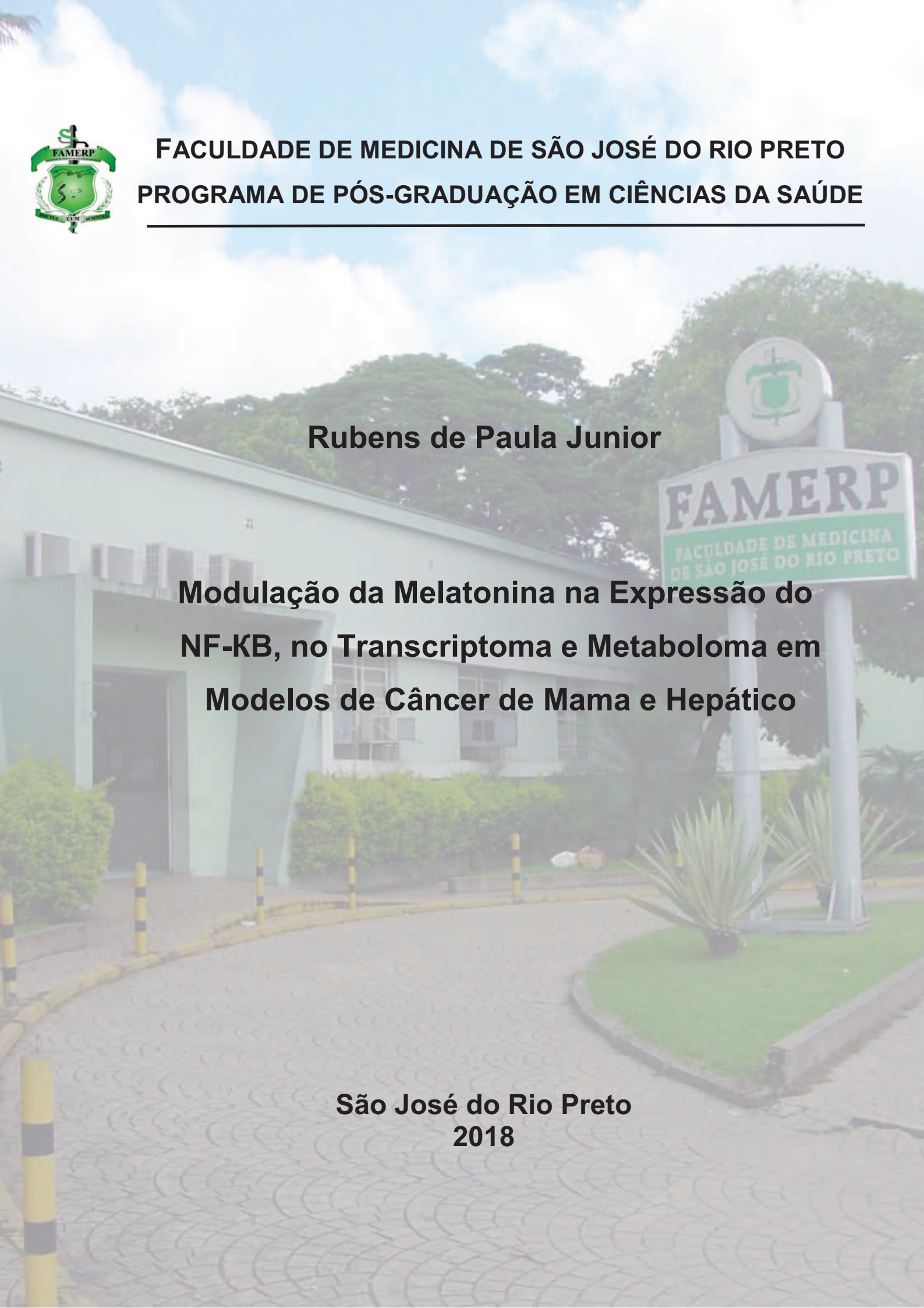

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Rubens de Paula Junior

Modulação da Melatonina na Expressão do NF-KB, no Transcriptoma e Metaboloma em Modelos de Câncer de Mama e Hepático

São José do Rio Preto
2018

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Tese apresentada à Faculdade de Medicina de São José do Rio Preto – FAMERP, para obtenção do título de Doutor em Ciências da Saúde junto ao Programa de Pós-Graduação em Ciências da Saúde, Área de Concentração: Medicina e Ciências Correlatas.

Orientadora: Profa. Dra. Debora Aparecida Pires de Campos Zuccari

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DEDICATÓRIA

Aos meus pais.

À minha orientadora.

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EPÍGRAFE

*"*Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muito conhecimento, que se sintam humildes. É assim que as espigas sem grãos erguem desdenhosamente a cabeça para o céu, enquanto as cheias as baixam para a terra, sua mãe*".*

Leonardo Da Vinci

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LISTA DE ABREVIATURAS E SÍMBOLOS

%	Porcentagem
°C	Grau célsius
µL	Microlitro
µm	Micrômetro
Aas	Aminoácidos
ALL	*Acute lymphoblastic leukemia*
am	*Ante Meridiem*
ANOVA	Análise de variância
ASS1	*Argininosuccinate synthase 1*
ATP	Adenosina Trifosfato
ATPase	Adenosinatrifosfatase
BCAA	*Branched Chain Amino Acids*
BSA	*Bovine Albumine Serum*
CAM	*Cell adhesion molecules*
cAMP	Adenosina 3',5'-monofosfato cíclico
CAT	Catalase
cDNA	DNA complementar
CD8+T	Célula T CD8+
CHC	Carcinoma Hepatocelular
CO2	Dióxido de Carbono
COX2	Ciclo-oxigenase 2
DE	Diferencialmente Expresso
DEPC	Dietil pirocarbonato
DMBA	7,12-di-methylbenz(a)anthracene
DMEM	*Dulbecco's Modified Eagle's Medium*
DMSO	Dimetilsulfóxido

DNA	Ácido Dexoxiribonucleico
ECM	*Extracelluar Matrix*
EMT	*Epithelial–mesenchymal transition*
ER	*Estrogene Receptor*
ERK	*extracellular signal–regulated kinases*
FBS	*Fetal bovine serum*
FC	*Fold Chang*
FDR	*False discovery rate*
g	Grama
GCS	Gânglio Cervical Superior
GPx	Glutationa peroxidase
GSA	Glutamic-c-semialdehyde
h	Hora
HepG2	Linhagem cellular de cancer hepático humano
HRP	*Horseradish peroxidase*
HSS T3 C18	Coluna de cromatrografia
IP	Intraperitoneal
IL	Interleucina
INCA	Instituto Nacional do Câncer
IU/mL	Unidades Internacionais por Mililitro
kDa	Quilodalton
kg	Kilograma
LAsn	L-asparagina
LC-MS	*Liquid chromatography–mass spectrometry*
Log	Logarítimo
MDSC	*Myeloid-derived Suppressor Cells*
mg	Miligrama
MHC	*Major Histocompatibility Complex*

min	Minuto
mL	Mililitro
mM	Micro molar
mm3	Milímetro cúbico
M.O.D.	*Mean Optical Density*
mRNA	RNA mensageiro
MT	Receptor de Melatonina
MyD88	*Myeloid differentiation primary response 88*
n	número amostral
NF-kB	*Nuclear Factor- kappa B*
ng	Nanograma
NK	*Natural Killer*
NSQ	Núcleo Supraquiasmático
OPLS	*Orthogonal Partial Least Squares*
OTC	*Ornithine Transcarbamylase*
p	Probabilidade de significância
PBS	Tampão fosfato salina
PC	*Principal component*
PCA	*Principal component analysis*
PCR	*Polymerase Chain Reaction*
PFAA	*Plasma Free Amino Acids*
PHGDH	*Phosphoglycerate Dehydrogenase*
pm	*Post Meridien*
PR	*Progesterone Receptor*
PRODH	*Proline dehydrogenase*
QC	*Quality control*
qPCR	*Quantitative Polymerase Chain Reaction*
RNA-Seq	Sequenciamento de RNA

rpm	Rotações por minuto
RPMI	*Roswell Park Memorial Institute*
RT-PCR	*Real Time Polymerase Chains Reaction*
RQ	*Relative Quantification*
SAM	*Senescence-accelerated mice*
SD	*Standard Deviation*
SOD	Superoxido Dismutase
TLR	*Toll-like receptors*
TLS	*Tertiary Lymphoid Structures*
TME	*Tumour Microenvironment*
TNBC	*Triple Negative Breast Cancer*
TNF	Fator de necrose tumoral
TQ-S	*Triple-quadrupole Spectrometer*
UPLC	*UPLC - Ultra Performance Liquid Chromatography*
WGCNA	*Weighted gene coexpression network analysis*

