Boston, v. 340, p. 1341-1348, 1999.

GLEIZES et al. TGF-beta latency: biological significance and mechanisms of activation. **Stem Cells,** Dayton, v.15, p. 190-197, 1997.

GRAINGER, D.J.; MOSEDALE, D.E.; METCALFE, J.C. TGF-beta in blood: a complex problem. **Cytokine & Growth Factor Reviews,** Oxford, v.11, p. 133-145, 2000.

GRAMMATICO, P. et al. Increased sensitivity to peroxidizing agents is correlated with an imbalance of antioxidants in normal melanocytes from melanoma patients. **Experimental Dermatology**, Malden, v.7, p. 205-212, 1998.

GRAY, C.P.; AROSIO, P.; HERSEY, P. Association of increased levels of heavy-chain ferritin with increased CD4+ CD25+ regulatory T-cell levels in patients with melanoma. **Clinical Cancer Research**, Philadelphia, v.9, p. 2551–2559, 2003.

GRAY-SCHOPFER, V.; WELLBROCK, C.; MARAIS, R. Melanoma biology and new targeted therapy. **Nature**, London, v. 445, p. 851-857, 2007.

GROSSMAN, D.; ALTIERI, D.C. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. **Cancer and Metastasis Reviews**, Dordrecht, v.20, p. 3-11, 2001.

GUPTA. A.; BHATT, M.L.; MISRA, M.K. Lipid peroxidation and antioxidant status in head and neck squamous cell carcinoma patients. **Oxidative Medicine and Cellular Longevity**, New York, v.2, p.68-72, 2009.

HAASS, N.K.; SMALLEY, K. S.; HERLYN, M. The role of altered cell–cell communication in melanoma progression. **Journal of Molecular Histology**, Dordrecht, v. 35, p. 309–318, 2004.

HALLIWELL, B. Free radicals and antioxidants - quo vadis? **Trends in Pharmacological Sciences**, Cambridge, v. 32, p. 125-130, 2011.

HALLIWELL, B.; GUTTERIDGE, J.M.C. **Free Radicals Biology & Medicine**. 4th ed. New York: Oxforf University Press, 2007.

HOHNHEISER, A.M. et al. Malignant melanoma of the skin: long-term follow-up and time to first recurrence. **World Journal of Surgery**, Dordrecht, v.35, p. 580-589, 2011.

HOUBEN, R. et al. High-Level Expression of Wild-Type p53 in Melanoma Cells is Frequently Associated with Inactivity in p53 Reporter Gene Assays. **Plos One**, San Francisco, v.6, p. e22096, 2011.

ICHIHASHI, M. et al. UV-induced skin damage. **Toxicology**, Amsterdam, v. 189, p. 21-39, 2003.

INCA, Instituto Nacional do Câncer (Brasil). **Estimativa 2014. Incidência do Câncer no Brasil**. Disponível em: <http://www.inca.gov.br/estimativa/2014/estimativa-24012014.pdf>. Acessado em: 22 fev 2014.

ISLAM, K.N. et al. TGF-beta1 triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase. **Free Radical Biology & Medicine**, New York, v.22, p. 1007–1017, 1997.

JACOBS, L.K.; LANGE, J.R.; BALCH, C.M. Sorting through the heterogeneity of recurrent melanoma. **Annals of Surgical Oncology**, Dordrecht, v.15, p.1280-1281, 2008.

JAVELAUD, D.; ALEXAKI, V.I.; MAUVIEL, A. Transforming growth factor-beta in cutaneous melanoma. **Pigment Cell & Melanoma Research**, Oxford, v.21, p.123-132, 2008.

JEFFS, A.R. et al. A Gene Expression Signature of Invasive Potential in Metastatic Melanoma Cells. **Plos One**, San Francisco, v.4, p.e8461, 2009.

JONES, D.P. Redefining oxidative stress. **Antioxidant and Redox Signaling**, New Rochelle, v.8, p. 1865 - 1879, 2006.

KAI, Y.; FUJIWARA, S. Management of Acral Lentiginous Melanoma. In: **Melanoma - From Early Detection to Treatment**. DUC, H (ed). Rijeka: InTech Open; 2013. Disponível em: <http://www.intechopen.com/books/melanoma-from-early-detection-to-treatment/management-of-acral-lentiginous-melanoma>. Acesso em: 26 fev. 2014.

KLUGER, H.M. et al. Plasma markers for identifying patients with metastatic melanoma. **Clinical Cancer Research**, Philadelphia, v.17, p. 2417-2425, 2011.

KLUZZA, J. et al. Mitochondrial metabolic reprogramming of melanoma cells exposed to BRAFV600E inhibitor. **Annals of Oncology**, Dordrecht, v.24, p. i30–i32, 2013.

KOHEN, R.; NYSKA, A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. **Toxicology Pathology**, Thousand Oaks, v. 30, p. 620-650, 2002.

KORKMAZ, G.G. et al. The association of oxidative stress markers with conventional risk factors in the metabolic syndrome. **Metabolism**, New York, v. 62, p. 828-835, 2013.

KRASAGAKIS, K. et al. Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta 2 in patients with disseminated malignant melanoma. **British Journal of Cancer***,* London, v.77, p.1492-1494, 1998.

LAZAR, A.J.F.; MURPHY, G.F. A pele. In: KUMAR, V. et al. (eds). **Robins & Cotran - Patologia - Bases Patológicas das doenças.** 8 ed. Rio de Janeiro: Elsevier, 2010. p. 1165 - 1204.

LE, Y. et al. TGF-beta1 disrupts endotoxin signaling in microglial cells through Smad3 and MAPK pathways**. Journal of Immunology**, Baltimore, v.15, p.962-968, 2004.

LEBRUN, J.J. The Dual Role of TGF-beta in Human Cancer: From Tumor Suppression to Cancer Metastasis*.* **ISRN Molecular Biology,** New York, Article ID 381428, 2012.

LETTERIO, J.J.; ROBERTS, A.B. Regulation of immune responses by TGF-beta. **Annual Review of Immunology**, Palo Alto, v.16, p.137-161, 1998.

LIU, X. et al. Heavy chain ferritin siRNA delivered by cationic liposomes increases sensitivity of cancer cells to chemotherapeutic agents. **Cancer Research**, Philadelphia, v.71, p. 2240-2249, 2011.

MALAPONTE, G. et al. Co-regulated expression of matrix metalloproteinase-2 and transforming growth factor-beta in melanoma development and progression. **Oncology Reports**, Athens, v.24, p. 81-87, 2010.

MANTOVANI, G. et al. Quantitative evaluation of oxidative stress, chronic inflammatory indices and leptin in cancer patients: correlation with stage and performance status. **International Journal of Cancer**, New York, v.98, p. 84-91, 2002.

MARTENSON, E.D. et al. Serum S-100b protein as a prognostic marker in malignant cutaneous melanoma. **Journal of Clinical Oncology**, Alexandria, v.19, p. 824-831, 2001.

MARTIN, R.C. et al. Association between manganese superoxide dismutase promoter gene polymorphism and breast cancer survival. **Breast Cancer Research: BCR**, London, v. 8, p. R45, 2006.

MARTINDALE, J.L.; HOLBROOK, N.J. Cellular response to oxidative stress: signaling for suicide and survival. **Journal of Cellular Physiology**, Philadelphia, v.192, p. 1-15, 2002.

MEYSKENS, F.L. Jr et al. Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. **Free Radical Biology & Medicine**, New York, v.31, p. 799-808, 2001.

MILLER, A.J.; MIHM, M.C. Jr. Mechanism of disease: Melanoma. **The New England Journal of Medicine**, Boston, v. 355, p. 51-65, 2006.

MORETTI, S. et al. Immunohistochemical evidence of cytokine networks during progression of human melanocytic lesions. **International Journal of Cancer**, New York, v.84, p.160-168, 1999.

MOUSTAKAS, A. TGF-beta targets PAX3 to control melanocyte differentiation. **Developmental cell**, Cambridge, v.15, p. 797-799, 2008.

NATIONAL CANCER INSTITUTE AT THE NATIONAL INSTITUTES OF HEALTH, United States of America. **Stages of Melanoma**. Disponível em: <http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/Patient/page2>. Acesso em: 25fev. 2014.

NORDENFELT, P.; TAPPER, H. Phagosome dynamics during phagocytosis by neutrophils. **Journal of Leukococytes Biology**, Bethesda, v. 90, p. 271-84, 2011.

PAEK, S.C. et al. Cutaneous Melanoma. In: WOLFF, K. et al. (eds). **Fitzpatrick's Dermatology in General Medicine**. 7° ed. New-York: McGraw-Hill, 2008. p. 1134-1157.

PANIS, C. et al. Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. **Breast Cancer Research and Treatment**, Boston, v.133, p. 89-97, 2012 a.

PANIS, C. et al. Differential oxidative status and immune characterization of the early and advanced stages of human breast cancer. **Breast Cancer Research and Treatment**, Boston, v. 133, p. 881-888, 2012b.

PANIS, C. et al. Screening of circulating TGF-β levels and its clinicopathological significance in human breast cancer. **Anticancer Research**, Athens, v.33, p. 737-742, 2013.

PHAM, C.G., et al. Ferritin heavy chain upregulation by NF-kappaB inhibits TNF-alpha-induced apoptosis by suppressing reactive oxygen species. **Cell**, Cambridge, v.119, p. 529–542, 2004.

PICARDO, M. et al. Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma. **Journal of Investigative Dermatology**, New York, v.107, p.322-326, 1996.

RAHA, S.; ROBINSON, B.H. Mitochondria, oxygen free radicals, disease and ageing. **Trends in Biochemical Sciences**, Cambridge, v. 25, p. 502-508, 2000.

RAMONT, S.P. et al. Transforming growth factor-β1 inhibits tumor growth in a mouse melanoma model by down-regulating the plasminogen activation system. **Experimental Cell Research**, Amsterdam, v. **291**, p. 1–10, 2003.

SAINTOT, M. et al. Tumor progression and oxidant-antioxidant status. **Carcinogenesis**, Oxforf, v.17, p.1267-1271, 1996.

SAINTOT, M. et al. Oxidant-antioxidant status in relation to survival among breast cancer patients. **International Journal of Cancer**, New York, v.97, p.574-579, 2002

SANDER, C.S. et al. Oxidative stress in malignant melanoma and non-melanoma skin cancer. **British Journal of Pharmacology**, Malden v. 148, p. 913-922, 2003.

SALZMAN, R. et al. High perioperative level of oxidative stress as a prognostic tool for identifying patients with a high risk of recurrence of head and neck squamous cell carcinoma.International. **Journal of Clinical Oncology**, Tokyo, v.15, p. 565-570, 2010.

SCHMID, P.; ITIN, P.; RUFLI, T. In situ analysis of transforming growth factor-beta s (TGF-beta 1, TGF-beta 2, TGF-beta 3), and TGF-beta type II receptor expression in malignant melanoma. **Carcinogenesis**, New York, v. 16, p. 1499-1503, 1995.

SCHRIEK, G. et al. Human SK-Mel 28 melanoma cells resume neural crest cell migration after transplantation into the chick embryo. **Melanoma Research**, London, v.15, 225-234, 2005.

SIES, H. Oxidative Stress: Oxidants and Antioxidants. **Experimental Physiology**, New York, v. 82, p. 291-295, 1997.

SIMON, H.U.; HAJ-YEHIA, A.; LEVI-SCHAFFER, F. Role of reactive oxygen species (ROS) in apoptosis induction. **Apoptosis**, Amsterdam, v.5, p. 415-418, 2000.

SOENGAS, M.S.; LOWE, S.W. Apoptosis and melanoma chemoresistance. **Oncogene**, New York, v. 22, p. 3138-3151, 2003.

SOONG, S.J. et al. Factors affecting survival following local, regional, or distant recurrence from localized melanoma. **Journal of Surgical Oncology**, Malden, v. 67, p. 228-233, 1998.

STEEGMANN-OLMEDILLAS, J.L. The role of iron in tumour cell proliferation. **Clinical and Translational Oncology**, Dordrecht , v.13, p. 71-76, 2011.

SUN, Z.; ANDERSSON, R. NF-kappaB activation and inhibition: a review. **Shock**, Baltimore, v. 18, p. 99-106, 2002.

TADOKORO et al. UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin. **The FASEB Journal**, Bethesda, v.17, p. 1177-1179, 2003.

THANNICKAL, V.J.; FANBURG, B.L. Activation of an H2O2- generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. **The Journal of Biological Chemistry**, Baltimore, v.270, p. 30334-30338, 1995.

THÖRN, M. et al. Clinical and histopathologic predictors of survival in patients with malignant melanoma: a population based study in Sweden. **Journal of the National Cancer Institute**, Oxford, v. 86, p. 761-769.

TURRENS, JF. Superoxide Production by the Mitochondrial Respiratory Chain. **Bioscience Reports**, Dordrecht, v.17, p. 3-8, 1997.

VALAVANIDIS, A.; VLACHOGIANNI, T.; FIOTAKIS, C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. **Journal of environmental science and health. Part C, Environmental carcinogenesis & ecotoxicology reviews**, New York, v.27, p.120-139, 2009.

VAN BELLE, P. et al. Melanoma-associated expression of transforming growth factor-beta isoforms. **The American Journal of Pathology**, Philadelphia, v.148, p. 1887–1894, 1996.

VAZQUEZ, F. et al. PGC1α expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. **Cancer Cell**, Cambridge, v.23, p. 287-301, 2013.

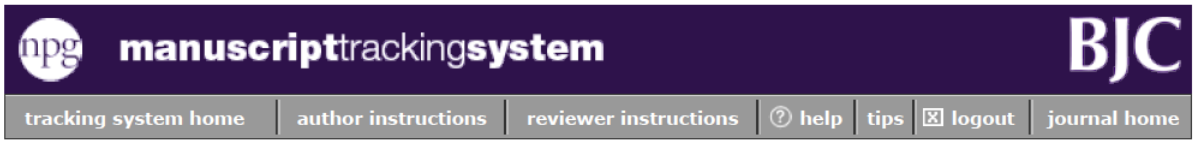
WITTGEN, H.G.; VAN KEMPEN, L.C. Reactive oxygen species in melanoma and its therapeutic implications. **Melanoma Research**, Pittsburgh, v. 17, p. 400-409, 2007.

ZHELEVA, A.; GADJEVA, V. Spin labeled nitrosoureas and triazenes and their nonlabeled clinically used analogues – a comparative study on their physicochemical properties and antimelanomic effects**. International Journal of Pharmaceutics**, Amsterdam, v. 212, p.257–266, 2001.

ANEXOS

ANEXO A

Oxidative stress in patients in surveillance after tumor removal of cutaneous melanoma.



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Abstract	Background: Systemic oxidative stress was reported in patients with cancer, but data about it in melanoma is sparse. Here, we evaluate systemic oxidative stress and three inflammatory mediators in patients with cutaneous melanoma in surveillance. We also evaluate 3-nytrotyrosine in tumor to verify its relationship with melanoma recurrence. Methods: 43 patients with melanoma in surveillance without recurrence episode, and 50 healthy volunteers were recruited. Patients were divided in two groups according to tumor Breslow thickness: T1/T2 (< 2 mm) and T3/T4 (≥ 2 mm). Were analyzed systemic oxidative stress and inflammatory biomarkers. 3-nytrotyrosine was measured in 28 randomized paraffin embedded tumor tissues from patients with melanoma, with or without recurrence, by immunohistochemistry. Results: Patients had decreased GSH, increased malondialdehyde, thiol and total radical antioxidant parameter:uric acid ratio levels. C-reactive protein (CRP), an inflammatory marker, and γ-glutamyl transferase were increased only in T3/T4 group. Furthermore, as greater tumor thickness, higher malondialdehyde and CRP levels were found. High Breslow tumors presented more 3-nytrotyrosine expression, and no relationship was found between 3-nytrotyrosine and the neoplasm recurrence. Conclusions: Surveillance patients had high systemic oxidative stress, and the tumor thickness correlates with CRP and malondialdehyde levels. High Breslow melanoma had increased 3-nytrotyrosine, despite no correlation found with tumor recurrence.
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Oxidative stress in patients in surveillance after tumor removal of cutaneous melanoma

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Abbreviations: ALM, acral lentiginous melanoma; CRP, C-reactive protein; GSH, reduced Glutathione; IL-1β, interleukin-1 beta; MDA, malondialdehyde; NM, nodular melanoma; OS, oxidative stress; SOD, superoxide dismutase; SSM, superficial spreading melanoma; TNF-α, tumor necrosis factor alpha; TRAP, total radical-trapping antioxidant parameter.

ABSTRACT

Breslow thickness is highly related with melanoma aggressiveness. Here, we evaluate Breslow's influence in systemic and tumoral oxidative status in surveillance patients with melanoma. A total of 43 patients with cutaneous melanoma, and 50 healthy volunteers were recruited. Patients were divided in two groups according to tumor Breslow thickness: T1/T2 (< 2 mm) and T3/T4 (≥ 2 mm). Systemic oxidative stress and three inflammatory mediators in the blood were analyzed. 3-nytrotyrosine was measured by immunohistochemistry in 28 tumor tissues from patients with melanoma, with or without recurrence, participants in the study of systemic oxidative stress evaluation or not. Patients showed decreased GSH, increased malondialdehyde, thiol and total radical-trapping antioxidant parameter : uric acid ratio levels. C-reactive protein (CRP), an inflammatory marker, and γ-glutamyl transferase were increased only in T3/T4 group. High Breslow tumors presented more 3-nytrotyrosine expression, and no relationship was found between 3-nytrotyrosine and the neoplasm recurrence. Even in surveillance, melanoma patients had high systemic oxidative stress, and the tumor thickness correlates with CRP and malondialdehyde levels. High Breslow melanoma had increased 3-nytrotyrosine, but tumoral oxidative stress is not related to the disease recurrence.

Keywords: Melanoma, Neoplasm recurrence, Oxidative stress, Inflammation, Immunohistochemistry, 3-nitrotyrosine, Breslow tickness.

INTRODUCTION

Cutaneous melanoma is one of the most aggressive solid tumors, with origin in epidermal melanocytes. When diagnosed early, melanoma is curable by surgery, with 80 percent of patients relapse free 10 years after surgery (Korn *et al*, 2008). Besides, the risk of recurrence after cutaneous melanoma removal remains for years, making post-treatment follow-up an important component of cancer care (Hohnheiser *et al*, 2011). The surveillance period recommended in melanoma is 5 years, but it could be extended to 10-15 years (Balch *et al*, 2009; Bichakjian *et al*, 2011; Dummer *et al*, 2008; Garbe and Eigentler, 2007; Rychetnik *et al*, 2012).

One of the most accepted correlation among tumor characteristics and aggressiveness in cutaneous melanoma is Breslow thickness, where high tumor thickness values at diagnosis was strongly indicative of melanoma recurrence and death even after longer disease free interval (Bichakjian *et al*, 2011; Breslow, 1970; Soong *et al*, 1992). In two years surveillance period, patients with Breslow > 2.5 mm have chance of recurrence starting from 23% and death from 14% (Soong *et al*, 1992).

Oxidative stress is a disruption of redox signaling and control, and is involved in the pathophysiology of several human diseases (Jones, 2006). Systemic oxidative stress (OS) is widely reported in patients with different types of cancer, and are closely related with chronic inflammation in this disease (de Cavanagh *et al*, 2002; Gadjeva *et al*, 2008; Gerber *et al*, 1997; Gupta *et al*, 2009; Mantovani *et al*, 2002; Panis *et al*, 2012a; 2012b; Reuter et al., 2010; Saintot *et al*, 2002). In general, the role of OS in cancer progression is still poorly understood. Besides systemic OS is considered bad prognosis in patients with cancer (Gerber *et al*, 1997; Saintot *et al*, 1996; 2002; Salzman

et al, 2010), some authors related pro-oxidative status, such as high systemic malondialdehyde (MDA) levels, and polymorphisms in antioxidant genes, as protective factor to cancer recurrence (Ambrosone *et al*, 2005; Gago-Dominguez *et al*, 2007; Martin *et al*, 2006).