RESUMO

INTRODUÇÃO: O câncer de mama é a neoplasia mais prevalente em mulheres e o subtipo Triplo Negativo (TNBC) é considerado o mais grave por possuir um fenótipo metabólico agressivo. O Carcinoma Hepatocelular (HCC) é a neoplasia primária mais frequente no tecido hepático e junto com as neoplasias mamárias lidera as causas de óbito associadas ao câncer no mundo. Sabe-se que o comportamento tumoral é influenciado pela inflamação local no microambiente intrínseco do hospedeiro, bem como pela reprogramação do metabolismo. Células imunológicas no microambiente tumoral são moduladas por citocinas e fatores de transcrição como o NF-kB. Além da inflamação, o metabolismo do hospedeiro está sobre o controle cíclico diurno, o qual pode controlar os níveis circulantes de aminoácidos, tornando assim um alvo importante para análises do câncer. **OBJETIVOS:** Avaliar o efeito da melatonina no perfil de transcritos e na modulação do NF-kB em modelos de câncer de mama e hepático, e também o perfil metabólico no plasma durante uma variação diurna em modelos de câncer de mama. **MATERIAL E MÉTODOS:** *In vitro,* foram utilizadas células HepG2 e MDA-MB-231. *In vivo,* o tumor foi desenvolvido pela implantação de células tumorais MDA-MB-231 em camundongos fêmeas nude atímicos. Amostras de plasma foram coletadas sob rigorosas condições em oito tempos durante um dia completo e os tumores foram retirados para análises de expressão gênica, sequenciamento de RNA e Espectrometria de Massas. **RESULTADOS:** Foi observada redução do volume tumoral e também da expressão de NF-kB nos animais com câncer de mama e tratados com melatonina; por outro lado em células de hepatocarcinoma, a melatonina aumentou a expressão do NF-kB. No microambiente tumoral mamário, a melatonina aumentou a expressão dos genes Tnfaip8l2 and Il1f6, que são importantes na resposta imune local. No plasma, dentre os 20 aminoácidos detectados, 10 tiveram comportamento similar durante o dia. Na maioria dos horários do dia, os animais com tumor apresentaram níveis plasmáticos reduzidos desses aminoácidos. Além disso, houve uma inversão no perfil desses

aminoácidos entre os grupos, observado nos primeiros horários da manhã. **CONCLUSÕES:** O efeito da melatonina sobre o NF-kB no câncer de mama parece estar melhor definido, porém o seu papel permanece controverso no câncer hepático. Da mesma forma a ação anti-tumoral desse hormônio foi comprovada pela alta expressão dos genes Tnfaip8l2 e Il1f6 no microambiente tumoral. No plasma, as alterações dos metabólitos são um reflexo do metabolismo alterado, determinado pelas células tumorais mamárias na matriz dos camundongos. Enquanto que, essa inversão do perfil dos aminoácidos sugere que um evento cíclico do metabolismo do hospedeiro nesses horários aparece influenciando os níveis de aminoácidos circulantes. Desse modo, os nossos resultados apontam que durante a carcinogênese existe uma forte associação entre a resposta imune e a cronobiologia do hospedeiro que exige futuras investigações.

Palavras-chave: Câncer, Microambiente, Inflamação, Metabolismo, Aminoácidos.

ABSTRACT

INTRODUCTION: Breast cancer is the most prevalent neoplasm in women and the subtype Triple Negative (TNBC) is one of the most serious because of its aggressive metabolic phenotype. Hepatocellular carcinoma (HCC) is the most common primary neoplasm in liver tissue and, together with breast neoplasms, leads to the causes of death associated with cancer worldwide. It is known that tumor behavior is influenced by the host local intrinsic inflammation in the tumor microenvironment, as well as, by the metabolism reprogramming. Immunological cells in the tumor microenvironment are modulate by cytokines and transcription factors such as NF-kB. In addition to inflammation, host metabolism is under the diurnal control, which can control circulating levels of amino acids thus making it an important target for cancer analysis. **OBJECTIVES:** To evaluate the effect of melatonin on the transcript profile, on the modulation of NF-kB in breast and hepatic cancer models as well as on the metabolic profile in plasma during a diurnal variation in breast cancer. **MATERIALS AND METHODS:** *In vitro* was used HepG2 and MDA-MB-231. *In vivo* the tumor was developed by implantation of MDA-MB-231 tumor cells in nude athymic female mice. Plasma samples were collected under strict conditions at eight times during a full day and the tumors were removed for gene expression, RNA sequencing and Mass Spectrometry. **RESULTS:** It was observed a reduction in tumor volume and the expression of NF-kB in the animals with breast cancer and treated with melatonin. On the other hand, melatonin increased the expression of NF-kB in hepatocarcinoma cells. In the mammary tumor, microenvironment melatonin increased the expression of the Tnfaip8l2 and Il1f6 genes, which are important in the local immune response. In plasma, among the 20 amino acids detected, 10 had similar behavior during the day. At most times of the day, animals with tumor had reduced plasma levels of these amino acids. In addition, there was an inversion in the profile of these amino acids between the groups observed in the first hours of the morning. **CONCLUSIONS:** The effect of melatonin on NF-kB in breast cancer appears to be better defined, but in liver

cancer its role remains controversial. In the same way the anti-tumor action of melatonin was proven by the high expression of the Tnfaip8l2 and Il1f6 genes in the tumor microenvironment. In the plasma samples, the changes in metabolites are a reflex of the altered metabolism, determined by mammary tumor cells in the mice background. While this inversion of the amino acid profile in animals suggests that one cyclic event of host metabolism at these times appears to influence circulating amino acid levels. Thus, our results have pointed out that during carcinogenesis there is a strong association between the immune response and the chronobiology of the host that requires further investigations.

Key words: Cancer, Microenvironment, Inflammation, Metabolism, Amino acids.

1.INTRODUÇÃO

O câncer de mama é uma grande preocupação global confirmada pelas altas taxas de incidência e mortalidade. Mundialmente é a segunda neoplasia maligna mais frequente e o tipo mais comum entre mulheres correspondendo à quinta maior causa de óbitos relacionados ao câncer mundialmente[1]. A Organização Mundial da Saúde estima que, por ano, ocorram mais de 1.050.000 novos casos de câncer de mama em todo o mundo. No Brasil, dados do Instituto Nacional do Câncer (INCA) estimaram a ocorrência de 14.388 casos de óbito por câncer de mama em 2013 e para o ano de 2016 foram estimados 57.120 novos casos. Por outro lado, o Carcinoma Hepatocelular (HCC) é a neoplasia primária mais frequente no tecido hepático, o quinto tipo mais comum mundialmente e corresponde à segunda causa de óbito associada ao câncer. No Brasil os dados do INCA mostram que em 2013 houveram 8.772 casos de óbito por hepatocarcinoma, sendo 5.012 homens e 3.759 mulheres.

Depois de décadas de estudo sobre a biologia do câncer, sabe-se que o comportamento do tumor é influenciado pelo microambiente que é inerente à resposta imune do hospedeiro[2]. As células imunológicas que estão presentes no microambiente tumoral podem exercer funções ambíguas no processo de carcinogênese, e podem ser capazes de eliminar as células tumorais ou trabalhar em favor das mesmas potencializando o crescimento neoplásico[3-5]. Além disso, uma potente reação imunológica requer diversos passos que envolvem não somente as células como também outros componentes do microambiente como a própria matriz extracelular, que serve de barreira física prevenindo a infiltração inflamatória e escapando assim da resposta imune[6]. A conexão entre o câncer e a inflamação é amplamente reconhecida na literatura, mostrando que a ativação do sistema imune pode desencadear uma potente resposta anti-tumoral[7]. O processo inflamatório está intimamente ligado à progressão do tumor, angiogênese e metástase, desempenhando assim um importante papel na carcinogênese em vários tipos de câncer, incluindo câncer de mama e hepático[8, 9].

Vários estudos têm associado o aumento da expressão do NF-kB com o desenvolvimento do câncer de mama[10], no entanto, em casos de hepatocarcinoma, a

função do NF-kB permanece controversa[11]. Esse fator nuclear tem habilidade de modular a expressão de genes envolvidos em processos de proliferação celular, apoptose, inflamação, angiogênese e metástase. O NF-kB participa ativamente na homeostase dos tecidos, regulação da sobrevivência celular e também na ativação das respostas imunológicas inata e adquirida[12, 13]. Em níveis celulares, a ativação do NF-kB é fundamental para inibição de apoptose, bem como na manutenção da sobrevida celular. A atividade do NF-kB é controlada por várias proteínas regulatórias, e que alterações nessa regulação pode desencadear vários processos neoplásicos[14, 15]. Além do mais, o NF-kB é uma molécula chave para promover o processo inflamatório, pelo fato de induzir a produção de citocinas e enzimas pró-inflamatórias como as interleucinas (IL)-6, IL-8, fator de necrose tumoral (TNF)-α e ciclooxigenase 2 (COX2)[15, 16].

O NF-kB é um complexo proteico composto de um dímero formado por duas sub-unidades, as proteínas p50 e a p65, essa última também conhecida como RelA. Além dessas proteínas, outras sub-unidades também foram descritas, dentre elas c-Rel, RelB e p52. Quando na ausência de estímulo celular, o dímero permanece no citoplasma interligado à sua proteína inibidora, IkB[17]. Uma vez que citocinas pró-inflamatórias se ligam na membrana celular, o complexo IkB é fosforilado pela IKK (IkB quinase), que vai causar ubiquitinização e a degradação subsequente do IkB[18-20]. Com a degradação do IkB, o complexo NF-kB é liberado e vai para o núcleo, onde promove a expressão de genes relacionados com várias sinalizações celulares[21]. Sua ativação ocorre por meio dos receptores de membrana chamados TLRs (*Toll-like receptors*), que podem estimular a proliferação celular, resposta inflamatória e recrutamento de células imunológicas a favor da progressão tumoral[22]. O principal receptor do NF-kB é o *Toll-like receptor-4* (TLR-4), que é envolvido com o potencial metastático das células neoplásicas, e o estímulo desse receptor desencadeia uma cascata de sinalização mediada pela proteína *Myeloid differentiation primary response 88* (MyD88) que é a causadora da ativação do NF-kB[22, 23].

Pesquisas mostram que além desse fator de transcrição, a melatonina, um hormônio produzido pela glândula Pineal também é uma molécula muito importante em desempenhar

funções diversas na resposta imune. Células do sistema imunológico expressam tanto na membrana celular quanto nuclear, os receptores de melatonina[24, 25]. Além disso, células e órgãos do sistema imune, bem como outros órgãos, como a pele, têm a maquinaria biosintética de enzimas para produzir melatonina[26]. Esse importante hormônio pode ter efeito parácrino, intrácrino ou como agente autócrino[24, 25, 27], e dessa maneira é capaz de influenciar na resposta imune em diferentes níveis, os quais podem ser úteis no controle do crescimento neoplásico[9]. A melatonina possui também propriedades imunoterápicas[28], e a sua administração exógena pode estimular tanto a imunidade inata quanto adquirida[24], sendo capaz de modular citocinas e mediadores inflamatórios e a expressão gênica de importantes fatores de transcrição[9]. Sua aplicação terapêutica tem efeito positivo na produção de células do sistema imune: granulócitos, macrófagos, células *natural killer* e monócitos[24], bem como na síntese de citocinas incluindo interleucinas (*IL-2, IL-6, IL-12*), *interferon-gamma* and *TNF-alpha*[28, 29].

A melatonina é sintetizada principalmente pela glândula pineal e é considerada o "transmissor neuroendócrino" do ciclo diurno possuindo inúmeras funções fisiológicas[30]. Várias atividades anti-tumorais com múltiplos mecanismos subjacentes já foram propostos como efeitos da melatonina[31, 32]. No câncer de mama, sua eficácia é descrita principalmente na linhagem de células tumorais ER-positivas MCF-7, nas quais doses fisiológicas de melatonina podem ter efeitos anti-neoplásicos enquanto que na linhagem tumoral mamária triplo negativa MDA-MB-231, são necessárias doses farmacológicas de melatonina para exercer efeito anti-tumoral[33]. Em estudos prévios, nosso grupo de pesquisa tem mostrado um leque de atividades anti-tumorais da melatonina em vários modelos utilizando a linhagem triplo negativa MDA-MB-231[33-35]. Esse hormônio indólico é capaz de agir através dos receptores de membrana, *MT1* e *MT2*, os quais são expressos em diversos tecidos, incluindo o tecido mamário, pele, vasos sanguíneos e o sistema imunológico[36]. A melatonina também apresenta sua característica química como sendo uma molécula anfipática e pode agir de forma independente de receptores, atravessando a membrana

plasmática e interagindo com proteínas intracelulares e receptores nucleares[37], além de apresentar propriedades anti-oxidantes[38] e anti-inflamatórias[39].

Embora vários estudos na literatura mostrem que a melatonina é um regulador positivo do sistema imunológico, outros autores apresentam a melatonina como um agente anti-inflamatório[26]. Na fase aguda da inflamação, a melatonina é capaz de ativar mediadores pro-inflamatórios[24, 26], enquanto que na fase crônica seu efeito contribui para atenuação da inflamação inibindo certas citocinas e diminuindo o estresse oxidativo por efeito anti-oxidante[9, 40]. A exposição à melatonina exógena é capaz de reduzir os índices de mitose em linhagens de adenocarcinomas e também minimizar a expressão imuno-histoquímica de diversas moléculas incluindo Fator de transcrição nuclear kB (NF-kB), TNF-a, IL-1B e STAT3 em neoplasias epiteliais[9].

O efeito inibidor da melatonina sobre o NF-kB destaca ainda mais a ligação entre esse fator de transcrição e os efeitos anfi-inflamatórios da melatonina em tumores. A melatonina é capaz de suprimir a expressão da subunidade p65 e aumentar a expressão da proteína inididora IkB, e da mesma maneira, inibir a expressão do TLR-4 e reduzir os níveis da proteína MyD88 no citoplasma, prevenindo assim a ativação do NF-kB[23]. Além disso, a melatonina também pode prevenir a fosforilação da IkB pela IKK 5, o que evita a translocação do NF-kB para dentro do núcleo inibindo a ligação à região promotora de vários genes[18].

Além do processo inflamatório que ocorre no microambiente tumoral, o desequilíbrio do metabolismo celular assim como a sua reprogramação são fatores intrínsecos comuns durante a carcinogênese que também faz parte dos *hallmarks* do câncer[41-44]. Esse conceito teve início no século passado com o trabalho do cientista alemão Otto Heinrich Warburg em meados do século XX e continua gerando interesse na busca do entendimento do metabolismo no microambiente tumoral, que vai além do aumento de consumo de glicose e da glicólise aeróbica pelas células malígnas. As células normais quando passam pelo processo neoplásico sofrem alterações que conduzem para a disfunção de várias vias metabólicas. As novas células tumorais têm a demanda de grandes quantidades de energia

e de matéria de diferentes fontes para adaptar seu metabolismo buscando acelerar seu crescimento e proliferação[44-46]. As células malignas também adquirem dependência da disponibilidade de vários metabólitos como glicose, ácidos nucléicos, lipídeos, aminoácidos entre outras moléculas que são precursores de macromoléculas essenciais para o seu metabolismo[47, 48]. Dessa maneira, complementando a visão do câncer de mama como uma doença genética, ele é atualmente abordado como uma doença metabólica[41, 49-52].

O câncer de mama é conhecido por possuir caráter heterogêneo que pode ser observado em níveis histológicos e moleculares[53-55]. A classificação das neoplasias mamárias é fundamentada em análises moleculares de expressão gênica e protéica de receptores de estrógeno (RE), de progesterona (RP), do receptor tipo 2 do fator de crescimento epidérmico humano (HER2) e outros marcadores, como o receptor tipo 1 do fator de crescimento epidérmico (EGFR), a citoqueratina 5 (CK5) e o índice Ki-67 (índice de proliferação celular)[56]. Essas análises identificam diferentes subtipos que compõem os seguintes fenótipos: Luminal A (RE+ e/ou RP+, HER2- e Ki-67 < 14 %)[54, 56], Luminal B (RE+ e/ou RP+, HER2- e Ki-67 ≥ 14 %, se HER2+ pode ser chamado de Luminal HER2) [57, 58], Superexpressão HER2 (RE-, RP- e HER2+)[59], Basaloide, também chamado de Triplo-negativo (RE-, RP-, HER2-, CK5+ e/ou EGFR+)[60, 61] ou Triplo-negativo não basaloide (RE-, RP-, HER2-, CK5- e EGFR)[62, 63] dentre os quais cada um tem seu fenótipo clínico e histopatológico espectivo[45, 64-66] .

Aproximadamente 60% a 75% dos tumores de mama apresentam expressão do Receptor de estrógeno (ER) e/ou Receptor de Progesterona (PR) que são marcadores determinantes para o uso de terapia endócrina[67-69]. Por outro lado, o câncer de mama triplo negativo (*TNBC*) representa aproximadamente de 15 a 20% de todos os casos de câncer de mama, e geralmente é considerado o subgrupo com pior prognóstico pois apresenta fenótipo metabólico mais diferenciado e agressivo, não tendo assim tratamento específico[42, 70].

Tanto o metabolismo geral quanto vários outros aspectos fisiológicos específicos exibem variações, e inúmeras funções são coordenadas de acordo com a variação do ritmo

diurno ou circadiano. O ciclo diurno do metabolismo é modulado por características multifatoriais chamadas *Zeitgebers,* as quais incluem fatores exógenos como alimentação, atividades físicas, e também fatores endógenos controlados pelos genes do relógio biológico[71-74]. No cérebro de mamíferos os processos biológicos são coordenados pelo relógio biológico central localizado no Núcleo Supraquiasmático (NSQ) no Hipotálamo o qual é capaz de sincronizar o ritmo das outras células do corpo e os osciladores periféricos[75]. Esse "relógio mestre" é afetado pela informação luminosa ambiental de claro e escuro através do trato retino-hipotalâmico, que conecta as células ganglionares da retina ao NSQ e todos os mesmos com a glândula pineal através do gânglio cervical superior (GCS). No caso dos mamíferos, a informação luminosa ambiental, captada pela retina, é transmitida para os NSQs, os quais, pelas vias eferentes neuronais e hormonais, sincronizam a oscilação dos relógios periféricos ao ciclo claro/escuro ambiental. A variação de claro e escuro modula parte do metabolismo e também a concentração de várias substâncias circulante, como um dos *zeitgebers* mais importantes, tornando esse fluxo bidimensional essencial para manter a homeostasia[69, 76, 77].

Embora essa conexão entre o relógio circadiano e o metabolismo ainda permaneça pouco compreendida na literatura científica, vários estudos com vias metabólicas mostram que vários aspectos do metabolismo humano estão sob o controle diurno[73, 74, 78-80]. Incrivelmente, novas evidências sugerem que alterações em diversas vias metabólicas nas células tumorais têm se tornado alvo de pesquisas com câncer de mama[81, 82]. No entanto, as técnicas tradicionais buscam alterações a nível de DNA e proteínas, enquanto análises de moléculas com menor peso molecular, os metabólitos, têm se tornado fundamental na busca de pequenas alterações significantes em vias metabólicas específicas no câncer[83, 84]. Foi mostrado recentemente, em um estudo temporal sobre a influência do relógio biológico no fígado de roedores, que metade dos metabólitos encontrados mostraram sofrer variação circadiana. Outros estudos metabolômicos em humanos e animais também já identificaram uma gama de moléculas plasmáticas como açúcares, ácidos graxos, fosfolipídeos, nucleotídeos e aminoácidos exibindo oscilação no plasma dependente do relógio

biológico[73, 85-87]. O desequilíbrio do metabolismo pode afetar os níveis de diversas moléculas plasmáticos e esse perfil alterado é frequentemente observado em situações patológicas como Alzheimer, obesidade, diabetes, doença renal crônica, doença hepática, síndrome metabólica e vários tipos de tumores[86, 88-91]. Dessa forma o perfil metabolômico pode fornecer uma análise mais adequada do envolvimento do relógio biológico como fator preditivo em certas doenças[92-94].

Pesquisas buscando entender o metabolismo celular tumoral revelaram que existem características comuns no metabolismo dos aminoácidos, como por exemplo: (i) aumento na demanda de nitrogênio buscando suprir as reações biosintéticas; (ii) consumo elevado de aminoácidos e aumento na expressão de seus transportadores; (iii) demanda para aminoácidos não essenciais que excedem o suprimento intracelular, levando a dependência de fontes exógenas; e (iv) níveis alterados de enzimas que catalisam a síntese ou catabolismo de aminoácidos[95-97]. Assim estratégias que buscam alvos específicos no perfil de aminoácidos em células tumorais têm avançado de estudos pré-clínicos para a triagem clínica, mostrando eficácia como método de detecção de biomarcadores para aprimorar o diagnóstico do câncer[95, 98].

Dessa maneira, durante as recentes décadas o câncer de mama tem sido apontado como uma doença de etiologia complexa e assim explorado por meio de análises denominadas “ômicas” (genômica, transcriptômica, proteômica, entre outras). Esse campo procura identificar novos biomarcadores, bem como complementar os métodos diagnósticos usuais e a avaliação de resposta a terapias[50, 99-101]. A análise metabolômica foi introduzida neste grupo para fornecer uma visão global dos processos metabólicos. Através dessa análise é possível obter-se uma visão geral qualitativa e quantitativa dos metabólitos (< 1 kDa) presentes em um organismo vivo, além de verificar a influência de agentes endógenos e exógenos, fornecendo uma melhor representação do seu fenótipo funcional em relação às alterações no DNA, RNA e proteínas[85, 101-103].