Once high Breslow thickness is associated with aggressiveness and increased risk of disease recurrence, our aim were to characterize systemic OS and related biomarkers in patients in surveillance of melanoma according to tumor Breslow. In a second step, in order to understand OS influence in melanoma recurrence, we also evaluated OS in tumor samples – belonged to patients participants of systemic OS evaluation or not - through the expression of protein oxidation biomarker 3-nitrotyrosine.

MATERIALS AND METHODOS

Sample collection and study design

First, 43 patients between 24 to 71 years old in surveillance of cutaneous melanoma were recruited at the Londrina Cancer Hospital between April 2011 and November 2012. All patients had localized primary cutaneous melanoma stages I or II, with surgical removal between 6 months and 5 years, no recurrence episode, and were non-smoking or in antioxidant therapy treatment, not obese and with no hepatic, cardiac or renal dysfunction present. Control group comprised 50 healthy women and men volunteers, between 21 to 74 years old, non-smoking, not obese or in antioxidant therapy, and none had previous history of any type of cancer. This study was approved by the Research and Ethics National Council (CAAE 5831.0.000.268.10). Patients were

allocated into two groups according to Breslow thickness: T1/T2 (Breslow thickness < 2 mm, 24 patients) and T3/T4 (Breslow thickness ≥ 2 mm, 19 patients), as classified in TNM thickness category set by American Joint Committee on Cancer classification for melanoma (Balch *et al*, 2009). Venous blood was collected and an aliquot of heparinized blood was used to determine hemoglobin (Coulter STKS®, Hialeah, USA). The blood was centrifuged at 1,100 *x g*, separated plasma or serum were immediately frozen at -20°C and erythrocytes were immediately processed for antioxidant enzymes and reduced glutathione (GSH) measurement. The characteristics of the sample included in the systemic OS study are outlined in Table 1. We also evaluated the relationship between tumor thickness, tumor oxidative stress and recurrence in melanoma cells through 3-nitrotyrosine immunohistochemistry in 28 paraffin-embedded cutaneous melanoma obtained from database of the Department of Pathology at the Londrina Cancer Hospital. In the analysis of tumor oxidative stress, not all tumors belonged to the patients analyzed in the first step of this study. Here, comparisons were performed as follows: tumors with Breslow thickness < 2 mm x Breslow thickness > 2 mm and tumors with no recurrence in 3-years period x tumors with recurrence in 3 years period.

Serum and plasma biochemical analysis

Serum gamma-glutamyltranspeptidase was automatically performed in Dimension RxL® (Dade Behring/Siemens Healthcare Diagnostics, Dade Behring, Deerfield, USA), and results expressed in U x L^{-1}. C-reactive protein (highly sensitive CRP) was measured using a nephelometric assay (Behring Nephelometer II, Dade

Behring, Marburg, Germany) and results expressed in mg x L^{-1}. Plasmatic uric acid was performed by uricase method using a commercial kit (Laborclin®), and analyzed with a Multiskan GO® microplate reader (Thermo Fisher Scientific, Rockford, USA) at 510 nm, and results expressed in mg x dL^{-1}.

Plasma tumor necrosis factor (TNF-α) and interleukin 1-beta (IL-1β) levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturers' instructions (eBioscience®) at 450 nm. The results were expressed as pg x mL^{-1}.

Oxidative stress parameters

Total radical-trapping antioxidant parameter (TRAP) of plasma was determined as described by Repetto *et al*. (1998) modified by Panis et al (2012a). Chemiluminescence curves were obtained in a GloMax® luminometer 20/20 (Promega, Madison, USA), and the results are expressed in µM of Trolox.

Total thiols in plasma were measured by the method of Hu (1994) at 412 nm with a microplate reader. Total thiol groups were calculated using a calibration curve prepared by GSH (Sigma Aldrich®). Results were expressed in µM of thiol.

Plasma MDA was measured by High-performance liquid chromatography in a LC-20AT® HPLC system (Shimadzu, Kyoto, Japan), as described by Victorino e*t al.* (2012). Readings were obtained at 535 nm over 11 min at a flow rate of 0.8 mL/minute at 35°C, and results were expressed as nM of MDA.

Erythrocytes antioxidant analysis

Erythrocytes were obtained from heparinized blood and washed three times with 0.9% saline solution at 4ºC. Catalase activity was determined as described by Aebi (1984) modified by Panis *et al* (2012a). Absorbance disappearance kinetics was monitored in UV-1650 PC® UV-vis spectrophotometer at 240 nm (Shimadzu, Kyoto, Japan). The results are expressed in absorbance decreased in 1 minute (Absorption) per g Hemoglobin x 10^3.

Superoxide (SOD) activity was determined according to the method of Marklund and Marklund (1974) modified by Panis *et al* (2012a), at 420 nm. Final SOD results were expressed in U per g Hemoglobin x 10^3.

Total glutathione levels were determined with 30 µL of diluted erythrocytes at a ratio 1:100 in deionized water and incubated with 5, 5′-dithiobis (2-nitrobenzoic acid) 60 µM in 1 M TRIS buffer according to the method described by Tietze (1969). Kinetic curve was measured at 412 nm for 3 minutes in UV-vis spectrophotometer. The results were expressed in µM per g hemoglobin x 10^3.

Immunohistochemistry

The paraffin-embedded tumors were submitted to immunohistochemical analysis. Briefly, 3-µm-thick sections mounted on sylane-coated slides (Sigma Aldrich®) were deparaffinized, rehydrated, immersed in 10 mmol x L^{-1} citrate buffer, pH 6.0, and submitted to heat-induced epitope retrieval using a vapor lock for 45 min. The slides were rinsed with phosphate-buffered saline (PBS) and immersed in 3% hydrogen

peroxide for 20 min to block endogenous peroxidase. Non-specific protein binding was blocked with normal serum (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.®) for 30 min. The sections were then incubated with monoclonal primary antibodies specific for 3-nitrotyrosine 1:100 (clone HM11, Santa Cruz Biotechnology, Inc.®) for 2 hours at room temperature (25ºC) in a humid chamber. Following washes in PBS, biotinylated pan-specific universal secondary antibody (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.®) was applied for 30 min. Next, the slides were incubated with the avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.®) for 30 min and developed with NovaRed kit (Vector Laboratories Inc.®) for 5 min. The slides were counterstained by Giemsa, dehydrated and mounted with Permount (Biomeda®). As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. The immunolabeling was considered to be positive when distinct fuchsia staining was present homogenously. The percentage of positive cells was obtained blindly, for each case, by two of the authors (LZR and SSB) at ten representative higher-power field (×400). For statistical purposes, the samples were scored as follows: -, Nonstainer; +, Non-majority stainer (< 10% per field); ++, Stainer (10-50% per field); +++, Majority-stainer (> 50% per field).

Statistical analysis

Comparisons were performed as follows: Control x T1/T2 group and Control x T3/T4 group. All data are expressed as arithmetic means and standard errors of means

(SEM). The Shapiro–Wilk W test verified the normality of the data. For quantitative analysis, data with normal distribution were compared by t-test and data with non-normal distribution by Mann–Whitney test. The median of age in the groups was analyzed by One-way ANOVA with Tukey's post test. Pearson's correlation test was used in data with normal distribution and Spearman's correlation test in data with non-normal distribution. Qualitative data were measured by Chi-square (X^2) or Fisher's test. Differences were considered statistically significant when $p<0.05$. All the statistical analyses were performed using Graphpad Prism® version 5.0 (GraphPad Software).

RESULTS

Table I shows characteristics of studied subjects. There were no significant difference in age, gender, Fitzpatrick skin classification, surgical removal tumor time and location of tumor among groups. The Fitzpatrick Skin Phototype Classification (FSPC) is the most commonly used measure of skin type, and classifies a person's complexion and their tolerance of sunlight. In questionnaire-based surveys, self-rated FSPC is often used as a measure of respondents' skin type (Fitzpatrick, 1988). The patients with more invasive tumors (T3/T4 group) showed increased severe sunburn history, contrasting from control and T1/T2 groups. The histologic type of tumor differs statistically between melanoma groups, it being evident the predominance of Superficial Spreading Melanoma (SSM) type in T1/T2 group, and Nodular melanoma (NM) and Acral Lentiginous Melanoma (ALM) in T3/T4 group. As expected, Breslow thickness were lower in T1/T2 in relation to T3/T4 group. All p and X^2 values are presented in Table I.

Figure 1a shows increased CRP levels in T3/T4 group (T1/T2: 2.714±0.508 mg x L^{-1}, p=0.686; T3/T4: 5.553±1.150 mg x L^{-1}, p=0.0229; Control: 2.410±0.304 mg x L^{-1}) and no alterations in IL-1β (Figure 1b) (T1/T2: 8.626±1.147 pg x mL^{-1}, p=0.100; T3/T4: 7.205±1.203 pg x mL^{-1}, p=0.8255; Control: 8.339±1.254 pg x mL^{-1}) and TNF-α levels (Figure 1c) (T1/T2: 14.44±2.825 pg x mL^{-1}, p=0.4738; T3/T4: 19.03±3.612 pg x mL^{-1}, p=0.9081; Control: 19.36±2.508 pg x mL^{-1}). Erythrocyte antioxidant enzymes SOD (T1/T2: 7.376±0.479 U SOD / g Hb x 10^3, p=0.2730; T3/T4: 9.037±1.026 U SOD / g Hb x 10^3, p=0.9531; Control: 8.551±0.499 U SOD / g Hb x 10^3) and catalase (T1/T2: 10.23±0.603 Absorption/ min/ g Hb x 10^3, p=0.9097; T3/T4: 10.74±0.653 Absorption/ min/ g Hb x 10^3, p=0.6761; Control: 10.70±0.534 Absorption/ min/ g Hb x 10^3) no showed significant difference to respective control groups (Figures 1d and 1e). Figure 1f shows a decreased erythrocyte GSH levels in both melanoma groups (T1/T2: 80.27±6.836 μM x g Hb^{-1}, p=0.0006; T3/T4: 73.06±5.227 μM x g Hb^{-1}, p=0.0005) than in control group (169.3±23.02 μM x g Hb^{-1}).

Significantly higher levels of plasma MDA were found in both groups of melanoma patients in surveillance (T1/T2: 281.2±17.36 nM, p=0.0001; T3/T4: 377.9±36.15 nM, p<0.0001) than in control group (191.7±15.01 nM) (Figure 2a). Melanoma patients also presented increased plasma Thiol levels (T1/T2: 207.2±7.514 μM, p=0.0482; T3/T4: 217.5±11.39 μM, p=0.0097) than in control group (190.1±4.662 μM) (Figure 2b). In addition, gamma-glutamyltranspeptidase levels are increased in T3/T4 group compared to control group levels (T1/T2: 39.52±4.304 U x L^{-1}, p=0.1819; T3/T4: 53.44±8.105 U x L^{-1}, p=0.0211; Control: 33.82±2.954 U x L^{-1}) (Figure 2c). Figure

2d and 2e shows no significant difference in TRAP (T1/T2: 38.31±4.209 µM Trolox, p=0.118; T3/T4: 35.72±4.469 µM Trolox, p=0.1969; Control 30.38±2.70 µM Trolox) and uric acid levels (T1/T2: 6.445±0.174 mg x dL^{-1}, p=0.1787; T3/T4: 6.410±0.203 mg x dL^{-1}, p=0.1818; Control: 6.770±0.144 mg x dL^{-1}), although decreased TRAP:uric acid ratio was found in both melanoma groups in relation to control group (Figure 2f) (T1/T2: 5.90±0.6246, p=0.0480; T3/T4: 6.125±0.7130, p=0.0321; Control: 4.45±0.3504). Positive correlations were found in Breslow thickness x plasma MDA levels and Breslow thickness x serum CRP levels (See table 2).

The characterization of tumor samples used in immunohistochemical analysis was presented on Table 3. In the 28 samples analyzed, sixteen were classified as SSM, six as NM, four as ALM and two were not classified (NC). Ten of sixteen SSM had Breslow thickness < 2 mm, all NM had Breslow thickness > 2 mm and Breslow < 2 mm or > 2 mm had equal distribution in ALM. About 50% of SSM and NM (Nodular Melanoma) were positive for 3-nytrotyrosine, and 100% of ALM was positive for 3-nytrotyrosine. Figure 3 shows semi-quantitative Immunohistochemistry for 3-nytrotyrosine increased nitrated protein in high Breslow thickness cutaneous melanoma compared to thin Breslow samples, predominantly in collagen fibers on dermis (X^2 = 7.169; p = 0.0277) (Figure 3a and 3b). No statistical significant difference (X^2 = 0.566; p = 0.7536) was found when compared cutaneous melanoma with no recurrence in 3-years period and cutaneous melanoma with recurrence in 3-years period after surgical tumor removal (Figure 3c and 3d).

DISCUSSION

The difference found in primary tumor histological type and Breslow thickness among the melanoma groups was expected, once NM melanoma and ALM are more invasive. Together with red hair, numbers of nevi and hereditary melanoma, sun exposure is one of the risk factors for the disease development (Erdmann *et al*, 2012). The sun exposure as risk of melanoma is associated specifically with sunburn, and a history of severe sunburns mainly during childhood and adolescence increases significantly the risk (Erdmann *et al*, 2012; Gilcherest *et al*, 1999; Veierød *et al*, 2010). In this study, most of patients with melanoma reported severe sunburn history in these life periods.

Malignant melanoma is an explored cancer but it lacks the understanding when it concerns OS. It is know that OS is present in melanoma tissue and melanoma cells isolated from patients. Sander *et al* (2003) showed an increase of enzymatic antioxidant defenses, Cu-ZnSOD, Mn-SOD and catalase in melanoma skin biopsies. Furthermore, the higher the tumor invasion, more glutathione S-transferase activity was found in human melanoma cells, and alterations in SOD and catalase activities were involved in malignant transformation of melanocytes (Grammatico *et al*, 1998; Nogués *et al*, 2002).

Our findings showed that even after surgical removal of the primary tumor, an important systemic oxidative alteration in melanoma patients occurs, and that the higher Breslow thickness, higher is the 3-nytrotyrosine expression in tumoral tissue. Patients with melanoma have high systemic Mn-SOD levels in plasma, and as the disease advances, higher are these levels (Schadendorf *et al*, 1995). Furthermore, Gadjeva *et al* (2008) showed that the activity of erythrocyte antioxidant enzymes, catalase and SOD, increased and decreased, respectively, even after 20 days of melanoma surgical

removal, while plasmatic thiobarbituric acid-reactive substances (TBARS) was increased only before surgery.

Our results, on the other hand, showed that, in erythrocytes, SOD and catalase content did not change, and only GSH reduction was significant when compared to healthy volunteers. Similar GSH results were found in studies with breast cancer patients. While Yeh *et al* (2005) showed 60% decreased in erythrocytes GSH levels in all breast cancer patients studied Panis *et al* (2012a) found GSH reduction only in patients in early stages of disease. Furthermore, even after surgical tumor removal, patients with breast cancer showed a GSH decrease of about 90% in platelets (Kedzierska *et al*, 2012). In diseases associated with increased systemic production of ROS there were erythrocytes GSH depletion (Pace *et al*, 2003), and in this study, high increased plasma MDA levels supports the result found.

MDA is one of the most frequently used indicators of lipid peroxidation, and can be used as OS indicator. MDA is the final breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids (Bartsch and Nair, 2004; Halliwell and Gutteridge, 2007). Since pro-inflammatory status increased availability of polyunsaturated fatty acids, an increase in MDA levels was expect in inflammatory diseases, like cancer (Bartsch and Nair, 2004). Plasma thiol groups are represented mainly by proteins and by a smaller part from free-SH group containing substances (Halliwell and Gutteridge, 2007). Increased thiols in plasma of both melanoma groups can indicate a modulated response front enhanced MDA levels. Once gamma-glutamyltranspeptidase represents an important role for the intracellular re-synthesis of GSH, the enzyme enhancement on T3/T4 patients may occur as compensatory mechanism to decrease intracellular GSH or to correspond the enhanced extracellular

thiols levels (Lee *et al*, 2004). No alterations were found in TRAP and uric acid levels in cutaneous melanoma groups, despite TRAP:uric acid ratio was enhanced in both groups. Usually, uric acid is the major contributor to the total antioxidant capacity of plasma (Halliwell and Gutteridge, 2007), and the increase of this ratio indicates the presence of other low-molecular-weight antioxidants mobilized to plasma, such as thiol proteins, as shown.

An inflammatory biomarker on serum in cancer disease is CRP, which is synthesized by the liver following pro-inflammatory stimuli (Heikkila *et al*, 2007). Our results show increased serum CRP levels only in T3/T4 group, where Breslow thickness > 2 mm, supports the inflammatory status in these patients. Findeisen *et al* (2009) showed that CRP was related with high risk of melanoma recurrence, and combined with serum amyloid A protein, CRP increased prognostic impact in early-stage patients, helping to discriminate low-risk recurrence patients from high-risk recurrence patients. High plasma pro-inflammatory cytokines, like IL-1β and TNF-α, are found in untreated patients in all stages of the disease (Neagu *et al*, 2013; Yurkovetsky *et al*, 2007). In our study, no difference in plasma IL-1β and TNF-α were found in surgically treated patients. We found a moderate correlation in Breslow and CRP levels and a weak but statistically significant correlation in Breslow thickness and plasma MDA levels, indicating that the higher the primary tumors Breslow,higher is the systemic inflammation and OS.

To evaluate tumor Breslow influence in OS in tumoral tissue and the possible role in the melanoma recurrence, we also measured 3-nytrotyrosine by immunohistochemistry. 3-nytrotyrosine is a product from peroxynitrite ($ONOO^-$) - formed by nitric oxide ($NO^\bullet$) reaction with superoxide ($O_2^{\bullet-}$) – with tyrosine residues of proteins

(Halliwell and Gutteridge, 2007). Thus, the occurrence of 3-nytrotyrosine in tissues is a marker of peroxynitrite formation, therefore, the presence of $NO^{\bullet}$ and $O_2^{\bullet-}$ (Ischiropoulos *et al*, 1992). We found a increase of 3-nytrotyrosine expression in high than thin melanoma, but no there was no statistical significance when was compared to cutaneous melanoma with no recurrence in 3-years period and with recurrence in 3-years period. Furthermore, we observed enhanced 3-nytrotyrosine expression mainly in dermis in patients with Breslow > 2 mm, indicating elevated OS in tumor surrounding tissue. Moreover, the 100% (4/4) ALM tumors positive for 3-nytrotyrosine could be related with increased aggressiveness and poor prognosis of this melanoma histological type (Bello *et al*, 2013). Evidences points 3-nytrotyrosine is not expressed in normal skin, so, we believe that 3-nytrotyrosine non evaluation it is not a limitation for our results (Terra et al., 2012).

Melanoma cells are equipped with a high antioxidant capacity compared to cells of surrounding tissue, and might use their ability to generate ROS for damaging them and thus supporting tumour progression and metastasis (Meyskens *et al*, 1999; 2001; Sander *et al*, 2003). Although no statistical evidence was found in no recurrence and recurrence groups, increased ROS in surrounding tissue in high Breslow melanomas supports disease aggressiveness in patients with ticker tumors (Soong *et al*, 1992; 1998).