No câncer, o objetivo da metabolômica é ajudar na compreensão dos processos bioquímicos que estão alterados nos diferentes estágios da carcinogênese e/ou na

avaliação da resposta à determinada terapia. Embora a aplicação da metabolômica na pesquisa do câncer de mama esteja em um estágio inicial, alguns estudos demonstram a possibilidade de utilizar perfis metabólicos como biomarcadores de detecção precoce, de caracterização do tumor, assim como, preditivos de resultados clínicos[101, 104]. Análises metabolômicas utilizando técnicas como cromatografia líquida acoplada à espectrometria de massas em conjunto com análises estatísticas multivariadas, podem fornecer informações sobre um grande número de metabólitos, contribuindo para a descoberta de novas vias bioquímicas e consequente compreensão de seu papel em diversas doenças[100, 105]. Além disso, permite a identificação de biomarcadores e o direcionamento para novos agentes terapêuticos[101, 106].

Pacientes com câncer de mama que apresentam receptores de estrógeno (RE) e de progesterona (PR) negativos possuem altos níveis de glicina, glicerol, fosfocolina e colina comparados com pacientes RE e PR positivos. Nos tecidos tumorais a baixa concentração de glicina está associada ao bom prognóstico das pacientes[107, 108]. Em um estudo realizado por Shen et al. (2013) foi demonstrado que pacientes com câncer de mama triplo negativo (RE, RP e HER2 (Receptor de Fator de Crescimento Epidermal Humano) negativos) possuem 10 aminoácidos em baixas concentrações quando comparados com pacientes saudáveis, como por exemplo, alanina, asparagina, histidina, tirosina, metionina entre outros, sugerindo o consumo desses aminoácidos no metabolismo das células desse subtipo tumoral. Existem três etapas principais que constituem as análises metabolômicas: a primeira permite o reconhecimento de padrões considerados como normais, em seguida, a identificação de metabólitos resultando em possíveis biomarcadores e por último, a quantificação e validação desses metabólitos[108]. Além disso, é importante ressaltar que a análise do perfil metabólico associado á variação do ciclo diurno, quando realizada em períodos limitados de um dia pode ser incompleta, pois alguns metabólitos sofrem variações significantes em suas concentrações, de acordo com a variação diária[85].

Da mesma maneira, as técnicas de sequenciamento de RNA (*RNA-Seq*) é amplamente utilizada nos campos da genômica e transcriptômica, tornando-se uma ferramente padrão

da comunidade científica[109]. Essa técnica aborda diferentes aplicações as quais podem incluir a detecção de genes que se apresentam diferencialmente expressos ou se comportam em módulos, em determinadas situações, mostrando alta amplitude e acurácia nos resultados. Além de identificar os possívei genes diferencialmente expressos, uma nova técnica de análise de redes de coexpressão de genes chamada *weighted gene coexpression network analysis (WGCNA)* foi recentemente proposta para explorar a relação entre genes e identificar módulos intrínsecos de genes coordenadamente expressos[110, 111]. A aplicação de *RNA-Seq* utilizando amostras de modelos xenográficos de câncer pode ser de grande utilidade para potencialmente identificar biomarcadores e mudanças diretas em resposta à terapias[112].

Nesse amplo cenário que envolve a busca de biomarcadores no câncer, a utilização de modelos xenográficos subcutâneos de tumores humanos é ampla na avaliação das características tumorais e também da resposta terapêutica específica em várias patologias. Além disso, podermos avaliar alterações primárias no tumor pois esse modelo proporciona implantes com volumes de fácil medição[113]. O crescimento tumoral nesse tipo de modelo experimental depende da relação entre as células humanas e o estroma dos animais[112]. Camungondos atímicos são comumente usados para o modelo xenográfico de câncer, os quais são deficientes para linfócitos T e permitem o enxerto e crescimento das células após a implantação[114]. Embora nesse modelo exista uma severa imunodeficiência, ela não é absoluta, pelo fato dos camundongos atímicos permanecerem com o sistema imune inato inalterado[115]. Além do mais, apesar da deficiência imunológica, os modelos xenográficos preservam outras características importantes do microambiente tumoral e da matrix extracelular. As interações no estroma, vascularização e estrutura tridimensional do tumor são capazes de reproduzir de forma fidedigna a situação biológica em outros organismos, como em humanos[114].

Portanto, pelo fato do câncer de mama e hepatocarcinoma terem altos índices de morbidade e mortalidade, existe um interesse na identificação de novas terapias e associação com novos biomarcadores moleculares específicos na progressão tumoral.

Diante do exposto, o objetivo desse trabalho foi verificar o perfil de transcritos e a modulação do NF-kB sob o efeito exógeno da melatonina e também avaliar o perfil metabólico de modelo xenográfico de câncer de mama durante uma variação diurna. Os resultados podem mostrar alterações mínimas na variação de metabólitos no plasma durante um dia completo e potenciais biomarcadores melhores caracterizados na doença, assim como fundamentar o uso da melatonina como terapia adjuvante no câncer de mama e hepático.

2. ARTIGOS CIENTÍFICOS

Os resultados do projeto original de doutorado, juntamente com outros trabalhos com as mesmas amostras e nos quais o doutorando participou das análises e desenvolvimento dos manuscritos, originaram três artigos científicos para publicação em revista indexada.

3.1. Artigo I

Título: *Melatonin differentially modulates Nf-Kb expression in breast and liver cancer cells.*

Autores: Jucimara Colombo; Bruna Victorasso Jardim Perassi; João Paulo de Senna Ferreira; Cristine Zampieri Braga; Nathália Martins Sonehara; Rubens de Paula Júnior; Marina Gobbe Moschetta; Ana Paula Girol; Debora Aparecida Pires de Campos Zuccari.

Periódico: *ACAMC - Anti-Cancer Agents in Medicinal Chemistry* ***[Has been accepted for publication].***

3.2. Artigo II

Título: *Melatonin treatment in breast cancer: the impact in transcriptomic networks of a xenograft model.*

Autores: Bruna Victorasso Jardim-Perassi1; Pâmela A. Alexandre2; Nathalia M. Sonehara1; Rubens de Paula-Junior1; Osvaldo Reis Júnior3, Roger Chammas4; Luiz Lehmann Coutinho5; Debora Aparecida Pires de Campos Zuccari1.

Periódico: *Journal of Pineal Research*

3.3. Artigo III

Título: *Modifications of the diurnal profile of plasma amino acids in mice bearing the human breast cancer xenograft MDA-MB-231.*

Autores: Rubens Paula Junior; Nathália Martins Sonehara; Akos Pal; Yasmin Asad; Roger Chammas; Florence Raynaud; Debora A. P. C. Zuccari.

Periódico: *PNAS - Proceedings of the National Academy of Sciences*

3. ARTIGO CIENTÍFICO I

MELATONIN DIFFERENTIALLY MODULATES NF-ΚB EXPRESSION IN BREAST AND LIVER CANCER CELLS

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ABSTRACT

Background NF-kB (nuclear factor kappa B) is a transcription factor composed of two subunits, p50 and p65, which plays a key role in the inflammatory process. Melatonin has oncostatic, antiangiogenic and antimetastatic properties, and some recent studies have indicated an inhibitory effect of melatonin on NF-kB in some types of cancer. This work aims to investigate the effects of melatonin treatment on the expression of NF-kB in breast and liver cancer models.

Methods The breast cancer xenographic model was performed using female Balb/c nude athymic mice injected with MDA-MB-231 cells. The animals were treated with 40 mg/Kg of melatonin for 21 days. Volume of the tumors was measured with a digital capiler. Hepatocarcinoma model was developed by using the HepG2 cells in vitro, treated with 1 mM melatonin for 24 h. The expression of NF-kB protein was verified by immunohistochemistry and immunocytochemistry and quantified by optical densitometry*, in vivo* study and *in vitro* study, respectively. NF-kB gene expression was performed by quantitative RT-PCR.

Results The breast cancer xenografts nude mice treated with melatonin showed reduced tumor size (*P*=0.0022). There was a decrease in NF-kB protein staining (*P*=0.0027) and gene expression (*P*=0.0185) in mice treated with melatonin. The opposite results were observed for the hepatocarcinoma model. HepG2 cells treated with melatonin showed an increase in the NF-kB immunostaining when compared to control cells (*P*=0.0042).

Conclusion Our results indicated that treatment with melatonin was able to decrease both gene and protein expressions of NF-kB in breast cancer cells and, conversely, increase the transcription factor protein expression in hepatocarcinoma cells. These data highlighted a double role in the expression of NF-kB, depending on the cell type. Further studies are needed to better elucidate the action of melatonin in NF-kB, since this transcription factor acts on different signaling pathways that are fundamental for carcinogenesis.

KEYWORD: NF-kB; breast cancer; hepatocellular carcinoma; melatonin

INTRODUCTION

The nuclear transcription factor kappa B (NF-kB) is composed of two subunits, p50 and p65 (RelA). In addition, other subunits have also been described, such as c-Rel, RelB, and p52. In the absence of cellular stimuli, the dimer remains in the cytoplasm bound to its inhibitory protein, IkB [1]. When proinflammatory cytokines bind to receptors on the cell membrane, IkB is phosphorylated by IKK (IkB kinase), which causes ubiquitination of IkB and its subsequent degradation in the proteosome [2,3,4]. With the degradation of IkB, the NF-kB complex is released and goes to the nucleus, where it promotes the expression of genes related to several cell-signaling pathways [5].

NF-kB regulates the expression of genes involved in cell proliferation, apoptosis, inflammation, angiogenesis and metastasis. This transcription factor plays a key role in the maintenance of tissue homeostasis, regulation of cellular survival, and activation of innate and adaptive immune responses [6,7]. At the cellular level, activation of NF-kB is important for inhibition of apoptosis, as well as for maintenance of cell survival. NF-kB activity is controlled by several regulatory proteins, and alterations in its regulation may lead to the development of various neoplasms [8,9].

NF-kB is a key molecule in promoting the inflammatory process, as it induces the production of cytokines and proinflammatory enzymes, such as interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α and cyclooxigenase (COX) 2 [9,10]. Activation of NF-kB occurs through receptors on the cell membrane such as TLRs (Toll-like receptor 4), which can stimulate cell proliferation, inflammatory response, and recruitment of immune system cells for cancer progression [11]. The main one is TLR-4, which is associated with the metastatic potential of tumor cells. Stimulation of this receptor triggers a cascade signaling mediated by the MyD88 protein, which causes the activation of NF-kB [11,12].

The inflammatory process is involved in tumor invasion, angiogenesis and metastasis, and therefore plays an important role in breast and liver carcinogenesis [13,14]. Several studies have associated NF-kB overexpression with the development of breast cancer [15]. However, in relation to hepatocarcinoma, the role of NF-kB remains controversial [16]. Breast

cancer is the leading cause of cancer-related death among females worldwide; in 2012, there were an estimated 1.7 million cases, with 521,900 deaths [17]. Among liver tumors, hepatocarcinoma, or hepatocellular carcinoma (HCC), is the most common, occuring in about 70 % to 90 % of cases. It is an aggressive tumor, and most often caused by chronic infection related to the hepatitis B or hepatitis C virus [17]. With regard to its incidence, liver cancer occupies sixth place, and represents the third highest cause of cancer mortality in the world [18].

Since breast cancer and hepatocarcinoma have high mortality and morbidity rates, there is a growing interest in the identification of new therapeutic agents that may interact with specific molecular markers to inhibit tumor progression. Melatonin, a hormone produced naturally by the pineal gland, appears to play an important role in inhibiting tumor growth [19,20]. This hormone has anti-tumor activities through a variety of mechanisms, including anti-proliferative, anti-neoplastic, anti-inflammatory, antiangiogenic, anti-metastatic, immune stimulation and modulation of proto-oncogenes expression [21].

Recently, some studies have indicated the inhibitory effect of melatonin on NF-kB, evidencing the link between this transcription factor and the anti-inflammatory effects of melatonin in tumors. Melatonin can suppress the expression of p65 subunit and increase the expression of the inhibitory IkB protein. It can also inhibit TLR-4 expression and decrease MyD88 protein levels in the cytosol, thus preventing the activation of NF-kB [12]. Additionally, melatonin can prevent the phosphorylation of IkB by IKK [5], which prevents the translocation of NF-kB into the nucleus and its binding to the promoter region of several genes [2].

Consequently, the present study aimed to investigate the gene and protein expressions of NF-kB in a breast cancer mice model, and a HepG2 hepatocarcinoma *in vitro* model after treatment with melatonin.

METHODS

Ethical Considerations

The *in vivo* study was approved by the Ethics Committee on the Use of Animals of the School of Medicine of São José do Rio Preto (Prot. 001-003336/2014 - License CEUA 06/2014). The study was carried out in accordance with the national and international standards of ethics in animal experimentation.

Development of the animal model and cell culture

A total of 10 female Balb/c nude athymic mice, 7-8 weeks of age and body weight of 25 g were maintained under pathogen free conditions, and kept in individual cages in a controlled environment (24 to 25 °C, 12h light/dark cycle) with water and food *ad libitum*.

Triple-negative human breast cancer cells (MDA-MB-231) and hepatocarcinoma cells (HepG2) came from ATCC (American Type Culture Collection, Manassas, VA, USA). HepG2 cell line is derived from the liver tissue of a 15-year-old Caucasian male with differentiated hepatocellular carcinoma. The cell lines were incubated in a 37 °C and 5 % CO_2 in DMEM (Cultilab, Campinas, SP, Brazil), supplemented with 10 % of fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), 1 % penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

For breast tumor implantation, the mice received an injection of 50 µL of culture medium without FBS containing 3 x 10^6 viable cells in the mammary gland. Animal weight and tumor volume were monitored weekly.

Melatonin administration in animal model

Mice were randomly separated into two groups (n=5/ each group). Control group received 100 µL of vehicle solution (8 PBS: 1 DMSO: 1 Cremophor/Sigma) by intraperitoneal injection, and the melatonin treated group received 100 µL of melatonin, (40 mg/kg of mouse body weight) [22].

Melatonin treatment started on the same day as tumor implantation, administered 5 days a week, 1 hour before the room lights were turned-off, for a period of 21 days [23]. After euthanasia in a CO_2 chamber, tumor excision was performed. Tumor fragments were cut longitudinally and stored in liquid nitrogen or 4 % formaldehyde.

Tumor measurement by caliper

Tumor volume was measured by digital caliper (Thermo Fisher Scientific, Rockford, IL, USA) on day 21 after tumor implantation. The major longitudinal diameter (length) and the minor transverse diameter (width) were determined. Tumor volume was calculated based on caliper measurements by the modified ellipsoidal formula [24] where: Tumor volume = ½ (length × width^2).

Melatonin treatment of cells *in vitro*

Cells were divided into the following groups: Control cells (untreated), and cells treated with 1 mM melatonin for 24 h [25]. It should be emphasized that the melatonin concentration of 1 mM melatonin used for the treatment of the cells in this study was defined according to the literature relating to the pharmacological concentration administered in several studies concerning the effects of melatonin in neoplastic cells [25,26,27,28,29,30].

Immunohistochemistry and Immunocytochemistry

Immunohistochemistry was performed on breast tumor tissue to evaluate the NF-kB protein expression. After euthanasia, tumor was extracted and cut longitudinally, and tissue was histologically processed. Histological sections of 4 μm were obtained from the paraffin embedded material. Subsequently, deparaffinization of the slides was performed, initially in an oven at 60 °C, followed by xylol and hydration with decreasing ethanol concentrations.

For immunocytochemistry, 0.5 x 10^6 HepG2 cells were placed on a slide with coupled bottle (Thermo Fisher Scientific, Grand Island, NY, USA) and incubated at 37 ºC for 24 h. After this period, the culture medium was removed, and the cells were washed with PBS. For fixing the cells, the bottle was incubated with 1 mL of 4 % formaldehyde for 20 min.

Next, slides were incubated with 10 % of hydrogen peroxide for 30 min to block endogenous peroxidase. Antigen retrieval was performed in a pressure cooker (ARNO, São Paulo, SP, Brazil) at 95 °C with citrate buffer (pH 6.0) for 30 min. After cooling, the slides were covered with a solution of BSA and incubated for 30 min at 4 °C, overnight with NF-KB antibody (Abcam 7970) (1:700). Subsequently, they were washed with PBS for 15 min and incubated with the Starr TREK Universal HRP Detection Kit (Biocare Medical, Concord, CA, USA) for 20 min, and streptavidin-peroxidase complex for 10 min, followed by washing with PBS for 15 min. Next, 0.5 % tetracloridrate of 3,3-diaminobenzidine (DAB; Signet Laboratories, Dedham, MA, USA) was applied for 2 min. The slides were counterstained with Harris's hematoxylin (Carlo Eerba Reagents, Milan, Italy) for 40 sec.

All experiments were accompanied by a positive control for the antibody tested and a negative control (no primary antibody). At the end of the procedure, the protein expression of NF-kB was quantified by the optical densitometry using ImageJ.

Various fields on each slide were examined. The slides were photographed, and immunostaining was quantified by Image J software (NIH, Bethesda, MD, USA) in the objective lens 40x of a Nikon Eclipse E200 microscope. For each slide, 3 photos were taken, scoring 20 points each, totaling 60 points for the calculation of the average relative intensity of immunoreactivity. The values were obtained as Arbitrary Units (AU), and the Mean Optical Density (MOD) revealed the intensity of staining in specifically immunoreactive areas.

RT-PCR quantitative (qPCR)

Total RNA was extracted from tumors and from cell culture through the method TRIZOL (Invitrogen Life Technologies® - Sao Paulo, SP, Brazil), and the cDNA was synthesized using the High Capacity cDNA Kit (Applied Biosystems®, Foster City, CA, USA) in a total volume of 20 µL of reaction mixtures at final concentration of 100 ng/µL.

The quantitative expression was performed through real time PCR in triplicate, using the Step One Plus System (Applied Biosystems®, Foster City, CA, USA), and a negative control was included in each reaction. PCR reactions containing 100 ng of cDNA, 10 µL of

TaqMan Universal Master Mix, 8 µL of DEPC solution of water, 1 µL of TaqMan Gene Expression for NF-KB, GAPDH, ACTB, were submitted to the following scheme amplification: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min.

The levels of mRNA of NF-kB, were normalized by GAPDH and ACTB genes, used as internal control of expression. For the analysis of gene expression, the TaqMan assays (Applied Biosystems®, Foster City, CA, USA) were used: NF-kB (Hs007657_m1), GAPDH (Hs02758991-g1), ACTB (Hs01060665-g1).

The relative expression of the genes of interest was determined by the comparative method ΔΔCt, which relates the average of expression of normalizer genes used as endogenous control and the average of expression of genes of interest in each sample [31].

The value of RQ (Relative Quantification) of the control group (used as reference) was established as the unit for analysis of the expression of NF-kB gene.