Up to date, it is the first report that relates alterations in systemic OS and related biomarkers according to primary tumor Breslow thickness, even after surgical removal of cutaneous melanoma. Furthermore, CRP was altered in high risk recurrence patients (Cutaneous melanoma Stage II; T3/T4 group), and together with MDA results correlates

positively with tumor thickness. We also showed increased OS in tumor environment and surrounding tissue in high Breslow thickness melanoma. Despite that no statistic correlation between 3-nytrotyrosine expression and tumor recurrence was found, this point deserves further studies for possible use of this oxidized protein as biomarker for melanoma prognosis, once it is more expressed in thick tumors.

We believe this is a new research area in cancer prognosis, once there are few reports of oxidative and inflammatory profile after tumor removal in cancer patients and their relationship with disease prognosis. Now that is showed OS involvement in patients with cutaneous melanoma, more robust studies are needed to understand the role of free radicals and their products in tumor recurrence mechanisms and aggressiveness.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aebi H (1984). Catalase in vitro. *In: Methods Enzymol 105* (Sies H, Kaplan N, Colowick N eds). Academic Press: California, 121 - 126.

Ambrosone CB, Ahn J, Singh KK, *et al* (2005). Polymorphisms in genes related to oxidative stress (MPO, MnSOD, CAT) and survival after treatment for breast cancer. *Cancer Res* 65: 1105 - 1111.

Balch CM, Gershenwald JE, Soong SJ, *et al* (2009). Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 27: 6199 – 61206, doi: 10.1200/JCO.2009.23.4799

Bartsch H, Nair J (2004). Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis. *Cancer Detect Prev* **28**: 385 - 391.

Bello DM, Chou JF, Panageas KS, *et al* (2013). Prognosis of acral melanoma: a series of 281 patients. *Ann Surg Oncol* 20: 3618 – 3625, doi: 10.1245/s10434-013-3089-0

Breslow A (1970). Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg* 172: 902 - 908.

de Cavanagh EM, Honegger AE, Hofer E, *et al* (2002). Higher oxidation and lower antioxidant levels in peripheral blood plasma and bone marrow plasma from advanced cancer patients. *Cancer* 94: 3247 -3251.

Dummer R, Hauschild A, Jost L, *et al* (2008). Cutaneous malignant melanoma: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* Suppl 2: ii86 – 88, doi: 10.1093/annonc/mdn100

Erdmann F, Lortet-Tieulent J, Schüz J, *et al* (2013). International trends in the incidence of malignant melanoma 1953-2008--are recent generations at higher or lower risk? *Int J Cancer* 132: 385 – 400, doi: 10.1002/ijc.27616

Findeisen P, Zapatka M, Peccerella T, *et al* (2009). Serum amyloid A as a prognostic marker in melanoma identified by proteomic profiling. *J Clin Oncol* 27: 2199 – 208, doi: 10.1200/JCO.2008.18.0554

Fitzpatrick TB (1988). The validity and practicality of sun-reactive skin types I through VI. *Arch Dermatol* 124: 869 - 871.

Gadjeva V, Dimov A, Georgieva N (2008). Influence of therapy on the antioxidant status in patients with melanoma. *J Clin Pharm Ther* 33: 179 – 85, doi: 10.1111/j.1365-2710.2008.00909.x

Gago-Dominguez M, Jiang X, Castelao JE (2007). Lipid peroxidation, oxidative stress genes and dietary factors in breast cancer protection: a hypothesis. *Breast Cancer Res* 9: 201.

Garbe C, Eigentler TK (2007). Diagnosis and treatment of cutaneous melanoma: state of the art 2006. *Melanoma Res* 17: 117 - 127.

Gerber M, Astre C, Ségala C, *et al* (1997). Tumor progression and oxidant-antioxidant status. *Cancer Lett* 114: 211- 214.

Grammatico P, Maresca V, Roccella F, *et al* (1998). Increased sensitivity to peroxidizing agents is correlated with an imbalance of antioxidants in normal melanocytes from melanoma patients*. Exp Dermatol* 7: 205 - 212.

Gupta A, Bhatt ML, Misra MK (2009). Lipid peroxidation and antioxidant status in head and neck squamous cell carcinoma patients. *Oxid Med Cell Longev* 2: 68 - 72.

Halliwel Bl, Gutteridge JMC (2007). Free Radicals in Biology and Medicine. Oxford University Press: Oxford, 704 pp.

Heikkila K, Ebrahim S, Lawlor DA (2007). A systematic review of the association between circulating concentrations of C reactive protein and cancer. *J Epidemiol Community Health* **61**: 824 - 833.

Hohnheiser AM, Gefeller O, Göhl J, *et al* (2011). Malignant melanoma of the skin: long-term follow-up and time to first recurrence. *World J Surg* **35**: 580 – 589, doi: 10.1007/s00268-010-0859-8

Hu ML (1994). Measurement of protein thiol groups and GSH in plasma. *In: Methods Enzymol 233* (Sies H, Abelson J, Simon M eds). Academic Press: California, 380 – 385.

Ischiropoulos H, Zhu L, Chen J, *et al* (1992). Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys* 298: 431 - 437.

Jones DP. Redefining oxidative stress (2006) *Antioxid Redox Signal* 8:1865-1879.

Kedzierska M, Olas B, Wachowicz B, *et al* (2012). Effects of the commercial extract of aronia on oxidative stress in blood platelets isolated from breast cancer patients after the surgery and various phases of the chemotherapy. *Fitoterapia* 83: 310 – 317.

Korn EL, Liu PY, Lee SJ, *et al* (2008). Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. *J Clin Oncol* 26: 527 – 534.

Lee DH, Blomhoff R, Jacobs DR Jr (2004). Is serum gamma glutamyltransferase a marker of oxidative stress? Free Radic Res 38: 535 - 539.

Mantovani G, Macciò A, Madeddu C, *et al* (2002). Quantitative evaluation of oxidative stress, chronic inflammatory indices and leptin in cancer patients: correlation with stage and performance status. *Int J Cancer* 98: 84 - 91.

Marklund S, Marklund G (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 47: 469 - 474.

Martin RC, Ahn J, Nowell SA, *et al* (2006). Association between manganese superoxide dismutase promoter gene polymorphism and breast cancer survival. *Breast Cancer Res* 8: R45.

Meyskens FL Jr, Buckmeier JA, McNulty SE, *et al* (1999). Activation of nuclear factor-kappa B in human metastatic melanoma cells and the effect of oxidative stress. *Clin Cancer Res* 5:1197-1202.

Meyskens FL Jr, McNulty SE, Buckmeier JA, *et al* (2001). Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. *Free Radic Biol Med* 31: 799 - 808.

Neagu M, Constantin C, Zurac S (2013). Immune parameters in the prognosis and therapy monitoring of cutaneous melanoma patients: experience, role, and limitations. *Biomed Res Int* 2013: 107940.

Nogués MR, Giralt M, Cervelló I, *et al* (2002). Parameters related to oxygen free radicals in human skin: a study comparing healthy epidermis and skin cancer tissue. *J Invest Dermatol* 119: 645 - 652.

Pace BS, Shartava A, Pack-Mabien A, *et al* (2003). Effects of N-acetylcysteine on dense cell formation in sickle cell disease. *Am J Hematol* 73: 26 - 32.

Panis C, Herrera AC, Victorino VJ, *et al* (2012a). Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. *Breast Cancer Res Treat* 133: 89 - 97.

Panis C, Victorino VJ, Herrera AC, *et al* (2012b). Differential oxidative status and immune characterization of the early and advanced stages of human breast cancer. *Breast Cancer Res Treat* 133: 881 - 888.

Repetto M, Reides C, Gomez Carretero ML, *et al* (1996). Oxidative stress in blood of HIV infected patients. *Clin Chim Acta* 255: 107 - 117.

Reuter S, Gupta SC, Chaturvedi MM, *et al* (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 49: 1603-1616. doi: 10.1016/j.freeradbiomed.2010.09.006

Rychetnik L, Morton RL, McCaffery K, *et al* (2012). Shared care in the follow-up of early-stage melanoma: a qualitative study of Australian melanoma clinicians' perspectives and models of care. *BMC Health Serv Res* 12: 468.

Saintot M, Astre C, Pujol H, *et al* (1996). Tumor progression and oxidant-antioxidant status. Carcinogenesis 17: 1267 - 1271.

Saintot M, Mathieu-Daude H, Astre C, *et al* (2002), Oxidant-antioxidant status in relation to survival among breast cancer patients*. Int J Cancer* 97: 574 - 579.

Salzman R, Pácal L, Kaňková K, *et al* (2010). High perioperative level of oxidative stress as a prognostic tool for identifying patients with a high risk of recurrence of head and neck squamous cell carcinoma. *Int J Clin Oncol* 15: 565 – 570.

Sander CS, Hamm F, Elsner P, *et al* (2003). Oxidative stress in malignant melanoma and non-melanoma skin cancer. *Br J Dermatol* 148: 913 - 922.

Schadendorf D, Zuberbier T, Diehl S, *et al* (1995). Serum manganese superoxide dismutase is a new tumour marker for malignant melanoma. *Melanoma Res* 5: 351 - 353.

Soong SJ, Shaw HM, Balch CM, *et al* (1992). Predicting survival and recurrence in localized melanoma: a multivariate approach*. World J Surg* 16: 191 - 195.

Soong SJ, Harrison RA, McCarthy WH, *et al* (1998). Factors affecting survival following local, regional, or distant recurrence from localized melanoma. *J Surg Oncol* 67: 228 - 233.

Terra VA, Souza-Neto FP, Pereira RC, *et al* (2012). Nitric oxide is responsible for oxidative skin injury and modulation of cell proliferation after 24 hours of UVB exposures. *Free Radic Res* 46: 872-882.

Tietze F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 27: 502 - 522.

Veierød MB, Adami HO, Lund E, *et al* (2010). Sun and solarium exposure and melanoma risk: effects of age, pigmentary characteristics, and nevi. *Cancer Epidemiol Biomarkers Prev* 19: 111 – 120.

Victorino VJ, Panis C, Campos FC, *et al* (2012). Decreased oxidant profile and increased antioxidant capacity in naturally postmenopausal women. *Age (Dordr)* 35: 1411 – 1421, doi: 10.1007/s11357-012-9431-9

Yeh CC, Hou MF, Tsai SM, *et al* (2005). Superoxide anion radical, lipid peroxides and antioxidant status in the blood of patients with breast cancer. *Clin Chim Acta* 361: 104 - 111.

Yurkovetsky ZR, Kirkwood JM, Edington HD, *et al* (2007). Multiplex analysis of serum cytokines in melanoma patients treated with interferon-alpha2b. *Clin Cancer Res* 13: 2422 - 248.

LEGENDS OF FIGURES

Figure 1. (a) Plasma C-reactive protein levels; (b) Plasma IL-1β levels; (c) Plasma TNF-α levels; (d) Erythrocyte superoxide dismutase; (e) Erythrocyte catalase; and (f) Erythrocyte GSH levels in cutaneous melanoma patients after surgical tumor removal. Results are expressed in mean ± SEM. T1: Breslow thickness < 1.0 mm; T2: Breslow thickness 1.01-2.0 mm; T3: Breslow thickness 2.01-4 mm; T4: Breslow thickness > 4 mm. Groups were compared with control group by Mann-Whitney test.* $p<0.05$.

Figure 2. (a) Plasma malondialdehyde levels; (b) Plasma Thiol levels; (c) Plasma gamma-glutamyl transferase; (d) Plasma Total radical antioxidant parameter levels; (e) Plasma Uric-acid levels; and (f) Total radical antioxidant parameter and Uric-acid ratio in cutaneous melanoma patients after surgical tumor removal. mean ± SEM. T1: Breslow thickness < 1.0 mm; T2: Breslow thickness 1.01-2.0 mm; T3: Breslow thickness 2.01-4 mm; T4: Breslow thickness > 4 mm. Groups were compared with control group by t-test in B, D, E, F and by Mann-Whitney in A and C analysis.* $p<0.05$.

Figure 3. Immunohystochemistry analysis of 3-nytrotyrosine in cutaneous melanoma tumors. (a) 3-nytrotyrosine in cutaneous melanoma with Breslow < 2 mm, case 13; (b) 3-nytrotyrosine in cutaneous melanoma with Breslow > 2 mm, case 18; (c) 3-nytrotyrosine in cutaneous melanoma with no recurrence in 3 years period, case 23; (d) 3-nytrotyrosine in cutaneous melanoma with recurrence in 3 years period, case 22. Data were compared by X^2 test.* $p<0.05$.

TABLES

Table 1. Characteristics of studied subjects in systemic oxidative stress and related biomarkers evaluation

	Control (n=50)	**T1/T2 (n=24)**	**T3/T4 (n=19)**	***p value***
Age (mean ± SEM)	54.5±1.8	54.9±2.4	55.7±3.2	0.9236
Gender				0.1033
Male	11 (22.0%)	6 (25.0%)	9 (47.4%)	
Female	39 (78.0%)	18 (75.0%)	10 (52.6%)	
Fitzpatrick skin classification				0.6085
I/II	15 (30.0%)	5 (20.8%)	4 (21.0%)	
III/IV/V	35 (70.0%)	19 (79.2%)	15 (79.0%)	
Severe sunburn history				0.0018*
No	29 (58.0%)	10 (41.7%)	2 (10.5%)	
Yes	21 (42.0%)	14 (58.3%)	17 (89.5%)	
Surgical removal tumor time				0.1161
< 2 years	-	13 (54.2%)	15 (78.9%)	
2 – 5 years	-	11 (45.8%)	4 (21.1%)	
Breslow (mean±SEM)		1.16±0.092	4.14±0.43	<0.0001*
Histological type				0.0173*
SSM	-	20 (83.3%)	8 (42.1%)	
NM	-	2 (8.35%)	7 (36.8%)	
ALM	-	2 (8.35%)	4 (21.1%)	

Location				0.7155
Lower extremities	-	7 (24.1%)	7 (36.8%)	
Upper extremities	-	4 (16.7%)	5 (26.3%)	
Trunk	-	9 (37.5%)	5 (26.3%)	
Head/neck	-	4 (16.7%)	2 (10.6%)	

Quantitative data were evaluated by Chi-square or Fisher's test, and quantitative data by One-way ANOVA test with Tukey's *post test* or t'test. $p < 0.05$ was considered significant. X^2 values: gender 2.571; Fitzpatrick skin classification 0.993; Severe sunburn history: 12.66; Histogenetic type: 8.116; Location: 1.358. SSM: Superficial spreading melanoma; NM: Nodular melanoma; ALM: Acral lentiginous melanoma. Breslow thickness was measured in millimeters.

Table 2. Correlation of Breslow thickness and oxidative stress parameters in cutaneous melanoma patients

Correlation	R-value	p-value
Breslow thickness x C-reactive protein	0.3604	0.0191*
Breslow thickness x MDA	0.3176	0.0404*
Breslow thickness x Thiol	0.0149	0.9432
Breslow thickness x TRAP : Uric-acid	0.0302	0.8477

MDA: malondialdehyde. TRAP: Total radical antioxidant parameter. * Statistical significant difference ($p < 0.05$).

Table 3. Cutaneous melanoma tumors characteristics and 3-nitroyrosine (3-NT) immunohystochemistry score

No	Histogenetic type	Breslow tickness	3-NT	Recurrence (3 years)
1	SSM	1.5	-	Yes
2	NC	1.5	++	No
3	NC	1.0	-	Yes
4	SSM	1.1	-	No
5	SSM	1.5	-	No
6	SSM	0.5	-	No
7	SSM	1.1	+	No
8	ALM	1.5	+	ND
9	SSM	0.7	-	ND
10	SSM	1.0	-	ND
11	ALM	1.2	+	ND
12	SSM	1.5	-	No
13	SSM	1.1	-	Yes
14	SSM	1.0	-	ND
15	SSM	15.0	+	Yes
16	NM	13.0	+	Yes
17	NM	5.1	-	Yes
18	SSM	3.0	++	Yes
19	NM	13.0	++	Yes
20	NM	3.0	++	Yes
21	NM	7.5	-	Yes
22	NM	6.5	-	Yes

23	SSM	4.0	++	No
24	SSM	3.0	+	No
25	SSM	2.0	+	No
26	ALM	3.5	+	Yes
27	ALM	3.0	+	ND
28	SSM	2.5	+	No

ALM: Acral lentiginous Melanoma; NM: Nodular Melanoma; SSM: Superficial Spreading Melanoma; NC: Not classified; ND: Not determined, tumor with < 3 years of surgery; -: Nonstainer; +: Non-majority stainer; ++: Stainer; +++: Majority-stainer.

FIGURES

Figure 1.

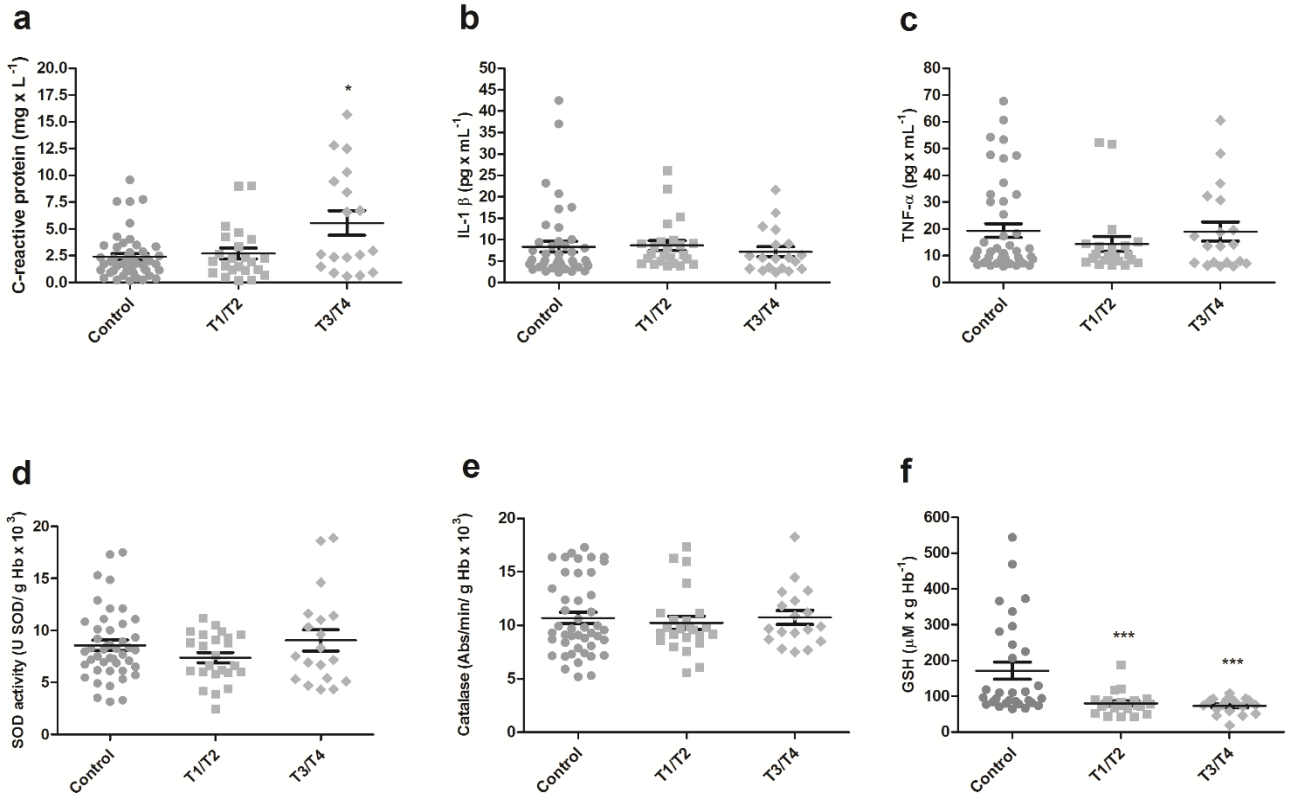
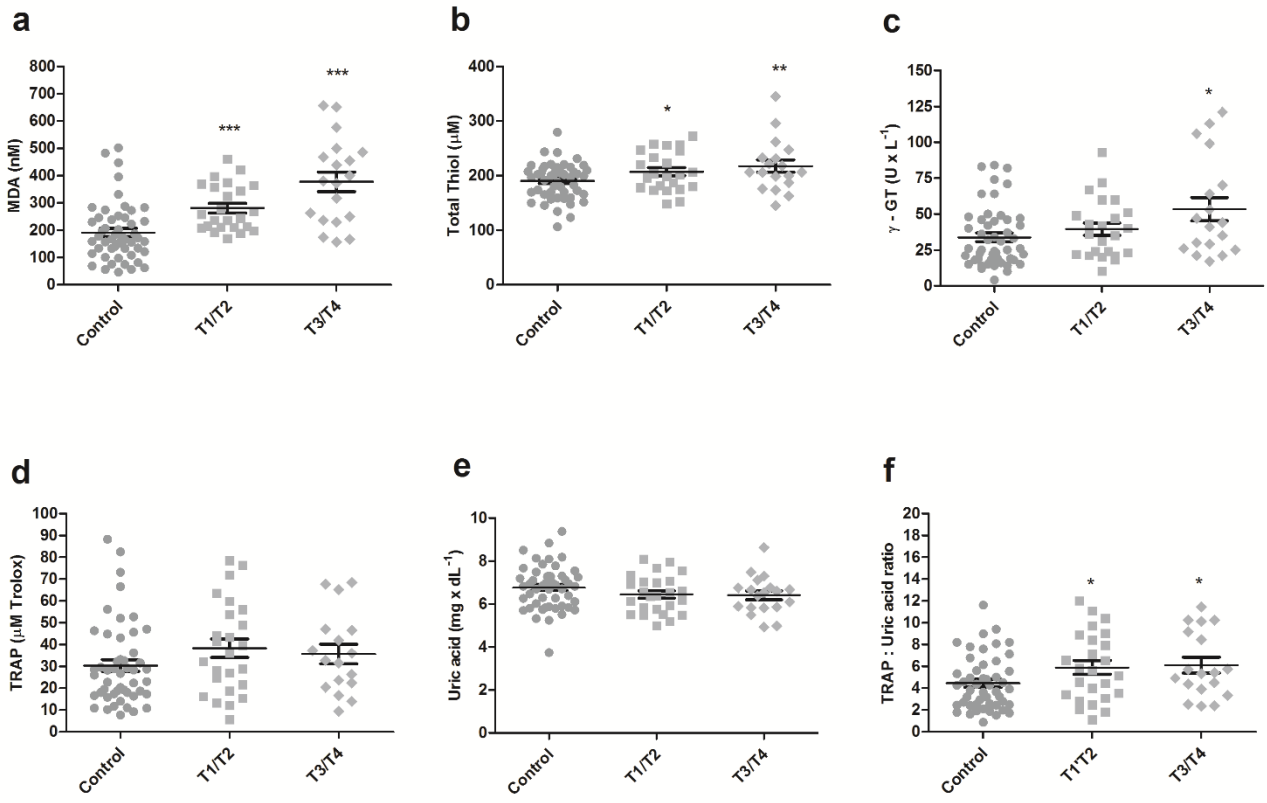