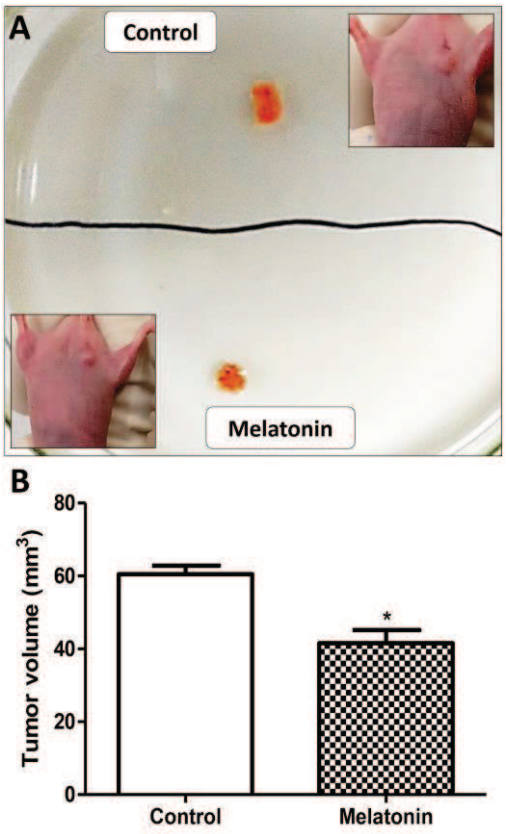
Statistical analysis

All values are expressed as the mean ± standard deviation. Comparison between melatonin and control groups was done by Student's t-test. Any $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed using GraphPad Prism 4 software version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

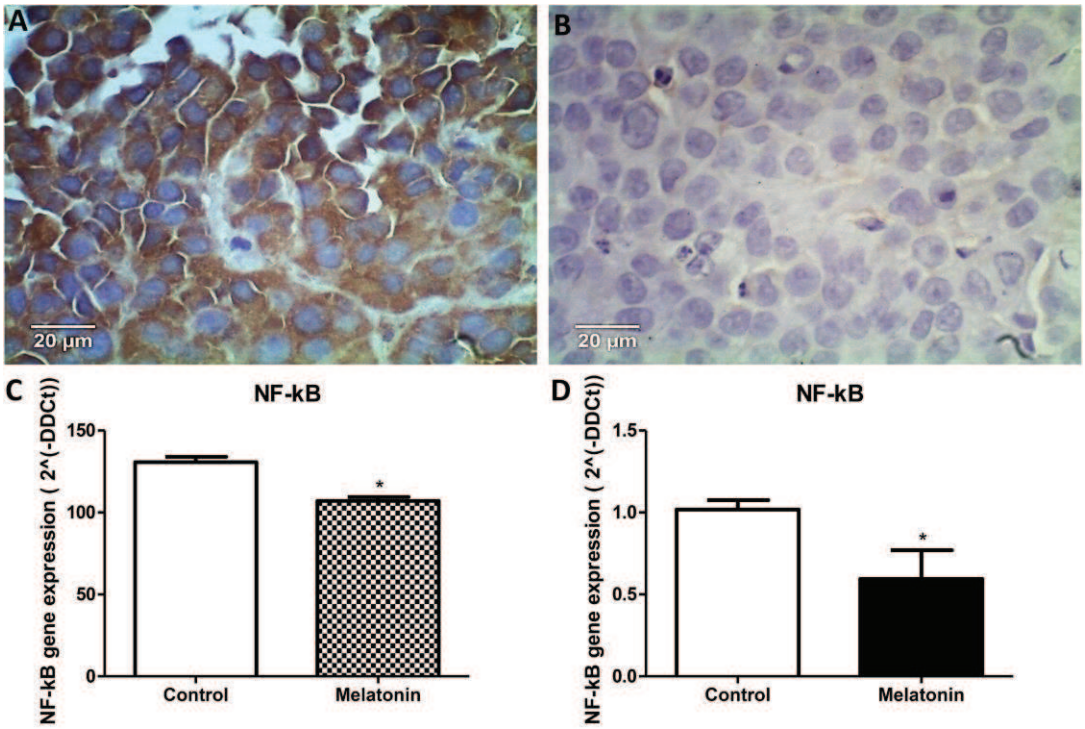
1. Breast cancer

To evaluate whether melatonin treatment reduces the breast tumor growth *in vivo*, we implanted MDA-MB-231 cells in athymic nude mice and treated then with melatonina (40 mg/kg) or vehicle for 21 days. Treated animals showed significantly smaller tumors after 21 days ($P=0.0022$) (Figure 1A-B). The mean tumor volume of control and treated animals were 60.50±2.302 mm^3 and 41.53±3.605 mm^3, respectively.



Immunohistochemistry, followed by the quantification of immunostaining by optical densitometry, was used to detect the NF-kB protein. The results were obtained in Arbitrary Units (AU) and demonstrated the value of the Mean Optical Density (MOD) ± standard error. The results showed a decreased NF-kB protein immunostaining in the group of animals treated with melatonin for 21 days (*P*=0.0027) (Figure 2A-C). Regarding the gene expression analysis, the melatonin treated group presented subexpression of NF-kB gene when compared with the vehicle treated group (*P*=0.0185) (Figure 2D), corroborating the immunohistochemical data.

According to the obtained results, it can be observed that the melatonin treatment was able to reduce both the protein and gene expression of NF-kB in breast tumors.

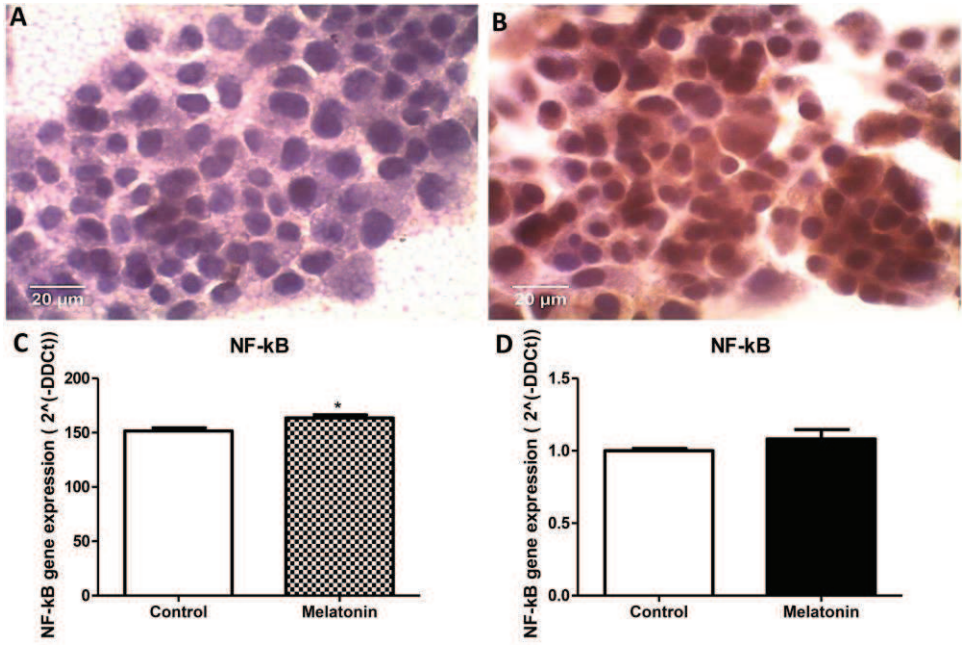


2. Hepatocarcinoma

In HepG2 cells treated with melatonin for 24 h, there was an increase in immunostaining of NF-kB when compared with control cells (*P*=0.0042) (Figure 3A-C).

The analysis of gene expression of NF-kB by qPCR indicated that the group of cells treated with 1 mM of melatonin did not show statistically significant difference in relation to the group without treatment (*P*=0.2224) (Figure 3D).

According to the results, melatonin treatment was able to increase only the NF-kB protein expression in hepatocarcinoma.



DISCUSSION

Our main proposal was to evaluate the effects of melatonin treatment on the NF-kB protein and gene expressions in breast and liver cancer models.

In the in vivo experiment set, we observed decreased protein and gene expression of NF-kB in breast cancer cells after treatment with melatonin. This result has also been observed in other types of neoplasia, such as ovarian cancer [11], pancreas [32], lung [33] and kidney [34]. According to some studies, the inhibitory effect of melatonin on NF-kB may be due to its ability to maintain this transcription factor in the cytoplasm, preventing the phosphorylation of IkB by IKK and consequently the nuclear translocation of NF-kB [2,21]. Therefore, melatonin inhibits the translocation of NF-kB to the nucleus, and consequently the expression of genes regulated by NF-kB [2].

Several studies have found that activation of NF-kB promotes aggressiveness in breast cancer [11,13,35,36]. According to Tewari et al. [37], this transcription factor has been implicated in mechanisms that promote inflammation in the tumor microenvironment, leading to metastasis in breast cancer. In addition to promoting inflammation, some studies have

shown that NF-kB participates in the angiogenesis process in breast cancer by increasing the expression of the vascular endothelial growth factor (VEGF) gene [38].

NF-kB activation has also been associated with the resistance to radiotherapy, as well as with tumor recurrence in breast cancer. According to Yu et al. [39], following exposure to radiation, TNFα secretion dependent on NF-kB is critical for the self-support of NF-kB by a mechanism of autocrine positive feedback, contributing to tumor resistance and recurrence. Therefore, the decrease in gene and protein expressions of NF-kB promoted by melatonin treatment in the studied model of breast cancer reinforces the beneficial pharmacological effects of melatonin

Interestingly, our in vitro studies showed that melatonin treatment was able to increase only the protein expression of NF-kB in liver cancer cells. It suggests that melatonin may regulate NF-kB expression by a post-transcriptional mechanism, since each NF-kB subunit is subject to a variety of post-transcriptional modifications, including phosphorylation, acetylation, ubiquitination, sumolization and nitrozylation [40,41]. Although most researches have observed a decrease in NF-kB activation after treatment with melatonin [2,21], some studies have also observed its activation after melatonin treatment [42,43].

According to Karin & Sun [16], different studies have described a double effect of NF-kB activation in hepatocytes during the process of liver carcinogenesis. In the early stages of tumorigenesis or viral infection, NF-kB acts as a cytoprotective agent, as it prevents apoptosis of hepatocytes. In the more advanced stages of carcinogenesis, NF-kB is involved in malignancy, as it promotes the survival of modified hepatocytes [16].

The development of hepatocarcinoma is related to the compensatory increase of hepatocytes, which is a direct consequence of the high apoptosis of these cells, associated with the absence of IKK and deficient activation of NF-kB [10,44]. Thus, in the early stages of tumorigenesis, the cytoprotective effect of NF-kB is beneficial, by preventing death of hepatocytes, thereby preventing the release of IL-1α by necrotic hepatocytes, and leading to compensatory proliferation. According Karin [10], compensatory proliferation is the main

cause of increased hepatocarcinogenesis in IKK deficient mice ($IKK\beta^{\Delta hep}$), the main NF-kB activating protein.

In addition, the loss of hepatic NF-kB function may increase hepatocarcinogenesis through the activation of JKK1 (N-terminal kinase c-Jun), which results in increased expression of cyclin D, important for the progression of the cell cycle, and consequently to proliferation of hepatocytes. [45].

It is also known that the activation of NF-kB can be induced by oxidative stress. Thus, since the cells are stimulated by an oxidative stress inducer, IkB is phosphorylated and NF-kB is translocated to the nucleus. There, it binds to the ĸB response element in the enhancer and promoter regions of its target genes. An initial response to oxidative stress is the activation of antioxidant enzymes, including superoxide SOD (superoxide dismutase), GPx (glutathione peroxidase) and CAT (catalase). In addition, genes encoding antioxidant enzymes are present in the promoter gene region of the responsive elements of kB [41].

Delerive et al. [43] have shown that the orphan nuclear receptor RORα interferes negatively with NF-kB signaling, by inhibiting its translocation to the nucleus. RORα acts on NF-kB through the induction of its inhibitory protein IkB. Melatonin is a repressor of the transcriptional activity of RORα by promoting antioxidant action and thus allowing NF-kB to activate the transcription of antioxidant genes [43,46]. This action would contribute to blocking the progression of hepatic carcinogenesis in its early stage.

Similar to our work, Caballero et al. [46] also observed differential modulation of NF-kB expression between SAMP8 (Senescence-accelerated mice) and SAMR1 (senescence-accelerated resistant mice) after treatment with melatonin. In this study, the authors observed increased expression of NF-kB in SAMR1 mice and, on the other hand, decreased expression of this nuclear factor in SAMP8. According to the authors, both responses (activation or inactivation of NF-kB) may exist in SAM mice, but always correlate with a beneficial effect on brain aging, corroborating the use of melatonin in the treatment of brain dysfunction caused by aging and damage of oxidative stress.

In addition to the gene and protein analysis of NF-Kb, we also performed breast tumors size analysis after treatment with melatonin. Ours results showed that melatonin treatment reduced the tumor growth. In studies by Jardim-Perassi et al. [23] and Cos et al. [47] nude mice bearing MDA-MB-231 and MCF-7, respectively, treated with melatonin also showed smaller tumor size comapred to control animals.

It should be noted that, there are multiples means by which melatonin can influence cancer development, some of these actions are mediated by well-known melatonin receptors, that are members of the G protein coupled, while others are receptor-independent [48]. MDA-MB-231 cells, used in the *in vivo* study, is a triple negative breast cancer cell line, which express low levels of the MT1 receptor. Melatonin can exert antitumoral properties by a set of complex mechanisms of action, not necessary involving the receptor pathway. Thus, melatonin may act directly, independently of its receptors or via them, making it difficult to understand the action of melatonin at the cellular level [21,49]. With respect to HepG2 cells, Carbajo-Pescador et al. [50] reported that melatonin acts in hepatocarcinoma HepG2 cells through MT1 melatonin receptor by modulation of cAMP basal levels and ERK kinase activation.

In conclusion, melatonin seems to play a double role in the expression of NF-kB, depending on the cell type. The role of melatonin on NF-kB in breast carcinogenesis seems to be better defined. However, in hepatic carcinogenesis, its role remains controversial. Further studies are necessary to better elucidate the action of melatonin in NF-kB, since this transcription factor acts on different signaling pathways that are fundamental for carcinogenesis. Such studies may reveal that, depending on the tissue type as well as the tumor stage, the activation or inhibition of NF-kB may be beneficial.

ABBREVIATIONS

NF-kB, factor nuclear kappa B; CHC, carcinoma hepatocelular; HepG2, hepatocyte carcinoma.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGMENT

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AUTHORS' CONTRIBUTIONS

DAPCZ conceived the study, designed the experiments and drafted the manuscript. JC, JPSF and CZB carried out the *in vitro* experiments, immunohistochemistry**,** immunocytochemistry and qPCR. BVJP, RPJ and NMS helped with *in vivo* model. BVJP and RPJ helped with the production of the manuscript. MGM helped in qPCR analysis. APG helped in immunohistochemistry and immunocytochemistry. JC assisted in the analyses of data and with the production of the manuscript. All authors read and approved the final manuscript.

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LEGENDS

Figure 1. Photographs showing (A) vehicle-treated tumor (control group) and tumor treated with melatonin for 21 days (B) Antitumor effects of melatonin on mammary tumor growth. Melatonin reduced the tumor growth in breast cancer nude mice.

*P<0.05 statistically significant difference when compared to control.

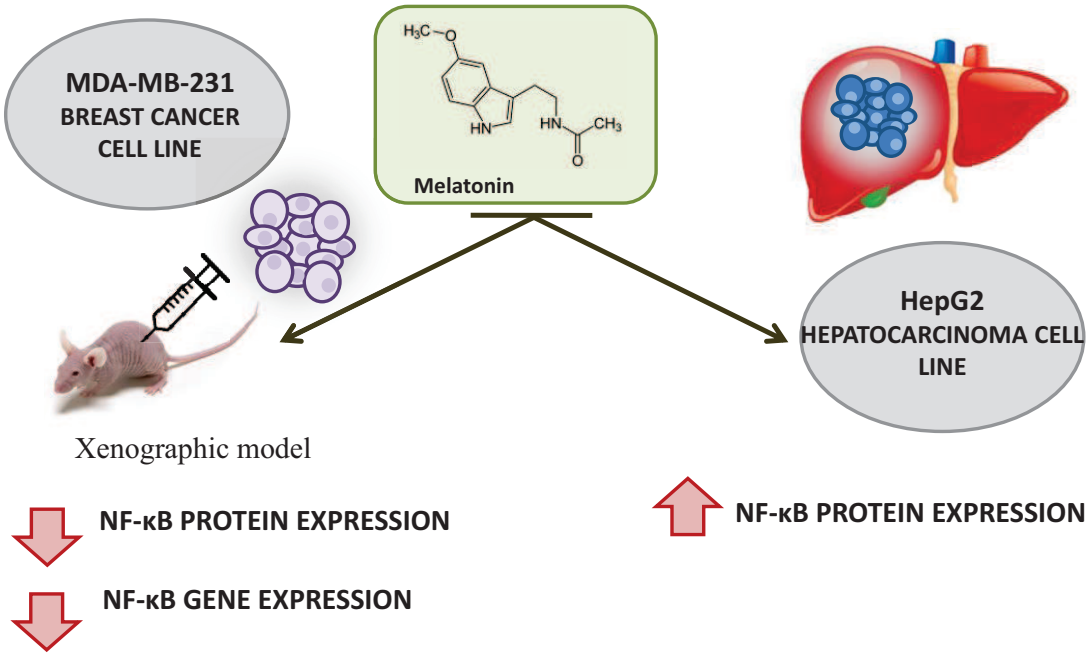
Figure 2. Immunohistochemistry of the protein NF-kB in breast cancer , showing breast tumors treated with vehicle (control) and treated with melatonin (A) Vehicle-treated tumor (control group). (B) Tumor treated with melatonin for 21 days. (magnification, x40) (C) Densitometric analysis of NF-kB protein expression in the experimental groups. Data are presented as the mean optical density ± standard error, and are shown in arbitrary units. (D) Analysis of gene expression of NF-kB in breast cancer tumors after treatment with melatonin. Data are expressed in 2^(-DDCt).

*P<0.05 statistically significant difference when compared to control.

Figure 3. Immunocytochemistry of the protein NF-kB in HepG2 cells, (magnification, x40) (A) Vehicle-treated cells (control group). (B) Cells treated with melatonin (1 mM) for 24 h. (C) Densitometric analysis of protein expression of NF-kB in the experimental groups. Data are presented as the mean optical density ± standard error, and are shown in arbitrary units. (D) Analysis of gene expression of NF-kB in HepG2 cells after treatment with melatonin (1 mM). Data are expressed in 2^(-DDCt).

*P<0.05 statistically significant difference when compared to control.

Graphical Abstract



ARTIGO CIENTÍFICO II

Melatonin treatment in breast cancer: the impact in transcriptomic networks of a xenograft model

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Running title: Transcriptomic data in response to melatonin

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Key words: Melatonin; RNA-Seq; Next generation sequencing; xenograft; breast cancer

ABSTRACT

Melatonin is a pleiotropic anti-cancer molecule that control cancer growth by multiple mechanisms. RNA-Seq is a useful tool to potentially evaluate response to therapy and its use in xenograft tumor models can differentiate the changes that occur specifically in tumor cells or in the tumor microenvironment (TME). The aim of this study was to perform the transcriptome analysis by RNA-Seq to identify melatonin actions in a xenograft model of triple-negative breast cancer. MDA-MB-231 cells were injected into the mammary gland of the Balb/c nude mice, which were treated with melatonin or vehicle during 21 days. RNA-Seq was performed on the Illumina HiSeq2500 and data were mapped against human and mouse genomes separately to differentiate species-specific expression. Differentially expressed (DE) genes were identified (Padj<0.1) and Weighted Gene Co-expression Network Analysis (WGCNA) was used to detect clusters of highly co-expressed genes. Results showed that melatonin treatment reduced tumor growth ($p<0.05$). 57 DE genes were identified in murine cells, which represented the TME, and were mainly involved in immune response. The WGCNA detected co-expressed genes in tumor cells and in TME, which are also related to immune system among other biological processes. In addition, we validated the upregulation of two genes (Tnfaip8l2 and Il1f6) by melatonin in the TME, which play an important role in the immune system. Taken together, transcriptomic network suggest that melatonin anti-tumor actions are especially by the modulation of immune response in the TME in this xenograft tumor model.

INTRODUCTION

Breast cancer is the most common type of cancer among women and it shows high incidence and mortality rates[1]. Among the phenotypic subtypes of breast cancer, the triple-negative (negative for estrogen (ER), progesterone receptor (RP) and human epidermal growth factor receptor 2 (HER-2/neu)) lacks specific targets[2] and had the poorest survival rate[3]. Thus, it remains dependent of conventional chemotherapy, radiation and surgery[4], and therefore therapeutics agents that show effectiveness in this subtype are of special interest.

Melatonin is a hormone synthesized mainly by the pineal gland and is considered a "neuroendocrine translator" of the light-dark cycle, displaying several physiological functions[5]. Several anti-tumors actions were ascribed to melatonin with multiple underlying mechanisms being proposed[6,7]. In breast cancer, its efficacy is mainly described in ER-positive breast cancer cell line MCF-7, in which physiological doses can exert anti-tumor effects, whereas in triple negative mammary tumors, such as the MDA-MB-231 cell line, pharmacological doses are needed to exerts anti-tumor effects[8]. In previous studies, our group has shown several anti-tumor effects of melatonin in MDA-MB-231 models[8–10]. Its molecule can act by the membrane receptors, MT1 and MT2, which are expressed in a variety of tissues, including breast, skin, blood vessels and immune system cells[11]. Melatonin can also act by receptors-independent mechanisms by crossing the membrane and interacting with intracellular proteins and nuclear receptors RZR / ROR (retinoid Z receptor / orphan receptor for retinoid)[12], and exhibit antioxidants [13]and anti-inflammatory properties[14] .