Figure 2.


a
MDA (nM)
800
700
600
500
400
300
200
100
0
***
***
Control
T1/T2
T3/T4
b
Total Thiol (μM)
400
300
200
100
0
*
**
Control
T1/T2
T3/T4
c
γ - GT (U x L-1)
150
125
100
75
50
25
0
*
Control
T1/T2
T3/T4
d
TRAP (μM Trolox)
100
90
80
70
60
50
40
30
20
10
0
Control
T1/T2
T3/T4
e
Uric acid (mg x dL-1)
10
8
6
4
2
0
Control
T1/T2
T3/T4
f
TRAP : Uric acid ratio
20
18
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12
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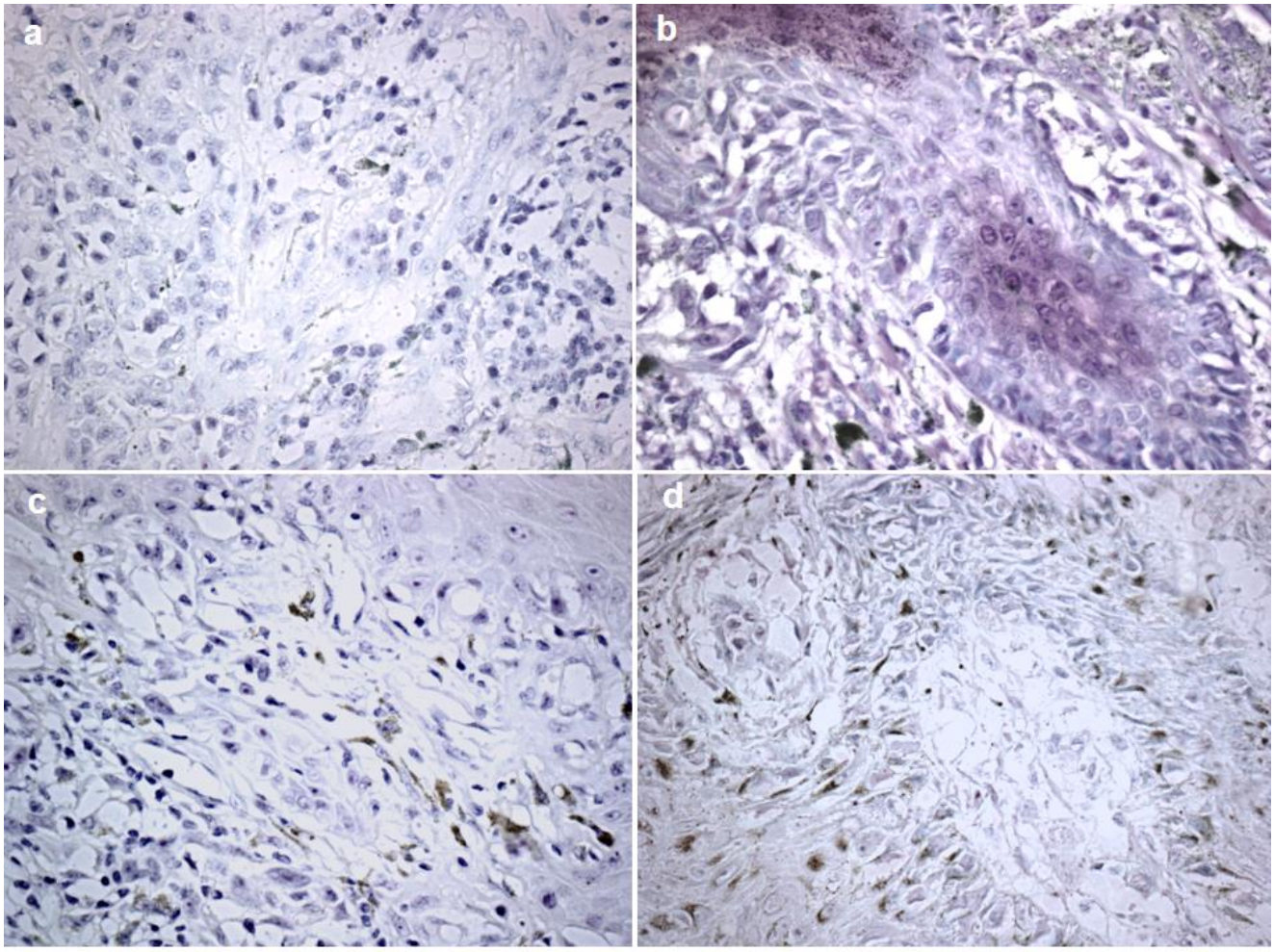
Control
T1/T2
T3/T4

Figure 3.



ANEXO B

Lipid peroxidation, ferritin-heavy-chain levels and p53 expression in patients with melanoma

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Title: Lipid peroxidation, ferritin-heavy-chain levels and p53 expression in patients with melanoma

Article Type: Original Research Paper

Keywords: Melanoma; Ferritin-heavy-chain; Oxidative stress; p53; cancer

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Lipid peroxidation, ferritin-heavy-chain levels and p53 expression in patients with melanoma

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ABSTRACT

Objective: Verify the influence of serum ferritin-heavy chain (FHC) in systemic oxidative stress in patients in different clinical stages of melanoma, and in p53 expression in primary and metastatic tumors. **Methods:** 57 patients were divided in Control; Cutaneous (< 2 mm) - Low recurrence risk; Cutaneous (> 2 mm) - High recurrence risk; and Recurrence – Metastatic disease. Systemic oxidative stress was evaluated by highly sensitive chemiluminescence induced by tert-butyl hydroperoxide in erythrocytes, and ferritin-light chain (FLC) and FHC in serum by ELISA. FHC is a p53 regulated gene, and in addition to FHC we also evaluated p53 expression in 49 cutaneous and metastatic melanoma tumors by immunohistochemistry. Not all tumors belonged to the patients analyzed in the first step of this study. Tumors were divided in Cutaneous < 2 mm, Cutaneous > 2 mm and Metastasis groups. **Results:** FHC is increased in serum of high risk recurrence and recurrence melanoma patients, and all patients showed increased serum FHC: FLC ratio compared to Control. The increase FHC in serum was accompanied by increase in systemic oxidative stress. Increased p53 expression in metastasis compared to Cutaneous < 2mm melanoma was found, although there was no statistical difference when analyzed nuclear p53 positivity. FHC was expressed in different stages of human melanoma tumor, but the groups were equal among them. **Conclusions:** High risk recurrence and recurrence groups showed similar pattern, indicating that FHC, oxidative stress and cell p53 expression may influence in melanoma progression and prognosis. Probable mechanisms involved deserves further investigation.

Key-words: Melanoma; Ferritin-heavy-chain; Oxidative stress; p53; Neoplasm.

Introduction

Serum markers related with the risk of recurrence in cutaneous localized melanoma patients is still poorly understood. Ferritin is an important iron-binding protein, formed by 24 subunits and consists of heavy chain (FHC) and light chain (FLC) [1]. The proportion of each of them varies in each tissue. Increased total ferritin levels in tumoral tissue were reported in several cancer types, such as colon cancer, breast cancer, and seminoma [3]. Ferritin is a key protein of iron metabolism that is capable of sequestering large amounts of iron, and thus serves the dual function of iron detoxification and iron storage [2]. Furthermore, intracellular FHC has an important ferroxidase activity, that play a role in rapid iron detoxification, as ferroxidase activity oxidizes iron to Fe(III) decreasing hydroxyl radical (•OH) formation by Fenton reaction, diminishing oxidative stress [4].

It has been observed that the frequency of p53 mutations in melanocytic tumors ranges from 5-25%, which is considerably lower than other types of cancers. Mutational inactivation of p53 in melanoma is uncommon and wild-type p53 is frequently expressed at high levels [5,6]. Recently, Avery-Kiejda et al [7] showed that the ability of p53 to regulate genes involved in the cell cycle was significantly reduced in melanoma cells. FHC gene is regulated by the p53 gene, and *in vitro*, the overexpression of p53 induced by reactive oxygen species (ROS) is able to repress FHC endogenous gene diminishing protein levels [8].

Extracellular (serum) total ferritin is composed mostly, but not exclusively, of FLC, and is relatively iron-poor [9, 10]. The source and detailed secretory pathway of total serum ferritin are not completely understood, but is well established that hepatocytes, macrophages and Kupffer cells are able to secrete ferritin [11]. As an acute-phase protein, its expression is upregulated in inflammation, infection, and

malignancies. Increased total serum ferritin was related to bad prognosis in breast cancer, melanoma, childhood Hodgkin's disease, squamous cell carcinoma of the head and neck and primary lung cancer [12, 13, 14, 15, 16]. Furthermore, it has been showed that ROS are able to induce intracellular total ferritin synthesis [17, 18]. Although tumor ferritin is an important protecting factor against oxidative stress, the FHC subtype inhibits cell death by TNF-α induced-apoptosis, through ROS suppression [19]; however there is no evidence about the total extracellular ferritin [17, 18].

Some melanoma cells release predominantly FHC, whereas others contained more equal proportions of FHC and FLC [20]. In a study with primary melanoma culture cells, Baldi et al. [21] showed that melanoma metastases display high levels of FLC expression when compared with their corresponding primary melanomas. Furthermore, elevated total serum ferritin levels, compared with healthy volunteers, were reported in melanoma patients with progressive metastatic disease [14].

These findings suggest a potential relationship between FHC, oxidative stress and p53 in malignant melanoma. Our aim in this work was to associate the serum FHC with systemic oxidative stress in patients in different clinical stages of melanoma. Since FHC is regulated by p53 gene, we also evaluate FHC relationship with p53 expression in primary and metastatic tumors, that belonged to patients participants of systemic study or not.

Materials and methods

Sample collection and study design

A total of 57 melanoma patients between 28 to 71 years old were recruited at the Londrina Cancer Hospital between April 2011 and November 2012. Twenty-four patients had localized primary cutaneous melanoma stage I, considered with low recurrence risk according the Breslow thickness (Cutaneous < 2 mm); Nineteen patients had localized cutaneous melanoma stage II, considered high recurrence risk according the Breslow thickness (Cutaneous > 2 mm), both with surgical tumor removal between 6 months and 5 years; six patients had surgical metastasis removal, and eight patients had active metastasis, all without chemotherapy or immunotherapy (Recurrence group, n=14). Control group comprised 30 healthy women and men volunteers. The characteristics of the sample included in the study are outlined in Table I. This study was approved by the Research and Ethics National Council (CAAE 5831.0.000.268.10), all the practices were approved by the institutional board, and all patients and controls signed informed consent. Patients and controls were non-smoking or in antioxidant therapy treatment, not obese and did not present hepatic, cardiac or renal dysfunction. In addition, control subjects had no previous history of any type of cancer. Venous blood was collected with and without heparin and centrifuged at 1,100 *x g*. Separated serum was immediately frozen at -80°C until biochemical or enzyme immunoassay (ELISA) analysis and erythrocytes used freshly to access erythrocyte chemiluminescence. To assess the presence of FHC and p53 in cutaneous and metastatic melanoma samples, 49 randomized paraffin-embedded tumor blocks were obtained from database of the Department of Pathology of Londrina Cancer Hospital (See Table II). Not all tumors belonged to the patients analyzed in the first step of this study. Here, comparisons were performed as follows: Cutaneous < 2 mm x Metastasis and Cutaneous > 2 mm x Metastasis.

Serum ferritins analysis

Serum ferritin-heavy chain and ferritin light-chain levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturers' instructions (Uscn life science Inc®) and analyzed with a microplate reader at 450 nm. Standard curves were constructed by a five-parameter regression formula and plotted as a linear curve (log-log). The Lower Limit of Detection of these assays was less than 5.5 pg x mL^{-1}l and 14.7 pg x mL^{-1}, respectively, and the results were calculated in pg x mL^{-1}.

Determination of oxidative stress by highly sensitive chemiluminescence induced by tert-butyl hydroperoxide

Systemic lipoperoxidation was evaluated in erythrocytes by chemiluminescence (CL). Erythrocytes were obtained from heparinized blood and washed three times with 0.9% saline solution at 4ºC. An aliquot was used to determine hemoglobin (Coulter STKS®, Hialeah, USA). Erythrocytes lipoperoxidation was evaluated according Panis et al. [22]. Briefly, 30 µl of packed erythrocytes was added to 3 ml of phosphate buffer, and 1 ml of this solution was diluted in 12.3 ml of the same buffer. The chemiluminescent reaction was initiated by the addition of tert-butyl (10 µl) at a final concentration of 3 mM in 1 mL of erythrocytes samples. Chemiluminescence curves were obtained in a GloMax® luminometer 20/20 (Promega, Madison, USA), and the results are expressed in relative light units (RLU) x g Hb^{-1} or area under curve (AUC) as median ± SEM. The obtained curve was used as a qualitative indicator of lipoperoxidation, and quantitative results were

obtained after area under curve integration using Graphpad Prism® version 5.0 (GraphPad Software).

Immunohistochemistry

The paraffin-embedded tumors were submitted to immunohistochemical analysis. Briefly, 3-µm-thick sections mounted on sylane-coated slides (Sigma Aldrich®) were deparaffinized, rehydrated, immersed in 10 mmol x L^{-1} citrate buffer, pH 6.0, and submitted to heat-induced epitope retrieval using a vapor lock for 45 min. The slides were rinsed with phosphate-buffered saline (PBS) and immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Non-specific protein binding was blocked with normal serum (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.®) for 30 min. The sections were then incubated with monoclonal primary antibodies specific for FHC (Dilution 1:200, clone H-53, Santa Cruz Biotechnology, Inc.®) and p53 (Dilution 1:100, clone DO-7, Santa Cruz Biotechnology, Inc.®) for 2 hours at room temperature (25ºC) in a humid chamber. Following washes in PBS, biotinylated pan-specific universal secondary antibody (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.®) was applied for 30 min. Next, the slides were incubated with the avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.®) for 30 min and developed with NovaRed kit (Vector Laboratories Inc.®) for 5 min. The slides were counterstained by Giemsa, dehydrated and mounted with Permount (Biomeda®). As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. The immunolabeling was considered to be positive when distinct fuchsia staining was present homogenously. The percentage of positive cells was obtained blindly, for each case, by two of the authors (LZR and

SSB) at ten representative higher-power field (×400). For statistical purposes, the samples were scored as follows: -, Nonstainer; +, Non-majority stainer (< 10% per field); ++, Stainer (10-50% per field); +++, Majority-stainer (> 50% per field). p53 expression in nuclei is considered positive (>10% of cell nuclei positively stained) and negative (< 1% of cell nuclei positively stained) according to Purdue et al [23]. Comparisons were performed as follows: cutaneous < 2 mm x metastasis; and cutaneous > 2 mm x metastasis.

Statistical analysis

Comparisons were performed as follows: Control x Cutaneous < 2 mm group; Control x Cutaneous > 2 mm group; and Control x Recurrence group. All data are expressed as means and standard errors of means (SEM). The Shapiro–Wilk W test verified the normality of the data. For quantitative analysis, data with normal distribution were compared by t-test and data with non normal distribution by Mann–Whitney test. The mean of age and Breslow thickness in the groups were analyzed by One-way ANOVA with Tukey's post test. CL curves were analyzed by Two-way ANOVA test. Qualitative data were measured by Chi-square (X^2) test. Differences were considered statistically significant when $p<0.05$. All the statistical analyses were performed using Graphpad Prism® version 5.0 (GraphPad Software).

Results

Table 1 shows characteristics of studied subjects. There were no significant difference in age, gender and Fitzpatrick skin classification among groups. The Fitzpatrick Skin Phototype Classification (FSPC) is the most commonly used measure of skin type, and classifies a person's complexion and their tolerance of

sunlight. In questionnaire-based surveys, self-rated FSPC is often used as a measure of respondents' skin type [24]. The histological type of primary tumor differs statistically among melanoma groups, it being evident the predominance of Superficial Spreading Melanoma (SSM) type in cutaneous tumors with Breslow thickness less 2 mm. Breslow thickness were lower in Cutaneous < 2 mm in relation to groups Cutaneous > 2 mm and Recurrence, as expected. All p and X^2 values are presented in Table 1 and its legend.

Figure 1A shows increased FHC in Cutaneous (> 2 mm) and Recurrence groups in relation to Control group (Control: 238.9±20.94 pg x mL^{-1}; Cutaneous (< 2 mm): 336.2±40.89 pg x mL^{-1}, p=0.123; Cutaneous (> 2 mm): 379.6±79.30 pg x mL^{-1}, p=0.0430; Recurrence: 445.1±78.51 pg x mL^{-1}, p=0.0025). No significant alterations were found in FLC (Control: 247.3±27.02 pg x mL^{-1}; Cutaneous (< 2 mm): 206.6±31.98 pg x mL^{-1}, p=0.1542; Cutaneous (> 2 mm): 207.4±62.74 pg x mL^{-1}, p=0.5105; Recurrence: 228.9±5924 pg x mL^{-1}, p=0.2775) (Figure 1B). However, FHC:FLC ratio was enhanced in all melanoma groups (Control: 1.136±0.08; Cutaneous (< 2 mm): 2.897±0.55, p=0.0292; Cutaneous (> 2 mm): 3.972±1.34, p=0.0136; Recurrence: 4.027±0.98, p=0.0101) (Figure 1C). CL curves showed significant difference between curves (p<0.001), in the peak height and in the curve rise time in Cutaneous (> 2 mm) and Recurrence groups (Figure 1D). The increased AUC was showed in Figure 1E, where high values were found in cutaneous (> 2 mm) and Recurrence groups in relation to Control group (Control: 60.990±2.986; Cutaneous (< 2 mm): 58.110±3.010, p=0.5019; Cutaneous (> 2 mm): 70.390±2.992, p=0.0344; Recurrence: 77.530±8.520, p=0.0292 RLU x Hb^{-1}).

The characterization of tumors used in immunohistochemical analysis is presented in Table 2. Figure 2A, 2B and 2C shows increased p53 expression in

metastasis cells in relation to cutaneous tumor with Breslow < 2 mm (X^2=7.266; p= 0.0264). Metastasis compared to Breslow > 2 mm had no statistical difference (X^2=0.4362; p= 0.8040). In the positive nuclei staining evaluation by Fisher test, no statistical difference was found in Breslow < 2 mm x Metastasis (p=0.0538) and Breslow > 2 mm x Metastasis (p=0.7303). Figure 2D, 2E and 2F shows no alterations found in FHC expression in metastatic tumors compared to Breslow < 2 mm (X^2=1.2428; p= 0.4897) and with Breslow > 2 mm (X^2=0.9037; p= 0.6365), although melanoma tumors showed different degrees of FHC expression (See Table 3).

Discussion

The difference found in primary tumor histological type and Breslow thickness among the melanoma groups was expected, as nodular melanoma and acral lentiginous melanoma are more invasive. The fact that Cutaneous > 2 mm group had similar Breslow values to the Recurrence group reinforce the highest risk recurrence shown by high Breslow thickness [25].

The role of ferritin in cancer is still poorly understood. It is known that in inflammatory states, cytokine stimulation of macrophages also induces serum FHC release [2, 26, 27]. Serum is rich in FLC type, and the alterations in FHC and FLC proportion seems to have immunosuppressive role in cancer. Gray, Arosio & Hersey [28] showed increase in FHC:FLC ratio in plasma of melanoma patients, and that it induced immunosuppressive responses for regulatory T cells proliferation. An interesting result is that plasma patients FHC:FLC ratio is high in all stages of the disease. In our study, we showed a significant enhancement of systemic FHC levels only in Cutaneous (> 2 mm) and Recurrence groups, though the FHC:FLC ratio were increased in all melanoma groups. It is well known that cancer patients often exhibit

an elevated level of total serum ferritin usually correlated with a poor prognosis [29, 30]. This is also true for human melanoma, where increased total serum ferritin concentrations are associated with progressive metastatic disease [11].

Together with serum FHC enhancement levels, systemic lipid peroxidation, evaluated in erythrocytes by CL, was increased in Cutaneous (> 2 mm) and Recurrence groups. Tert-butyl hydroperoxide-initiated CL was used to evaluate integral level of nonenzymatic antioxidant defense. Lower level of antioxidant as consequence of previous oxidative stress corresponds to an accentuated high and a shift to the left in CL curves, giving a positive correlation between CL and lipid peroxidation [31, 32]. In this study, we demonstrated a qualitative difference in all CL curves, indicating alterations in cell membrane properties, as antioxidant composition, which modifies the kinetics pattern by exhausting the oxidizable species [33]. The quantitative analysis of lipid peroxidation by CL confirms these changes, represented by AUC increased values in Cutaneous (> 2 mm) and Recurrence groups in relation to Control.

Despite that no studies have been made about serum FHC and oxidative stress in melanoma patients, recently it was showed an enhancement in systemic MDA, a lipid peroxidation biomarker, and total serum ferritin levels in patients with multiple myeloma and breast cancer [22, 34]. Systemic oxidative stress status is poorly explored in human melanoma, and there are some evidences that it has an important influence in the disease prognosis [35, 36]. Here we showed increased systemic lipid peroxidation and enhancement of serum FHC levels only in subjects with high risk of disease recurrence (Breslow > 2 mm) and in recurrence groups. As total ferritin, mainly FHC, increases in pro-oxidant conditions, we assume that

systemic oxidative stress in advanced stages of melanoma can induce FHC enhancement and probably are connected to the bad prognosis.

We evidenced the increased FHC levels in serum of patient´s with high Breslow or metastasis, but not in the tumor tissue. Tumor labeling for FHC was as follows: 5/15 in Breslow < 2 mm; 8/18 in Breslow > 2 mm, and 7/16 in metastatic tumors. This is the first time the presence of FHC in human melanoma tumors was evidenced. Mutation in p53 gene is controversial in melanoma, notwithstanding it alone there is no link with prognosis [37, 38]. In this work, only primary tumors with Breslow thickness < 2 mm showed less expression in p53 in relation to metastasis, besides not difference was found in p53 positive nuclei between groups. It is know that p53 has additional activities in the cytoplasm, where it triggers apoptosis through mitochondrial activation and inhibits autophagy [39]. Decreased cell p53 expression in tumors with Breslow < 2 mm compared to metastatic tumors can indicate a new approach in tumor p53 analyses to differentiate high risk and low risk recurrence patients with melanoma [36]. This data is compatible with the literature which demonstrated a lower rate of recurrence-free survival in this patient group, once increased p53 expression in cancerous cells generally indicates a bad prognosis [25, 40, 41, 42].

Summarizing, FHC was increased in serum of patients with melanoma in advanced stages, as systemic oxidative stress. Despite that the FHC gene is regulated by p53, no alterations between different tumor stages were found, notwithstanding we demonstrated FHC expression in human melanoma tumors for the first time. High risk recurrence and recurrence groups showed similar pattern, indicating that FHC, oxidative stress and cell p53 expression may influence in

melanoma progression and prognosis. Probable mechanisms involved deserves further investigation.

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References

[1] Arosio P, Ingrassia R, Cavadini P. Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochim Biophys Acta* 2009; **1790**: 589-599.

[2] Recalcati S, Invernizzi P, Arosio P, Cairo G. New functions for an iron storage protein: the role of ferritin in immunity and autoimmunity. *J Autoimmun* 2008; **30**: 84-89.

[3] Steegmann-Olmedillas JL. The role of iron in tumour cell proliferation. *Clin Transl Oncol* 2011; **13:** 71-76.

[4] Orino K, Lehman L, Tsuji Y, Ayaki H, Torti SV, Torti FM. Ferritin and the response to oxidative stress. *Biochem J* 2001; **357:** 241-247.

[5] Houben R, Hesbacher S, Schmid CP,Kauczok CS, Flohr U, Haferkamp S *et al.* High-Level Expression of Wild-Type p53 in Melanoma Cells is Frequently Associated with Inactivity in p53 Reporter Gene Assays. *Plos One*, 2011; **6**: e22096.

[6] Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. *Oncogene*, 2003; **22**: 3138-3151.

[7] Avery-Kiejda KA, Bowden NA, Croft AJ, Scurr LL, Kairupan CF, Ashton KA *et al*. p53 in human melanoma fails to regulate target genes associated with apoptosis and the cell cycle and may contribute to proliferation. *BMC Cancer*, 2011; **11**: 203.

[8] Faniello MC, Di Sanzo M, Quaresima B, Baudi F, Di Caro V, Cuda G *et al*. p53-mediated downregulation of H ferritin promoter transcriptional efficiency via NF-Y. *Int J Biochem Cell Biol*, 2008; **40**: 2110-2119.

[9] Linder MC, Schaffer KJ, Hazegh-Azam M, Zhou CY, Tran TN, Nagel GM. Serum ferritin: does it differ from tissue ferritin? *J Gastroenterol Hepatol*, 1996; **11**: 1033-1036.

[10] Ghosh S, Hevi S, Chuck SL. Regulated secretion of glycosylated human ferritin from hepatocytes. *Blood,* 2004; **103:** 2369-2376.

[11] Wang W, Knovich MA, Coffman LG, Torti FM, Torti SV. Serum ferritin: Past, present and future. *Biochim Biophys Acta*, 2010; **1800**: 760-769.

[12] Alkhateeb AA, Connor JR. The significance of ferritin in cancer: Anti-oxidation, inflammation and tumorigenesis. *Biochim Biophys Acta*, 2013; **1836**: 245-254.

[13] Hann HW, Lange B, Stahlhut MW, McGlynn KA. Prognostic importance of serum transferrin and ferritin in childhood Hodgkin's disease. *Cancer,* 1990; **66**: 313-316.

[14] Luger TA, Linkesch W, Knobler R, Kokoschka EM. Serial determination of serum ferritin levels in patients with malignant melanoma. *Oncology*, 1983; **40**: 263-267.

[15] Maxim PE, Veltri RW. Serum ferritin as a tumor marker in patients with squamous cell carcinoma of the head and neck. *Cancer*, 1986; **57**: 305-311.

[16] Milman N, Pedersen LM. The serum ferritin concentration is a significant prognostic indicator of survival in primary lung cancer. *Oncol Rep*, 2002; **9**: 193-198.

[17] Cairo G, Tacchini L, Pogliaghi G, Anzon E, Tomasi A, Bernelli-Zazzera A. Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. *J Biol Chem*, 1995; **270**: 700-703.

[18] Tsuji Y, Ayaki H, Whitman SP, Morrow CS, Torti SV, Torti FM. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol Cell Biol*, 2000; **20:** 5818-5827.

[19] Pham CG, Bubici C, Zazzeroni F, Papa S, Jones J, Alvarez K *et al*. Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. *Cell*, 2004; **119**: 529-542.

[20] Gray CP, Franco AV, Arosio P, Hersey P. Immunosuppressive effects of melanoma-derived heavy-chain ferritin are dependent on stimulation of IL-10 production. *Int J Cancer*, 2001; **92**: 843-850.

[21] Baldi A, Lombardi D, Russo P, Palescandolo E, De Luca A, Santini D *et al*. Ferritin contributes to melanoma progression by modulating cell growth and sensitivity to oxidative stress. *Clin Cancer Res*, 2005; **11**: 3175-3183.

[22] Panis C, Herrera AC, Victorino VJ, Campos FC, Freitas LF, De Rossi T *et al*. Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. *Breast Cancer Res Treat*, 2012; **133:** 89-97.

[23] Purdue MP, From L, Kahn HJ, Armstrong BK, Kricker A, Gallagher RP *et al*. Etiologic factors associated with p53 immunostaining in cutaneousmalignant melanoma. *Int J Cancer*, 2005; **117**:486-493.

[24] Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. *Arch Dermatol*, 1988; **124:** 869-871.

[25] Soong SJ, Shaw HM, Balch CM, McCarthy WH, Urist MM, Lee JY. Predicting survival and recurrence in localized melanoma: a multivariate approach. *World J Surg*, 1992; **16**: 191-195.

[26] Chen TT, Li L, Chung DH, Allen CD, Torti SV, Torti FM *et al*. TIM-2 is expressed on B cells and in liver and kidney and is a receptor for H-ferritin endocytosis. *J Exp Med*, 2005; **202**: 955-965.

[27] Tran TN, Eubanks SK, Schaffer KJ, Zhou CY, Linder MC. Secretion of ferritin by rat hepatoma cells and its regulation by inflammatory cytokines and iron. *Blood*, 1997; **90**: 4979-4986.

[28] Gray CP, Arosio P, Hersey P. Association of increased levels of heavy-chain ferritin with increased CD4+ CD25+ regulatory T-cell levels in patients with melanoma. *Clin Cancer Res*, 2003; **9:** 2551-2559.

[29] Bomford AB, Munro HN. Ferritin gene expression in health and malignancy. *Pathobiology*, 1992; **60:** 10-18.

[30] Hazard JT, Drysdale JW. Ferritinaemia in cancer. *Nature*, 1977; **265**: 755-756.

[31] Llesuy SF, Milei J, Gonzalez-Flecha B, Boveris A. Myocardial damage induced by doxorubicins: hydroperoxide-initiated chemiluminescence and morphology. *Free Rad Biol Med*, 1990; **8** 259-264.

[32] Oliveira FJA, Cecchini, R. Oxidative stress of liver in hamsters infected with Leishmania (L.) Chagasi. *J Parasitol,* 2002; **86**: 1067-1072.

[33] Azorin I, Bella MC, Iborra FJ, Fornas E, Renau-Piqueras J. Effect of tert-butyl hydroperoxide addition on spontaneous chemiluminescence in brain. *Free Rad Biol Med*, 1995; **19:** 795-803.

[34] Lodh M, Goswami B, Gupta N, Patra SK, Saxena A. Assessment of oxidative stress and inflammatory process in patients of multiple myeloma. *Indian J Clin Biochem*, 2012; **27**: 410-413.

[35] Gadjeva V, Dimov A, Georgieva N. Influence of therapy on the antioxidant status in patients with melanoma. *J Clin Pharm Ther*, 2008; **33**: 179-185.

[36] Joosse A, De Vries E, van Eijck CH, Eggermont AM, Nijsten T, Coebergh JW. Reactive oxygen species and melanoma: an explanation for gender differences in survival? *Pigment Cell Melanoma Res*, 2010; **23**: 352-364.

[37] Hieken TJ, Ronan SG, Farolan M, Shilkaitis AL, Das Gupta TK. Molecular prognostic markers in intermediate-thickness cutaneous malignant melanoma. *Cancer*, 1999; **85**: 375-382.

[38] Väisänen A, Kuvaja P, Kallioinen M, Turpeenniemi-Hujanen T. A prognostic index in skin melanoma through the combination of matrix metalloproteinase-2, Ki67, and p53. *Hum Pathol*, 2011; **42**: 1103-1111.

[39] Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. *Nature*, 2009; **458:**1127-1130.

[40] Bichakjian CK, Halpern AC, Johnson TM, Foote Hood A, Grichnik JM, Swetter SM *et al*. Guidelines of care for the management of primary cutaneous melanoma. American Academy of Dermatology. *J Am Acad Dermatol*, 2011; **65:** 1032-1047.

[41] Breslow A. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg*, 1970; **172**: 902-908.

[42] Soong SJ, Harrison RA, McCarthy WH, Urist MM, Balch CM. Factors affecting survival following local, regional, or distant recurrence from localized melanoma. *J Surg Oncol*, 1998; **67**: 228-233.

Figures Captions

Figure 1. (A) Serum ferritin heavy (FHC); and (B) Serum ferritin light (FLC) chain; (C) FHC:FLC ratio; (D) lipid peroxidation curve of erythrocytes by chemiluminescence (CL); (E) Analysis of area under the curve (AUC) of the lipid peroxidation assay. Cutaneous (< 2 mm): cutaneous melanoma patients with Breslow thickness < 2.0 mm; Cutaneous (> 2 mm): cutaneous melanoma patients with Breslow thickness > 2.0 mm. Lipid peroxidation curves were compared by Two-way ANOVA test, and all curves are different ($p<0.0001$). Groups were compared with control group by t'test or Mann-Whitney test.* $p<0.05$.

Figure 2. Immunohistochemistry analysis of melanoma tumors. (A) p53 in cutaneous < 2 mm, case 4; (B) p53 in cutaneous > 2 mm, case 26; (C) p53 in melanoma metastasis, case 49; (D) FHC in cutaneous < 2 mm, case 10; (E) FHC in cutaneous > 2 mm, case 26; (F) FHC in melanoma metastasis, case 37. Data relative to p53 and FHC expression in cells were compared by X^2 test. Positive and negative p53 in nuclei were compared by Fisher exact test. A and C had different p53 levels in cells ($X^2=7.266$; $p= 0.0264$), although no statistical significance was found in positive nuclei p53 staining ($p=0.0538$).

Tables

Table 1. Characteristics of studied subjects

	Control	Cutaneous < 2 mm	Cutaneous > 2 mm	Metastatic melanoma	p value
Age (mean±SEM)	57.85±2.2	54.9±2.4	55.7±3.2	52.64±4.015	0.7408
Gender					0.4626
Male	11 (36.6%)	6 (25.0%)	9 (47.4%)	6 (42.9%)	
Female	19 (64.4%)	18 (75.0%)	10 (52.6%)	8 (57.1%)	
Fitzpatrick skin classification					0.1266
II	8 (26.6%)	5 (20.8%)	2 (10.5%)	4 (28.6%)	
III	18 (60.0%)	19 (79.2%)	13 (68.4%)	6 (42.8%)	
IV/V	4 (13.4%)	-	4 (21.1%)	4 (28.6%)	
Primary tumor histological type					< 0.0001*
SSM	-	20 (83.3%)	8 (42.1%)	4 (28.6%)	
NM	-	2 (8.35%)	7 (36.8%)	3 (21.4%)	
ALM	-	2 (8.35%)	4 (21.1%)	1 (07.1%)	
ND	-	-	-	6 (42.9%)	
Primary tumor Breslow (mean±SEM)	-	1.16±0.092	4.14±0.43	4.29±1.62	< 0.0001*
Metastasis location					-
Lymph node	-	-	-	8 (57.2%)	-
Brain	-	-	-	2 (14.3%)	-
Skin	-	-	-	3 (21.4%)	-
Liver				1 (07.1%)	-

Quantitative data were evaluated by One-way ANOVA with Tukey's *post test*. Qualitative data were evaluated by Chi-square (X^2) test. $p < 0.05$ was considered significant. X^2 values: Gender 4.540; Fitzpatrick skin classification 9.953; Primary tumor histogenetic type 30.04. Tukey's *post test* of Primary tumor Breslow was signicant in Cutaneous < 2 mm x Metastatic melanoma and Cutaneous < 2 mm x Cutaneous 2 mm statistical analysis. SSM: Superficial spreading melanoma; NM: Nodular melanoma; ALM: Acral lentiginous melanoma. Breslow thickness was measured in millimeters.

Table 2. Cutaneous and metastatic melanoma tumors characteristics

Case No.	Histogenetic type	Breslow tickness	Case No.	Location	Primary tumor histogenetic type	Breslow thickness of primary tumor
1	SSM	1.1	**34**	Lymph node	SSM	15.0
2	SSM	< 1	**35**	Lymph node	NM	13.0
3	SSM	1.5	**36**	Lymph node	ALM	ND
4	SSM	0.5	**37**	Lymph node	SSM	1.5
5	SSM	1.1	**38**	Skin	NM	13
6	ALM	1.5	**39**	Lymph node	NM	5.1
7	SSM	0.7	**40**	Lymph node	ND	ND
8	SSM	1.0	**41**	Skin	NM	ND
9	ALM	1.2	**42**	Lymph node	NM	2,5
10	SSM	1.5	**43**	Skin	ALM	2,5
11	SSM	1.1	**44**	Skin	NM	ND
12	SSM	1.0	**45**	Lymph node	NM	< 0.5
13	SSM	0.9	**46**	Spine	ALM	>10.0
14	SSM	1.5	**47**	Skin	NM	0.9
15	ND	0.5	**48**	Subcutaneous	ND	ND
16	SSM	4.0	**49**	Lymph node	ND	ND
17	SSM	3.0				
18	NM	3.0				
19	SSM	2.0				
20	ALM	3.5				
21	NM	3.2				
22	ALM	3.0				
23	SSM	2.5				
24	SSM	15.0				
25	NM	13.0				
26	ALM	>10.0				
27	NM	13.0				
28	NM	5.1				
29	SSM	6.0				
30	NM	3.0				
31	NM	7.5				
32	SSM	3.0				
33	NM	6.5				

SSM: Superficial spreading melanoma; NM: Nodular melanoma; ALM: Acral lentiginous melanoma; ND: Not determined. Breslow thickness was measured in millimeters.

Table 3. p53 and ferritin heavy-chain (FHC) immunohystochemistry score in cutaneous and metastatic melanoma cells.

Cutaneous				Metastasis			
	p53		FHC		p53		FHC
Case No.	Cell	Nuclei	Total	Case No.	Cell	Nuclei	Total
1	-	< 1%	-	**34**	+	< 1%	-
2	-	< 1%	-	**35**	+++	> 10%	++
3	-	< 1%	-	**36**	+++	> 10%	-
4	-	< 1%	+	**37**	++	> 10%	-
5	+	< 1%	-	**38**	+++	> 10%	++
6	+	< 1%	+	**39**	+	< 1%	+
7	+	< 1%	-	**40**	+	< 1%	-
8	+	< 1%	++	**41**	+++	> 10%	-
9	+	< 1%	-	**42**	+++	> 10%	-
10	++	> 10%	-	**43**	-	< 1%	+
11	+	< 1%	-	**44**	+++	> 10%	++
12	-	< 1%	+	**45**	+	< 1%	+
13	+	< 1%	-	**46**	++	> 10%	-
14	+	< 1%	-	**47**	-	< 1%	-
15	-	< 1%	+	**48**	-	< 1%	-
16	+	< 1%	-	**49**	+++	> 10%	+
17	+++	> 10%	++				
18	+++	> 10%	-				
19	++	> 10%	-				
20	+++	> 10%	++				
21	+	< 1%	+				
22	+	< 1%	-				
23	+++	> 10%	-				
24	+++	> 10%	-				
25	-	< 1%	+				
26	+++	> 10%	++				
27	+	< 1%	+				
28	-	< 1%	-				
29	-	< 1%	-				
30	+	< 1%	-				
31	+++	> 10%	+				
32	+	< 1%	-				
33	+++	> 10%	++				

FHC: Ferritin-heavy-chain; -, Nonstainer; +, Non-majority stainer (< 10% per field); ++, Stainer (10-50% per field); +++, Majority-stainer (> 50% per field). For statistical analyses of p53 expression in nuclei, tumors with >10% field nuclei staining was considered positive, and with <1% field nuclei staining was considered negative.

FIGURES

Figure 1.

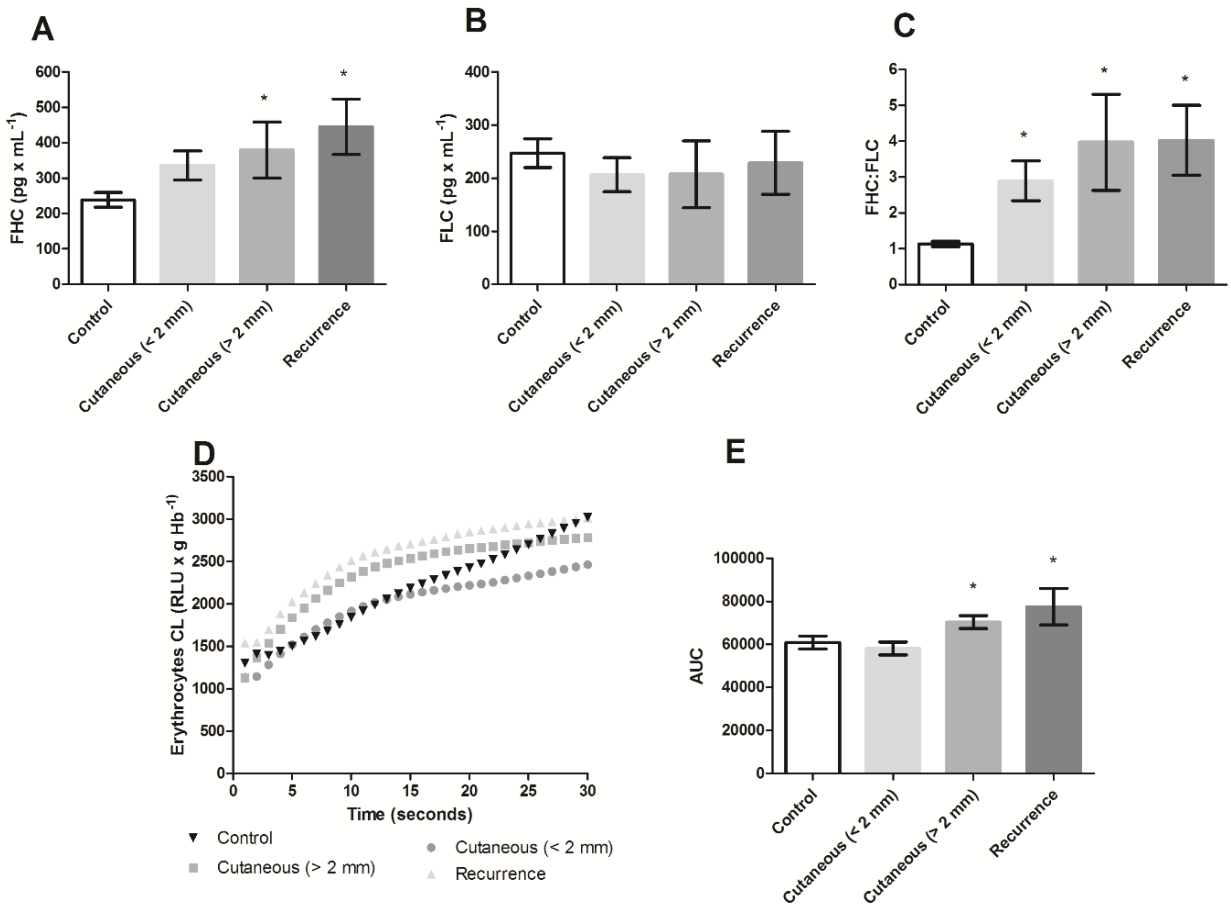
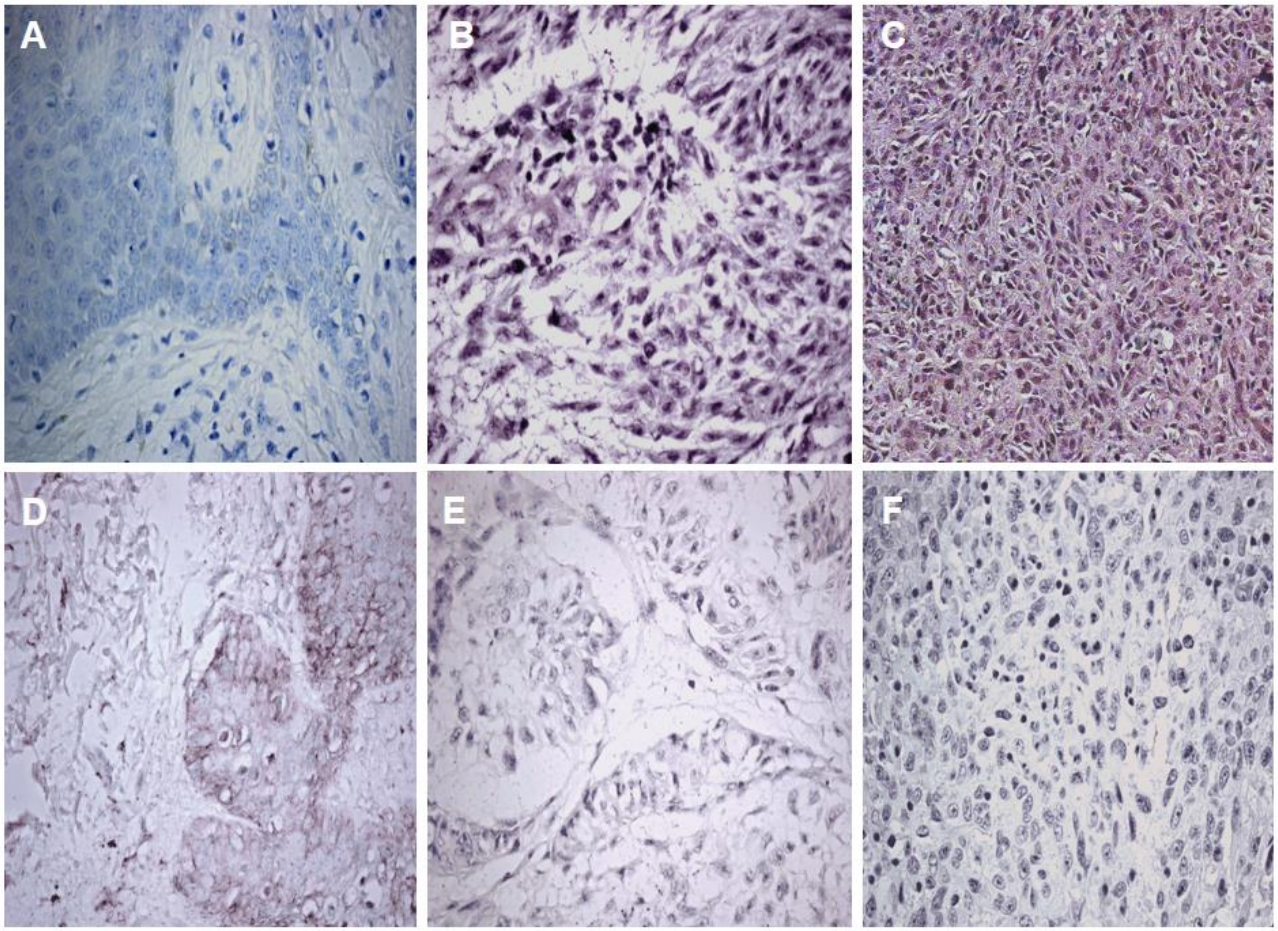


Figure 2.



ANEXO C

Relationship of TGF-β1 and oxidative stress in plasma of patient with melanoma: is there a clue to understand melanoma progression?

Melanoma Research

Relationship of TGF-β1 and oxidative stress in plasma of patient with melanoma: is there a clue to understand melanoma progression?

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Abstract:	Objective: TGF-β1 and oxidative stress are involved in cancer progression, but in melanoma their role is still controversial. Our aim was to correlate plasma TGF-β1 levels and systemic oxidative stress biomarkers in patients with melanoma, with or without disease recurrence, to understand its' participation on melanoma progression. Methods: Were recruited 30 patients in melanoma surveillance and 50 healthy volunteers. Patients were divided into two groups: No Recurrence, comprised by patients with tumor removal and no recurrence episode in 3-years, and Recurrence, comprised by patients with spread disease. The plasmatic cytokines TGF-β1, IL-1 β and TNF-α were analyzed by ELISA. For oxidative stress evaluation, we measured lipid peroxidation in erythrocytes, and malondialdehyde (MDA), Advanced Oxidation Protein Products (AOPP) levels, Total Radical Antioxidant Parameter (TRAP) and Thiol in plasma. Furthermore, the systemic advanced disease indicators ferritin, C-reactive protein (CRP) and lactate dehydrogenase (LDH) was evaluated together with serum total ferritin. Results: Recurrence group had increased LDH levels. Patients with recurrence also had less circulating TGF-β1, and increased TRAP, Thiol, AOPP and lipid peroxidation levels. MDA was increased in both melanoma groups, and IL-1β only in No Recurrence group. Conclusions: Our findings reinforce the new evidences that in melanoma TGF-β acts a tumor suppressor, inhibiting tumor relapse episodes. Lower levels of TGF-β1 is related with increased oxidative stress in patients with disease

	recurrence. These results supplies new knowledge of this cancer pathophysiology, and opening up possibilities for investigation of new therapies based on this new evidences.

Relationship of TGF-β1 and oxidative stress in plasma of patient with melanoma: is there a clue to understand melanoma progression?

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Abstract

Objective: TGF-β1 and oxidative stress are involved in cancer progression, but in melanoma their role is still controversial. Our aim was to correlate plasma TGF-β1 levels and systemic oxidative stress biomarkers in patients with melanoma, with or without disease recurrence, to understand its' participation on melanoma progression. **Methods:** Were recruited 30 patients in melanoma surveillance and 50 healthy volunteers. Patients were divided into two groups: No Recurrence, comprised by patients with tumor removal and no recurrence episode in 3-years, and Recurrence, comprised by patients with spread disease. The plasmatic cytokines TGF-β1, IL-1 β and TNF-α were analyzed by ELISA. For oxidative stress evaluation, we measured lipid peroxidation in erythrocytes, and malondialdehyde (MDA), Advanced Oxidation Protein Products (AOPP) levels, Total Radical Antioxidant Parameter (TRAP) and Thiol in plasma. Furthermore, the systemic advanced disease indicators ferritin, C-reactive protein (CRP) and lactate dehydrogenase (LDH) were evaluated together with serum total ferritin. **Results:** Recurrence group had increased LDH levels. Patients with recurrence also had less circulating TGF-β1, and increased TRAP, Thiol, AOPP and lipid peroxidation levels. MDA was increased in both melanoma groups, and IL-1β only in No Recurrence group. **Conclusions:** Our findings reinforce the new evidences that in melanoma TGF-β acts a tumor suppressor, inhibiting tumor relapse episodes. Lower levels of TGF-β1 is related with increased oxidative stress in patients with disease recurrence. These results supplies new knowledge of this cancer pathophysiology, opening up possibilities for investigation of new therapies based on these new evidences. **Key Words:** Melanoma, Transforming Growth Factor beta, Oxidative stress, Neoplasm recurrence, Malondialdehyde.

Introduction

Transforming growth factor beta 1 (TGF-β1) is a secreted ligand that has been intimately linked to the regulation of tumor initiation, progression and metastasis. It has been reported that leukocytes cultured *in vitro* secrete large amounts of TGF--β into the medium. The regulation of tumorigenesis by TGF-β1 signaling is dependent on the ability to regulate the behavior of tumor and host cell populations [1]. The role of TGF-β1 in cancer is very complex and ranges from cell growth inhibition to regulation of cell migration and invasion. While TGF-β1 acts as a potent cell cycle inhibitor and pro-apoptotic factor in most of its target tissues, its regulatory role on cell migration and invasion is not well understood and appears to be tissue specific [1,2].

Cutaneous melanoma is a highly aggressive malignancy with increasing incidence, limited therapeutic options in the metastatic stage of disease. A primary cutaneous melanoma will not kill the patient, but its metastases will. The tumor suppression effects derived from epithelial, endothelial, myeloid and lymphoid cancer cell types is well understood, but in melanoma that arises from the transformation of neural crest derived melanocytes, the suppressor and pro-metastatic effects are still controversial and not well understood [1,3,4]. In melanoma, the TGF-β regulatory responses on the progression of the disease towards the metastatic stage are less well characterized [2,5]. Evidences show that TGF-β1 could inhibit tumor growth and migration of murine melanoma cells *in vivo*, and also in human melanoma cells *in vitro*, despite that TGF-β1 has been increased in plasma of melanoma patients in all stages of the disease [2,6,7,8].

Oxidative stress is present in all steps of melanoma development, and its participation was already demonstrated *in vitro* and *in vivo* [9,10,11]. The systemic

oxidative stress in melanoma patients is poorly investigated, but it is known that these patients have systemic and tumoral redox deregulation [10,11,12]. TGF-β1 has been shown to stimulate Reactive Oxygen Species (ROS) production and suppresses antioxidant enzymes expression in a variety of cell types [13,14,15]. The specific molecular targets of TGF-β1-stimulated ROS are unknown, but TGF-β1 pro-oxidant effect on various cells has been proposed to regulate a number of physiological actions, such as growth-inhibitory effects, apoptosis and activation of latent TGF-β1 [16,17,18,19].

In melanoma, TGF-β1 regulatory responses remain unclear, and the characterization of circulation levels is fairly explored. There is an important link between oxidative stress and this cytokine function; thus, in this study our aim was to correlate plasma TGF-β1 levels and systemic oxidative stress biomarkers in patients with melanoma, with or without disease recurrence, to understand its participation on melanoma progression.

Materials and Methods

Sample collection and study design

A total of 30 melanoma patients between 28 to 67 years old were recruited at the Londrina Cancer Hospital between April 2011 and November 2012. Sixteen patients had localized primary cutaneous melanoma stage I/II with surgical tumor removal and no recurrence episode in 3-years period (No recurrence group); and fourteen patients had stage III/IV melanoma, without chemotherapy or immunotherapy (Recurrence group). Control group comprised 30 healthy women and men volunteers. This study was approved by the Research and Ethics National Council (CAAE 5831.0.000.268.10), all the practices were approved by the

institutional board, and all patients and controls signed informed consent. Patients and controls were non-smoking or in antioxidant therapy treatment, not obese and did not present hepatic, cardiac or renal dysfunction. In addition, control subjects had no previous history of any type of cancer. All subjects had skin type classified by self-rated Fitzpatrick Skin Phototype Classification (FSPC) [20]. The Fitzpatrick Skin Phototype Classification (FSPC) is the most commonly used measure of skin type, and classifies a person's complexion and their tolerance of sunlight. Venous blood was collected with and without heparin and centrifuged at 1,100 *x g*. Separated serum or plasma were immediately frozen at -80°C until biochemical or enzyme immunoassay (ELISA) analysis, and erythrocytes used fresh to access lipid peroxidation in membrane.

Serum and plasma biochemical analysis

Systemic advanced melanoma biomarkers Ferritin, C-reactive protein (CRP) and Lactate dehydrogenase (LDH) were determined. Ferritin was determined by chemiluminescence microparticule immunoassay (Architect, Abbott Laboratory, Abbott Park, IL, USA). CRP (highly sensitive CRP) was measured using a nephelometric assay (Behring Nephelometer II, Dade Behring, Marburg, Germany). Plasmatic lactate dehydrogenase was performed by Whitaker (1969) method using a commercial kit (Laborclin®), and analyzed with a Multiskan GO® microplate reader (Thermo Fisher Scientific, Rockford, USA) at 510 nm.

Plasma Human TNF-α, Human interleukin 1-beta (IL-1β) and Human/mouse TGF-β1 levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturers' instructions (eBioscience®), and analyzed with a microplate reader at 450 nm. To TGF-β1 analysis, plasma samples were previously acidified to activate latent TGF-β1 to its

immunoreactive form. Standard curves were constructed by a five-parameter regression formula and plotted as a linear curve (log-log). The results were calculated in pg x mL^{-1}.

Erythrocyte chemiluminescence

Systemic lipoperoxidation was evaluate in erythrocytes by chemiluminescence (CL). Erythrocytes were obtained from heparinized blood and washed three times with 0.9% saline solution at 4ºC. An aliquot of heparinized blood was used to determine hemoglobin (Coulter STKS®, Hialeah, USA). Erythrocytes lipoperoxidation was evaluated according to Panis et al. (2012) [21]. Briefly, 30 µl of packed erythrocytes was added to 3 ml of phosphate buffer, and 1 ml of this solution was diluted in 12.3 ml of the same buffer. The chemiluminescent reaction was initiated by the addition of tert-butyl (10 µl) at a final concentration of 3 mM in 1 mL of erythrocytes samples. Chemiluminescence curves were obtained in a GloMax® luminometer 20/20 (Promega, Madison, USA), and the results are expressed in relative light units (RLU) x g Hb^{-1}. The obtained curve was used as a qualitative indicator of lipoperoxidation, and quantitative results were obtained after area under curve integration using Graphpad Prism® version 5.0 (GraphPad Software).

Evaluation of oxidative stress in plasma

Plasma malondialdehyde (MDA) as measured by High-performance liquid chromatography (HPLC), as described by Victorino et al. (2012) [22]. Briefly, 160 µL of plasma sample was mixed with 100 µL of 0.5 M perchloric acid and incubated for 10 min on ice to precipitate the proteins, and then mixed with 100 µL of 1% thiobarbituric acid. This reaction was incubated for 30 min in a boiling water bath and transferred to an ice bath to stop the reaction. A 100 µL volume of 1 M NaH_2PO_4, pH 7, was added to each sample to stabilize the sample pH. Samples were centrifuged

for 10 min at 5,000 × *g* at 4°C. The supernatants were filtered and injected into LC-20AT® HPLC system (Shimadzu, Kyoto, Japan) in a mobile phase consisted of 65% 50 mM KH_2PO_4 buffer and 35% HPLC-grade methanol at ShimPack® C18 reverse-phase column (Shimadzu, Kyoto, Japan). Acid hydrolyzed 1,1,3,3-tetraethoxypropane (TEP) were used as standard to obtain the linear regression equation and calculate the MDA concentration of the samples. Readings were obtained at 535 nm over 11 min at a flow rate of 0.8 mL/minute at 35°C, and results were expressed as nM of MDA.

Total radical antioxidant parameter (TRAP) of plasma was determined as described by Repetto et al. (1998) [23]. Briefly, the reaction medium contains 70 µL of plasma sample diluted 1:50 in glycine buffer (0.1 M, pH 8.6), 20 µM 2-azo-bis-(2-amidinopropane) and 200 µM luminol at 37ºC. Trolox, a soluble E vitamin analogue, was used as the reference antioxidant (1.75 µM in the reaction medium). Chemiluminescence curves were obtained in a GloMax® luminometer 20/20 (Promega, Madison, USA), and the results are expressed in µM of Trolox.

Total thiols in plasma were measured by the method of Hu (1994) [24]. In final volume of 400 µL, was added 40 µL of plasma, 320 µL of 0.25 M TRIS buffer containing 0.02 M EDTA, pH 8.2 followed by addition of 40 µL of 0.01 M 2,2-dithiobisnitrobenzoic acid (DTNB) in absolute methanol. The tubes were capped and color was developed for 15 min at room temperature. The tubes were then centrifuged at 3,000 *x g* for 15 min. Supernatant was collected and absorbance measured at 412 nm with a microplate reader. Total thiol groups were calculated using a calibration curve prepared by GSH (Sigma Aldrich®).

To evaluate oxidized protein formation by inflammatory response, Advanced Oxidation protein products (AOPP) levels was measured. AOPP levels

was measured as described by Descamps-Latscha et al. (2004) [25] in a microplate reader. Briefly, plasma was diluted 1:5 in phosphate-buffered saline (PBS). Supernatant samples or chloramine-T standard solutions (200 μL) were placed in the appropriate wells of a 96-well microtiter plate, 10 μL of 1.16 mol/L KI added to each well and 20 μL of acetic acid added two minutes later. The absorbance of the reaction mixture was immediately read at 340 nm (Multiskan GO, Thermo Scientific, Waltham, USA) against a blank containing 200 μL of PBS, 10 μL of KI and 20 μL of acetic acid. AOPP levels were calculated using a chloramine-T calibration curve (Sigma-Aldrich, St. Louis, USA). Total protein was measured by Lowry et al. (1951) [26] technique, modified by Miller (1959) [27], used bovine serum albumin (BSA) as standard. The results were expressed in $\mu M \cdot mg^{-1}$ protein.

Statistical analysis

Comparisons were performed as follows: Control x No recurrence group and Control x Recurrence group. All data were expressed as arithmetic means and standard errors of means (SEM). The Shapiro–Wilk W test verified the normality of the data. For quantitative analysis, data with normal distribution were compared by t-test and data with non normal distribution by Mann–Whitney test. CL curves were analyzed by Two-way ANOVA test. Spearman's correlation test was used because data analyzed had non normal distribution. Differences were considered statistically significant when $p<0.05$. All the statistical analyses were performed using Graphpad Prism® version 5.0 (GraphPad Software).

Results

Table 1 shows a characterization of studied subjects. Table 2 shows systemic advanced melanoma biomarkers Ferritin, CRP and LDH. Ferritin (No recurrence: p=0.3726; Recurrence: 0.1988 mg x mL^{-1}) and CRP (No recurrence:

p=0.8933; Recurrence: p=0.605) showed no alterations. LDH levels was increased in recurrence group (No recurrence: p=0.5541; Recurrence: p=0.0168) (For values, see Table 2).

Plasma TNF-α showed no difference among groups (Control: 11.92±1.243; No recurrence: 17.12±3.884, p=0.1817; Recurrence: 9.641±0.8492, p=0.6055 pg x mL^{-1}), IL-1β was increased in No recurrence group (Control: 6.533±0.9448; No recurrence: 11.21±2.120, p=0.0152; Recurrence: 6.375±1.048, p=0.8997 pg x mL^{-1}) and TGF-β was decreased in Recurrence group (Control: 4.902±498.6; No recurrence: 4.201±862.0, p= 0.1263; Recurrence: 2.985±276.7, p=0.0272 pg x mL^{-1}) (Figures 1A, 1B and 1C).

Recurrence patients with melanoma had increased antioxidant parameters in plasma. Figure 2A shows an enhancement in TRAP levels (Control: 28.72±3.098; No recurrence: 36.65±5.356, p=0.1484; Recurrence: 45.04±7.204, p=0.0472 μM Trolox) and Figure 2B in Thiol levels (Control: 194.7±5.733; No recurrence: 208.3±7.610, p=0.1612; Recurrence: 222.0±9.751, p= 0.0172 μM). AOPP, an oxidized protein generated by oxidative burst and inflammatory response, was increased only in Recurrence group (Control: 1.013±0.04838; No recurrence: 1.104±0.065, p=0.2662; Recurrence: 1.235±0.092, p=0.0243 μM • mg^{-1} protein) (Figure 2C).

All erythrocytes CL curves are different by 2-way ANOVA test (p<0.0001) (Figure 3A). Figure 3B shows recurrence group had increased AUC values (74.630±8298; p=0.0329) than control (60.440±2365), and No recurrence group was not different (63.160±3957; p=0.5328) to the control. Plasma MDA levels are increased in both melanoma groups (Control: 175.4±22.55; No recurrence: 294.2±24.53, p=0.0005; Recurrence: 336.5±38.78, p=0.0004 nM) (Figure 3C).

There were a negative significant correlation among TGF-β levels and systemic MDA, and TGF-β levels and systemic AOPP (See Table 3).

Discussion

Recent years have seen a dramatic increase in incidence of melanoma, that has not been matched by the development of effective therapies for patients with advanced disease [28,29]. While very early stage melanoma (localized, stage I) is >90% curable, disseminated stage IV melanoma leads to life expectancy of less than a year [30,31]. Melanoma patients with recurrence had increased of systemic LDH levels than control subjects. In melanoma, serum LDH is the only blood-based biomarker in clinical use, and elevated serum LDH occasionally leads to imaging studies that reveal metastatic recurrence. However, the sensitivity and specificity for LDH as a predictor of metastatic recurrence is low, and additional blood based biomarkers are clearly needed [32,33].

TGF-β1, inflammatory status and oxidative stress have been related to cancer promotion and progression, but its relationship is poorly understood in melanoma metastasis. Since TGF- β1 is secreted by leukocytes *in vitro*, it is possible to assume that leukocytes *in vivo* could secrete TGF—β1 into the plasma, although there is no direct evidence for this [34]. The presence of TGF- β in human melanoma tissue is extensively reported, and its expression is increased particularly in metastatic tumors [8,35,36]. Malaponte et al (2010) [8] showed that circulating TGF-β1 correlate with their tumor levels.

Here we found a decreased TGF-β1 in advanced disease but not in no recurrence melanoma. Recently, Humbert & Lebrun (2013) [2] showed a protective role of TGF-β1 in human melanoma cells migration and invasion *in vitro*, to strongly

up-regulate the Plasminogen Activator Inhibitor-1 (PAI-1). In a murine melanoma model, TGF-β1 was also able to decreased metastatic process [7]. Classically, TGF-β1 is thought to be an important anti-inflammatory mediator, in that TGF-β1–null mice and mice deficient in various TGF-β1 receptors and activators exhibit persistent inflammation [37,38]. In this study, the inflammatory biomarkers CRP and ferritin, besides being present in higher amounts than in control subjects, were not statistically significant. It is known that increased inflammatory response is related to bad prognosis in several cancers, include melanoma [39,40,41]. As an evidence of circulating TGF-β1 low levels have pro-inflammatory effect in cancer, patients with triple negative breast cancer and with metastasis shows low levels circulation of TGF-β1, and this fact was related with poor prognosis [42]. Through these evidences, it is possible that in advanced melanoma systemic low TGF-β1 has a pro-inflammatory role and stimulates metastatic process.

No alterations were found in TNF-α levels, although there were higher amounts in no recurrence group in relation to control levels. IL-1β, another pro-inflammatory cytokine, was higher in no recurrence group, and together TNF-α seems to have had a protective role against melanoma recurrence. *In vitro*, IL-1β recombinant or secreted by macrophages demonstrated cytotoxicity against melanoma cells, although *in situ* IL-1 β and TNF-α produced by macrophages in human melanoma tumors are correlated with angiogenesis and tumor depth [43].

TGF- β isoforms are synthesized as latent complex from which TGF-β must be released to elicit its biological activity. Although most studies on the regulation of TGF- β action have focused on TGF- β transcription and translation, latency appears to be a critical step in the control of TGF- β activity, as enhanced TGF- β expression does not always correlate with increased levels of active TGF-β

[44]. The antioxidant system has been described as a protective mechanism against latent TGF-β activation in pathological processes, indicating that the increased circulating antioxidant levels may reflect TGF-β redox regulation [14,15,45].

Increased antioxidant response with increased pro-oxidant products can indicate a compensatory mechanism under sustained oxidative stress conditions [46]. Our findings support this, as despite TRAP and thiol enhancement, patients with recurrence showed increased AOPP, MDA and erythrocyte CL levels. AAOP is formed primarily by chlorinated oxidants (including hypochloric acid and chloramines) which result from myeloperoxidase activity during the respiratory burst [47]. As TGF-β1 also suppresses expression of ROS intermediates and respiratory burst capacity by both resting blood monocytes and activated macrophages [48], is reasonable to suppose that decreased TGF-β stimulates respiratory burst, supporting the increased AOPP levels found in this group. Furthermore, increased AOPP levels were found in cancer patients with advanced disease [49], and no relationship was done among this observation, systemic antioxidant system and circulating TGF- β1 before.

There are no report about increased systemic lipid peroxidation in patients with melanoma recurrence, but unpublished data of our laboratory showed increased plasmatic MDA and erythrocytes CL levels in patients with cutaneous and recurrence of melanoma even after surgical tumor removal. The increased erythrocyte CL only in patients with recurrence can signify alterations in cell membrane properties, as antioxidant composition, which modifies the kinetics pattern by exhausting the oxidizable species [50].

Thus, as in melanoma circulating TGF-β and this cytokine role remains not understood, here we showed that decreased TGF-β in patients with melanoma was statistically inversely significant correlated with the systemic pro-oxidant biomarkers

MDA and AOPP. Our findings reinforce the new evidences that TGF-β1 may act as a tumor immunossupressive, inhibiting disease spread even in advanced disease [2,43]. Furthermore, the increased circulating pro-oxidant biomarkers may reinforce a pro-inflammatory role of low TGF-β1 levels. Together, our findings contribute to the better comprehension of the role of circulating TGF-β1 in melanoma progression, opening up possibilities for investigation of new therapies based on this new evidence.

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References

1. Lebrun JJ. The Dual Role of TGF-beta in Human Cancer: From Tumor Suppression to Cancer Metastasis*. ISRN Molecular Biology* 2012, **Article ID** 381428.

2. Humbert L, Lebrun JJ. TGF-beta inhibits human cutaneous melanoma cell migration and invasion through regulation of the plasminogen activator system. *Cell Signal* 2013; **25**: 490-500.

3. Moustakas A. TGF-beta targets PAX3 to control melanocyte differentiation. *Dev Cell* 2008;**15**: 797-799.

4. Schriek G, Oppitz M, Busch C, Just L, Drews U. Human SK-Mel 28 melanoma cells resume neural crest cell migration after transplantation into the chick embryo. *Melanoma Res* 2005;**15**: 225-234.

5. Perrot CY, Javelaud D, Mauviel A. Insights into the Transforming Growth Factor-β Signaling Pathway in Cutaneous Melanoma. *Ann Dermatol* 2013;**25**: 135-144.

6. Krasagakis K, Thölke D, Farthmann B, Eberle J, Mansmann U, Orfanos CE. Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta 2 in patients with disseminated malignant melanoma. *Br J Cancer* 1998;**77**: 1492-1494.

7. Ramont SP, Hornebeck W, Maquart FX, Monboisse JC. Transforming growth factor-β1 inhibits tumor growth in a mouse melanoma model by down-regulating the plasminogen activation system. *Exp Cell Res* 2003**;291**: 1–10.

8. Malaponte G, Zacchia A, Bevelacqua Y, Marconi A, Perrotta R, Mazzarino MC *et al*. Co-regulated expression of matrix metalloproteinase-2 and transforming growth factor-beta in melanoma development and progression. *Oncol Rep* 2010;**24**: 81-87.

9. Meyskens FL Jr, McNulty SE, Buckmeier JA, Tohidian NB, Spillane TJ, Kahlon RS *et al*. Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. *Free Radic Biol Med* 2001;**31**: 799-808.

10. Sander CS, Hamm F, Elsner P, Thiele JJ. Oxidative stress in malignant melanoma and non-melanoma skin cancer. *Br J Dermatol* 2003;**148**: 913-922.

11. Gadjeva V, Dimov A, Georgieva N. Influence of therapy on the antioxidant status in patients with melanoma. *J Clin Pharm Ther* 2008;**33**: 179-185.

12. Picardo M, Grammatico P, Roccella F, Roccella M, Grandinetti M, Del Porto G *et al*. Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma. *J Invest Dermatol* 1996;**107**: 322-326.

13. Kayanoki Y, Fujii J, Suzuki K, Kawata S, Matsuzawa Y, Taniguchi N. Suppression of antioxidative enzyme expression by transforming growth factor-beta 1 in rat hepatocytes. *J Biol Chem* 1994;**269**: 15488–1592.

14. Islam KN, Kayanoki Y, Kaneto H, Suzuki K, Asahi M, Fujii J *et al*. TGF-beta1 triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase. *Free Radic Biol Med* 1997; **22**: 1007–1017.

15. Thannickal VJ, Fanburg BL. Activation of an H2O2- generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. *J Biol Chem* 1995;**270**: 30334-30338.

16. Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor-beta 1. *Mol Endocrinol* 1996;**10**: 1077-1083.

17. Bauer G. Elimination of transformed cells by normal cells: a novel concept for the control of carcinogenesis. *Histol Histopathol* 1996;**11**: 237- 255.

18. Häufel T, Dormann S, Hanusch J, Schwieger A, Bauer G. Three distinct roles for TGF-beta during intercellular induction of apoptosis: a review. *Anticancer Res* 1999;**19**: 105-111.

19. De Bleser PJ, Xu G, Rombouts K, Rogiers V, Geerts A. Glutathione levels discriminate between oxidative stress and transforming growth factor-beta signaling in activated rat hepatic stellate cells. *J Biol Chem* 1999;**274**: 33881- 33887.

20. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. *Arch Dermatol* 1988;**124**: 869 - 871.

21. Panis C, Herrera AC, Victorino VJ, Campos FC, Freitas LF, De Rossi T *et al*. Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. *Breast Cancer Res Treat* 2012;**133**: 89-97.

22. Victorino VJ, Panis C, Campos FC, Cayres RC, Colado-Simão AN, Oliveira SR *et al*. Decreased oxidant profile and increased antioxidant capacity in naturally postmenopausal women. *Age (Dordr)* 2013;**35**: 1411-1421.

23. Repetto M, Reides C, Gomez Carretero ML, Costa M, Griemberg G, Llesuy S. Oxidative stress in blood of HIV infected patients. *Clin Chim Acta* 1996;**255**: 107-117.

24. Hu ML. Measurement of protein thiol groups and GSH in plasma. InL Sies H, Abelson J, Simon M (eds). *Methods Enzymol 233*, California: Academic Press, 2004, pp 380 – 385

25. Descamps-Latscha B, Witko-Sarsat V, Nguyen-Khoa T, Nguyen AT, Gausson V, Mothu N *et al*. Early prediction of IgA nephropathy progression: proteinuria and AOPP are strong prognostic markers. *Kidney Int* 2004;**66**: 1606-1612.

26. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;**193**: 265-2.

27. Miller G. Protein determination of large numbers of samples. *Anal Chem* 1959;**31**: 964.

28. Goodson AG, Cotter MA, Cassidy P, Wade M, Florell SR, Liu T *et al*. Use of oral N-acetylcysteine for protection of melanocytic nevi against UV-induced oxidative stress: towards a novel paradigm for melanoma chemoprevention. *Clin Cancer Res* 2009;**15**: 7434-7440.

29. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman J *et al*. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 2010;**363**: 809-819.

30. Kim CJ, Reintgen DS, Balch CM. The new melanoma staging system. *Cancer Control* 2002; **9**: 9–15.

31. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB *et al*. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 2009;**27**: 6199-6206.

32. Kluger HM, Hoyt K, Bacchiocchi A, Mayer T, Kirsch J, Kluger Y *et al*. Plasma markers for identifying patients with metastatic melanoma. *Clin Cancer Res* 2011;**17**: 2417-2425.

33. Schramm SJ, Menzies AM, Mann GJ. Molecular biomarkers of prognosis in melanoma: how far are we from the clinic? *Melanoma Res* 2013;**23**: 423-425.

34. Grainger DJ, Mosedale DE, Metcalfe JC. TGF-beta in blood: a complex problem. *Cytokine Growth Factor Rev* 2000;**11**: 133-145.

35. Schmid P, Itin P, Rufli T. In situ analysis of transforming growth factor-beta s (TGF-beta 1, TGF-beta 2, TGF-beta 3), and TGF-beta type II receptor expression in malignant melanoma. *Carcinogenesis*. 1995;**16**: 1499-1503.

36. Van Belle P, Rodeck U, Nuamah I, Halpern AC, Elder DE. Melanoma-associated expression of transforming growth factor-beta isoforms. *Am J Pathol* 1996;**148**: 1887–1894.

37. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J *et a*l. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;**96**: 319-328.

38. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M *et al*. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;**359**: 693-699.

39. Deichmann M, Kahle B, Moser K, Wacker J, Wüst K. Diagnosing melanoma patients entering American Joint Committee on Cancer stage IV, C-reactive protein in serum is superior to lactate dehydrogenase. *Br J Cancer* 2004;**91**: 699-702.

40. Bouwhuis MG, Collette S, Suciu S, de Groot ER, Kruit WH, Ten Hagen TL *et al*. Changes of ferritin and CRP levels in melanoma patients treated with adjuvant interferon-α (EORTC 18952) and prognostic value on treatment outcome. *Melanoma Res* 2011;**21**: 344-351.

41. Wu Y, Antony S, Meitzler JL, Doroshow JH. Molecular mechanisms underlying chronic inflammation-associated cancers. *Cancer Lett* 2013; **pii**: S0304-3835(13)00589-2.

42. Panis C, Herrera AC, Victorino VJ, Aranome AM, Cecchini R. Screening of circulating TGF-β levels and its clinicopathological significance in human breast cancer. *Anticancer Res* 2013;**33**: 737-742.

43. Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J *et al*. Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNF-alpha and IL-1 alpha. *Int J Cancer* 2000;**85**: 182-188.

44. Gleizes PE, Munger JS, Nunes I, Harpel JG, Mazzieri R, Noguera I, Rifkin DB. TGF-beta latency: biological significance and mechanisms of activation. *Stem Cells* 1997;**15**: 190-197.

45. Cui Y, Robertson J, Maharaj S, Waldhauser L, Niu J, Wang J *et al*. Oxidative stress contributes to the induction and persistence of TGF-β1 induced pulmonary fibrosis. *Int J Biochem Cell Biol* 2011;**43**: 1122-1133.

46. Halliwel Bl, Gutteridge JMC. *Free Radicals in Biology and Medicine*, 4th edn. Oxford: Oxford University Press, 2007.

47. Korkmaz GG, Altınoglu E, Civelek S, Sozer V, Erdenen F, Tabak O, Uzun H. The association of oxidative stress markers with conventional risk factors in the metabolic syndrome. *Metabolism* 2013;**62**: 828-835.

48. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 1998;**16**:137-161.

49. Suzuki Y, Ohno S, Okuyama R, Aruga A, Yamamoto M, Miura S *et al*. Determination of chronic inflammatory states in cancer patients using assay of reactive oxygen species production by neutrophils. *Anticancer Res* 2012;**32**: 565-570.

50. Azorin I, Bella MC, Iborra FJ, Fornas E, Renau-Piqueras J. Effect of tert-butyl hydroperoxide addition on spontaneous chemiluminescence in brain. *Free Rad Biol Med* 1995;**19**: 795-803.

Figures Captions

Figure 1. Systemic (A) TNF-α, (B) IL-1β and (C) TGF- β levels in no recurrence and recurrence patients with melanoma. No recurrence group: localized primary cutaneous melanoma stage I/II with surgical tumor removal and no recurrence episode in 3-years period; Recurrence: Patients with melanoma and disease recurrence, with or without surgical removal. Melanoma groups were compared with control group by Mann-Whitney test.* $p<0.05$.

Figure 2. Systemic antioxidant (A) TRAP, (B) Thiol and (C) AOPP levels in no recurrence and patients with melanoma recurrence. No recurrence group: localized primary cutaneous melanoma stage I/II with surgical tumor removal and no recurrence episode in 3-years period; Recurrence: Patients with melanoma and disease recurrence, with or without surgical removal. Groups were compared with control group by Mann-Whitney or t'test. AOPP: Advanced Oxidation protein products; TRAP: Total radical antioxidant parameter. * $p<0.05$.

Figure 3. (A) Erythrocyte lipid peroxidation by CL, (B) Erythrocyte lipid peroxidation analysis of the Area Under Curve (C) Plasmatic MDA levels in no recurrence and recurrence melanoma patients. No recurrence group: localized primary cutaneous melanoma stage I/II with surgical tumor removal and no recurrence episode in 3-years period; Recurrence: Patients with melanoma and disease recurrence, with or without surgical removal. Groups were compared with control group by Mann-Whitney test. AUC: Area Under Curve; CL: Chemiluminescence; MDA: Malondialdehyde. * $p<0.05$.

Tables

Table 1. Characteristics of studied subjects

	Control	**No Melanoma recurrence**	**Melanoma recurrence**
	(n=30)	**(n=16)**	**(n=14)**
Age (mean±SEM)	57.48±1.784	52.75±3.172	52.64±4.015
Gender			
Male	9 (30.0%)	5 (31.3%)	6 (42.9%)
Female	21 (70.0%)	11 (68.7%)	8 (57.1%)
Fitzpatrick skin classification			
II	9 (30.0%)	4 (25.0%)	4 (28.6%)
III	16 (53.3%)	11 (68.8%)	6 (42.8%)
IV/V	5 (16.7%)	1 (6.2%)	4 (28.6%)
Surgical tumor removal			
No	NA	0	8 (57.2%)
1- 2 years	NA	0	3 (21.4%)
3-4 years	NA	8 (50.0%)	3 (21.4%)
5 years	NA	8 (50.0%)	0
Primary tumor Breslow (mean±SEM)	NA	1.540±0.338	4.14±0.433
Metastasis location			
Lymph node	NA	NA	9 (64.3%)
Skin	NA	NA	3 (21.4%)
Brain	NA	NA	2 (14.3%)

ALM: Acral lentiginous Melanoma; NM: Nodular Melanoma; SSM: Superficial Spreading melanoma; NA: Not apply.

Table 2. Advanced melanoma biomarkers LDH, CRP, and ferritin in patients with melanoma with no recurrence and recurrence episode

	Control	No recurrence	Recurrence
Ferritin (mg x mL^{-1})	137.1±13.88	116.2±17.81	175.5±31.47
CRP (mg x L^{-1})	2.5±0.36	2.74±0.67	4.33±1.94
LDH (U x L^{-1})	197.7±9.63	191.0±16.59	246.9±20.39*

LDH: Lactate dehydrogenase; CRP: C-reactive protein. Groups were compared with control group by t'test or Mann-Whitney test.* $p<0.05$.

Table 3. Spearman's TGF-β correlation with systemic advanced melanoma biomarkers LDH and CRP, and oxidative stress parameters MDA, AOPP, TRAP and Thiol

Correlation	R-value	p-value
TGF-β1 x LDH	0.0889	0.5109
TGF-β1 x CRP	0.1642	0.2310
TGF-β1 x MDA	- 0.3443	0.0089*
TGF-β1 x AOPP	- 0.3057	0.0207*
TGF-β1 x TRAP	- 0.2491	0.0693
TGF-β1 x Thiol	- 0.1734	0.2012

LDH: Lactate dehydrogenase; CRP: C-reactive protein; MDA: malondialdehyde; AOPP: Advanced Oxidation protein products; TRAP: Total radical antioxidant parameter. *p<0.05.

FIGURES

Figure 1.

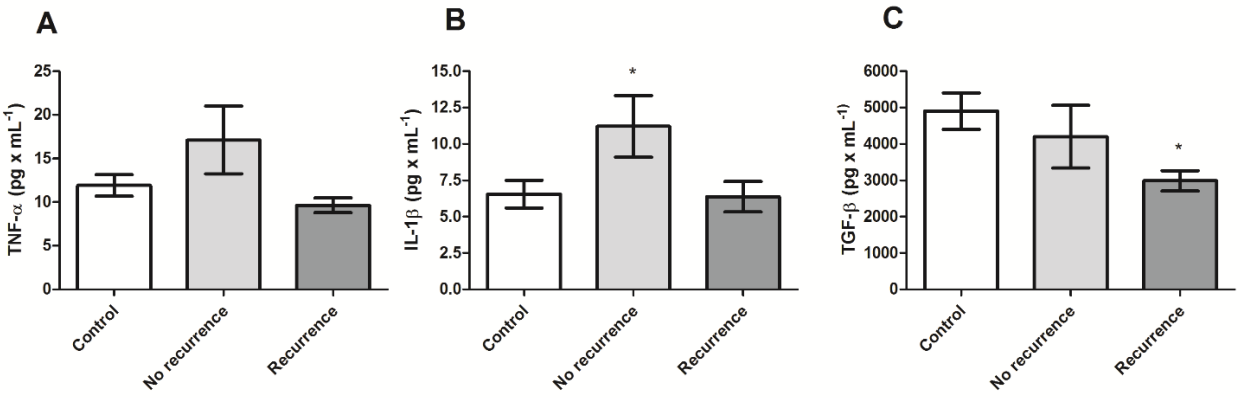


Figure 2.

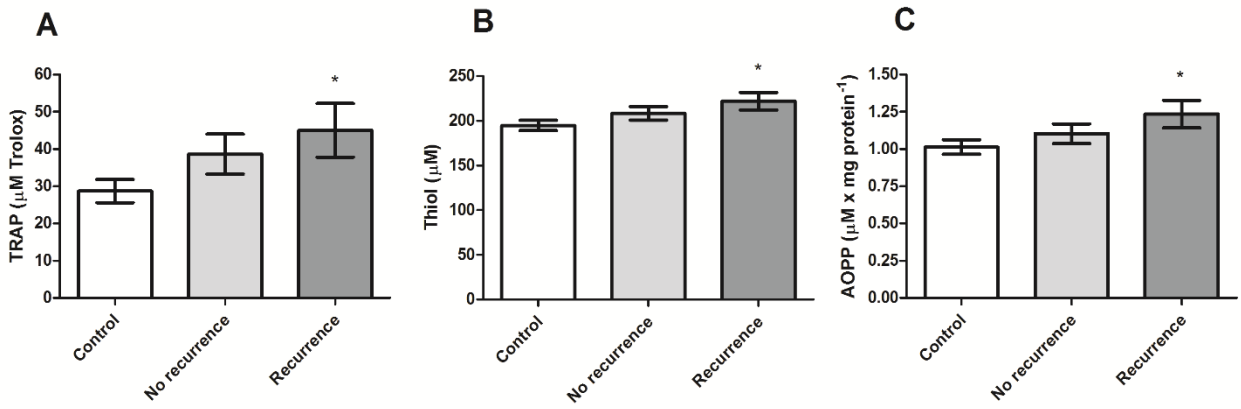
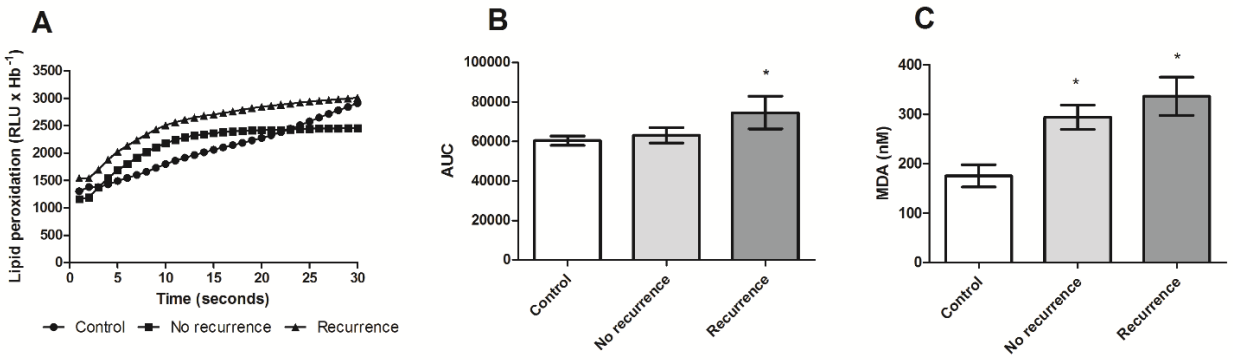

A
TRAP (μM Trolox)
60
50
40
30
20
10
0
*
Control
No recurrence
Recurrence
B
Thiol (μM)
250
200
150
100
50
0
*
Control
No recurrence
Recurrence
C
AOPP (μM x mg protein-1)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
Control
No recurrence
Recurrence

Figure 3.


A
Lipid peroxidation (RLU x Hb^{-1})
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Time (seconds)
Control
No recurrence
Recurrence
B
AUC
100000
80000
60000
40000
20000
0
*
Control
No recurrence
Recurrence
C
MDA (nM)
400
300
200
100
0
*
*
Control
No recurrence
Recurrence

ANEXO D

Endereço eletrônico do guia para autores das revistas científicas *British Journal of Cancer, Cancer Letters e Melanoma Research*

Guia para autores da *British Journal of Cancer*. Disponível em: < http://www.nature.com/bjc/authors/submit.html#manuscripts >. Acessado em: 27 fev 2014.

Guia para autores da *Cancer Letters*. Disponível em: < http://www.elsevier.com/journals/cancer-letters/0304-3835/guide-for-authors >. Acessado em: 27 fev 2014.

Guia para autores da *Melanoma Research.* Disponível em: < http://edmgr.ovid.com/mr/accounts/ifauth.htm >. Acessado em: 27 fev 2014.

ANEXO E

Parecer de aprovação do Comitê de Ética em Pesquisa Envolvendo Seres Humanos, Universidade Estadual de Londrina

Universidade Estadual de Londrina
COMITÊ DE ÉTICA EM PESQUISA

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 268

Parecer de Aprovação nº 263/2010 **CAAE nº** 5831.0.000.268-10 **Folha de Rosto nº** 381553 **Processo nº** 33138/2010	Londrina, 28 de fevereiro de 2011.

PESQUISADOR(A): **Alessandra Lourenço Cecchini Armani**
CCB – Departamento de Ciências Patológicas

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 268) – de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"FERRITINA E ESTRESSE OXIDATIVO SISTÊMICO COMO BIOMARCADORES DE PROGRESSÃO DO MELANOMA CUTÂNEO: estudo em pacientes do Instituto do Câncer de Londrina"

Situação do Projeto: **APROVADO**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL relatório final da pesquisa.

Atenciosamente,

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

ANEXO F

Termos de Consentimento livre e esclarecido - Pacientes portadores de melanoma

UNIVERSIDADE
ESTADUAL DE LONDRINA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Pacientes portadores de melanoma.

A – Informações sobre a pesquisa:

Você está sendo convidado(a) a participar, como voluntário(a), da pesquisa intitulada "FERRITINA E ESTRESSE OXIDATIVO SISTÊMICO COMO BIOMARCADORES DE PROGRESSÃO DO MELANOMA CUTÂNEO: ESTUDO EM PACIENTES DO INSTITUTO DO CÂNCER DE LONDRINA", que tem por objetivo do estudo avaliar os níveis séricos de H e L-ferritina e moléculas provenientes do estresse oxidativo sistêmico e relacioná-los como biomarcadores de progressão do melanoma cutâneo em pacientes com estadiamentos I a IV. Você será esclarecido(a) sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

B – Procedimentos do Estudo:

A pesquisa envolve a obtenção de uma amostra sanguínea de pacientes com diferentes estadiamentos do melanoma cutâneo, atendidos no Hospital do Câncer de Londrina. Este procedimento não trará qualquer risco para o(a) senhor(a), e, após as análises de ferritina e estresse oxidativo, as amostra serão desprezadas de acordo com as normas da Vigilância Sanitária. Nossa expectativa é de que este estudo possa fornecer ferramentas necessárias para correlacionar o estresse oxidativo e níveis de ferritina com a progressão da doença, bem como verificar se há diferenças no prognóstico do paciente com melanoma cutâneo em relação ao predomínio de L- ou H- ferritina e se o estresse oxidativo possui participação nesta diferença.

C – Confidencialidade da Pesquisa

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados do paciente serão armazenados em armários fechados à chave e não serão divulgados de forma a não possibilitar sua identificação.

Não serão realizados estudos sobre o DNA nem estudos sobre doenças genéticas e após a conclusão dos testes laboratoriais as amostras serão descartadas.

A participação no estudo não acarretará custos para você e não haverá nenhuma compensação financeira adicional. Você receberá uma cópia deste termo onde consta o telefone e o endereço da coordenadora do projeto de pesquisa, da pesquisadora responsável e do Comitê de Ética em Pesquisa Envolvendo Seres Humanos caso tenha dúvidas ou necessite de maiores esclarecimentos agora ou a qualquer momento. A coordenadora do projeto é a Profª. Drª. Alessandra Lourenço Cecchini Armani, que pode ser encontrada no endereço: Rod. Celso Garcia Cid, 445, Departamento de ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, tel. 3371-4529. O Comitê de Ética encontra-se no endereço: Avenida Robert Kock, n. 60, tel. 3371-2490.

D – Consentimento livre esclarecido e informado:

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecido(a) pelo(s) pesquisador(es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste serviço.

Londrina, ____ de _____, 20 ___.

Assinatura do pesquisador responsável:_____

Assinatura do doador: _____

ANEXO G

Termos de Consentimento livre e esclarecido – Indivíduos controles

UNIVERSIDADE
ESTADUAL DE LONDRINA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – Indivíduos controle.

A – Informações sobre a pesquisa:

Você está sendo convidado(a) a participar, como voluntário(a), da pesquisa intitulada "FERRITINA E ESTRESSE OXIDATIVO SISTÊMICO COMO BIOMARCADORES DE PROGRESSÃO DO MELANOMA CUTÂNEO: ESTUDO EM PACIENTES DO INSTITUTO DO CÂNCER DE LONDRINA", que tem por objetivo do estudo avaliar os níveis séricos de H e L-ferritina e moléculas provenientes do estresse oxidativo sistêmico e relacioná-los como biomarcadores de progressão do melanoma cutâneo em pacientes com estadiamentos I a IV. Você será esclarecido(a) sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

B – Procedimentos do Estudo:

A pesquisa envolve a obtenção de uma amostra sanguínea de pacientes com diferentes estadiamentos do melanoma cutâneo, atendidos no Hospital do Câncer de Londrina e de indivíduos saudáveis ditos "controle", cujo caso se aplica a voce. Este procedimento não trará qualquer risco para o(a) senhor(a), e, após as análises de ferritina e estresse oxidativo, as amostra serão desprezadas de acordo com as normas da Vigilância Sanitária. Nossa expectativa é de que este estudo possa fornecer ferramentas necessárias para correlacionar o estresse oxidativo e níveis de ferritina com a progressão da doença, bem como verificar se há diferenças no prognóstico do paciente com melanoma cutâneo em relação ao predomínio de L- ou H- ferritina e se o estresse oxidativo possui participação nesta diferença.

C – Confidencialidade da Pesquisa

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados do paciente serão armazenados em armários fechados à chave e não serão divulgados de forma a não possibilitar sua identificação.

Não serão realizados estudos sobre o DNA nem estudos sobre doenças genéticas e após a conclusão dos testes laboratoriais as amostras serão descartadas.

A participação no estudo não acarretará custos para você e não haverá nenhuma compensação financeira adicional. Você receberá uma cópia deste termo onde consta o telefone e o endereço da coordenadora do projeto de pesquisa, da pesquisadora responsável e do Comitê de Ética em Pesquisa Envolvendo Seres Humanos caso tenha dúvidas ou necessite de maiores esclarecimentos agora ou a qualquer momento. A coordenadora do projeto é a Profª. Drª. Alessandra Lourenço Cecchini Armani, que pode ser encontrada no endereço: Rod. Celso Garcia Cid, 445, Departamento de ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, tel. 3371-4529. O Comitê de Ética encontra-se no endereço: Avenida Robert Kock, n. 60, tel. 3371-2490.

D – Consentimento livre esclarecido e informado:

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarecido pelo(s) pesquisador(es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste serviço.

Londrina, ____ de _____, 20 ___.

Assinatura do pesquisador responsável:_____

Assinatura do doador: _____

ANEXO H
Questionário

1) Classificação do tipo de pele segundo Fitzpatrick

FOTÓTIPO	CABELOS	PIGMENTAÇÃO	SARDAS	QUEIMADURA	BRONZEADO
ZERO	BRANCO	ALBINA	AUSENTE	SEMPRE	ZERO
I	RUIVO	LEITOSA	NUMEROSAS	SEMPRE	ZERO
II	LOIRO	CLARA	BASTANTE	SEMPRE	LEVE
III	CASTANHO	CLARA/PARDA	POUCA	FREQUENTE	CLARO/ MÉDIO
IV	ESCURO	PARDA	ZERO	RARA	ESCURO
V	ESCURO	MORENA	ZERO	EXCEPCIONALMENTE	MUITO ESCURO
VI	NEGRO	NEGRA	ZERO	AUSENTE	NEGRO

2) Histórico de queimaduras solares graves __ vezes

3) É fumante? □ sim □ não

4) Ingere bebida alcoólica com frequência? □ sim □ não

5) Possui doença crônica? □ sim □ não

6) Faz uso contínuo de algum medicamento ou vitamina? □ sim □ não _____

7) Histórico de melanoma familial? □ sim □ não

8) Histórico de outro tipo de câncer? □ sim □ não

9) Peso _____ Altura _____