It is know that tumor biology is influenced by the intrinsic microenvironment and the host immune response[4]. Immune cells present in the tumor microenvironment (TME) can exert ambiguous functions during carcinogenesis, being able to eliminate tumor cells, or, conversely, can be recruited by cancer cells allowing the tumor growth[15–17]. A successful antitumor immune response requires many steps, involving not only the immune cells, but also others components of the TME, such as the extracellular matrix (ECM), which serves as a physical barrier to prevent immune infiltration and promote immune escape[18].

In this context, human tumor xenografts are commonly used to evaluate the response to therapy, as in these models the tumor growth is dependent on the interplay between the human tumor cells and murine stromal cells[19]. Athymic nude mice are commonly used to this purpose, which are T cells deficient, allowing the engraftment and growth of the tumor cells from the xenograft after the implantation[20]. Although in this model the immunodeficiency is severe, it is not absolute, as the nude mutation allows an intact humoral adaptive immune system and an intact innate immune system[21]. In addition, despite the deficient immunologic environment, xenograft models preserve other important characteristics of the TME as the ECM, tumor stroma-associations, vascularization and three-dimensional structure of the tumor[21], mimicking, at least to some extent, the biological situation in a human organism[20].

Currently, RNA-Seq technique has spread well beyond the genomics community and has become a standard part of the toolkit of the research community[22]. This approach can be used for different applications, which include the detection of differentially expressed (DE) genes, and shows high amplitude and accuracy. In addition to identifying DE genes, a weighted gene coexpression network analysis (WGCNA) has recently been proposed as a technique to explore the relationships between genes and identify intrinsic modules of coordinately expressed genes[23,24]. The use of RNA-Seq in xenograft samples can be a useful tool to potentially identify biomarkers and direct changes in response to therapy, differentiating the human tumor cells from the cells of the TME, characterized by the murine cells[19].

Thus, the aim of this study was to perform the transcriptome analysis to evaluate the pathways of melatonin action in this MDA-MB-231 breast cancer xenograft model previously established.

2. METHODS

2.1. Breast cancer xenograft model

All procedures were approved by the Ethics Committee on the Use of Animals of Faculdade de Medicina de São José do Rio Preto (001-003336/2014).

Human triple-negative breast *cancer cell line MDA-MB-231 (***American Type Culture Collection, Manassas, Virginia, USA**) was cultured in RPMI-1640 (Life Technologies, Carlsbad, California, United States) supplemented with 10% of fetal bovine serum (FBS) (Life Technologies, Carlsbad, California, United States) and 1% of penicillin/streptomycin (Life Technologies, Carlsbad, California, United States) at 37°C and 5% CO_2. Cells *were* detached using *trypsin*, suspended in the growth medium, washed with phosphate-buffered saline (PBS) twice and re-suspended at the 3 x 10^6 cells to be inoculated in the right 4th mammary gland of Balb/c athymic nude mice (n=10).

After tumor cells inoculation, mice were randomly divided into control and melatonin-treated groups (n=5 each). The treatment was performed in accordance with a previous study[8]. Control mice received an intraperitoneal injection (IP) of vehicle solution, and melatonin-treated mice received melatonin (Sigma, St. Louise, MO, USA) by IP at a dose of 40 mg/kg of body weight. Treatment started one day after tumor cell inoculation and was given five days a week, for 3 weeks. Mouse weight was periodically monitored during the experiment. Tumor growth was measured by digital caliper and tumor volume was calculated based on caliper measurements by the formula: Tumor volume =½ (length × $width^2$).

2.2. Samples collection and RNA extraction

At the end of the experiment, mice were euthanized using *ketamine* and *xylazine* and the tumors tissue were collected. Tumors were immediately frozen in liquid nitrogen and then kept at -80°C until RNA extraction. Total RNA was extracted from the tumors using TRIZOL reagent (Invitrogen Life Technologies® - Sao Paulo, SP, Brazil), following manufacturer's instructions.

RNA quality and quantity were assessed using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to the manufacturer's instructions (Agilent Technologies Ireland, Dublin, Ireland). All samples presented RNA integrity number (RIN) higher than 8.0 (Supplementary **Fig.** 1), and then were used in the transcriptomic analysis.

2.3. Preparation and Sequencing of Illumina RNA libraries

RNA-Seq libraries were created using Truseq RNA-Seq Library Prep Kit v2. The experiment was paired-end with 100nt read length, performed on the Illumina HiSeq2500 sequencer.

In summary, 1 µg of total RNA was used to isolate mRNA poli(A) by two rounds of purification using oligo dT magnetic beads followed by fragmentation and cDNA synthesis by random hexamer primers and reverse transcriptase. Next, end repair and 3' ends adenylation of the fragments was performed by adding a nucleotide A (*A-Tailing Mix)* to the 3' end in order to prevent them to binding to each other during the ligation of adapters. Bar-coded adapters were ligated to the cDNA fragments and a PCR reaction was performed to produce the sequencing libraries. The quality of libraries and quantification were performed using Agilent 2100 Bioanalyzer and qPCR with KAPA Library Quantification kit (KAPA Biosystems, Foster City, USA). Adapter-ligated cDNA fragment libraries were run on Illumina HiSeq 2500 equipment using TruSeq PE Cluster Kit and TruSeq SBS Kit (2 x 100 bp). All 10 samples were sequenced in one lane, producing about 30 million reads per library.

2.4. Reads alignment and differential expression analysis

Initially, sequencing quality was evaluated by FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adaptors and poor quality bases were trimmed by Trimmomatic software[25] and only reads with at least 50bp in length were kept for further analysis.

An alignment strategy was used to especially analyze data of xenograft samples, which contain two genomes (murine and human). Therefore, to differentiate human and mouse expression we used a strategy similar to the species-specific RNA-Seq workflow used by Bradford et al. (2013)[19]. Common reads between the two genomes (those aligning with equal similarity in humans and in mice) were discarded. Although some information may be lost by discarding the "common reads", it is an important step to avoid false positive.

To differentiate human and mouse expression, the alignment was performed to filter out mouse-like reads before mapping to the human reference and vice versa, and data were mapped against human (GRCh37/hg19) and mouse (NCBI37/mm9) genomes separately, using the STAR software. The HTSeq was employed for read counts and DESeq2 was used to identify DE genes between melatonin treated and control tumors for each species. DE genes were obtained for each organism: human characterizing the tumor cells MDA-MB-231 and mouse characterizing the TME. Genes with adjusted p-value ≤ 0.1 and fold change (FC) values ≤ -1.5 and ≥ 1.5 were considered as DE genes. Genes with FC ≥ 1.5 were considered up-regulated in the melatonin-treated group whereas genes with FC ≤ -1.5 were considered down-regulated in the melatonin group.

2.5. Differential connectivity analysis

First, as a quality control, genes with zero expression values in more than half (5) of the samples were excluded. Then, genes with average read counts less than 10 and standard deviation less than 5 were excluded. This because genes with low counts may represent a bias of sequencing and genes with little variation contribute less to the network analysis. A total of 13669 human genes and 13899 mouse genes remained. Data were then divided into 2 sub-networks, one composed of melatonin-treated animals and the other composed of control animals. The sub-networks were created using WGCNA package in R environment[26], and the total connectivity value of each gene within the network was calculated. Connectivity is the sum of the correlations between the expression of a given gene and the expression of each of the other genes in the network. Connectivity values of each gene in the two sub-networks were then divided by the maximum connectivity found in each sub-network, so that the two sub-networks could have comparable values. The connectivity value of each gene from the control group was then subtracted from the connectivity value of the same gene in the melatonin-treated group. The resulting values range from 1 to -1 and genes with values greater than | 0.6 | were considered to be differentially connected. Negative values are associated with highly connected genes in the

control group and poorly connected in the melatonin-treated group, just as positive differential connectivity values are associated with highly connected genes in the melatonin-treated group and poorly connected in the control group. A change in gene behavior, from highly connected to lowly connected and vice-versa can indicate an important role of this gene in response to melatonin treatment.

2.6. Module preservation analysis

For module preservation analysis human and mouse data were also analyzed separately. The same 13669 human genes and 13899 mouse genes which passed quality control were used to create a smaller set of genes, by selecting the 3000 most connected genes in each of the groups (melatonin vs control), which resulted in a total of 4557 human genes and 5383 mouse genes. A gene co-expression analysis was then performed using WGCNA package in R environment and modules of co-expressed gene were identified for the control group. Different color names were assigned to each module. Those modules were then compared with the expression data of the melatonin-treated group for the evaluation of module preservation between the two groups. The preservation measure is given by Zsummary and modules with values greater than 10 are highly preserved: modules with values between 2 and 10 have poor to moderate preservation and modules with values below 2 are not preserved. Modules which are not preserved can indicate sets of genes influenced by melatonin treatment.

2.7. Functional enrichment analysis

The online tool WEB-based GEne SeT AnaLysis Toolkit (Webgestalt) was used to perform the functional enrichment of GO terms and KEEG analysis for the DE genes, differentially connected genes and gene modules. Human and murine data were tested separately. For the DE genes lists, the specific genome for each organism was used as background, while a list of all genes that passed the quality control was used as background to each organism for the differentially connected genes and gene modules. P-values for each

term were obtained through hypergeometric analysis and corrected for FDR by Benjamini–Hochberg method. Terms were considered significant when Padj ≤ 0.1.

2.8. Real time PCR

Quantitative real-time PCR was used to validate selected DE genes by melatonin treatment in the TME. First, total RNA (100 ng) was used to generate cDNA by using the High-Capacity cDNA Reverse Transcription (Applied Biosystems). Then, gene expressions of tnfaip8l2, il1f6 and nfkb1 were detected by real-time PCR (*Step One Plus* (*Applied Biosystems*) using SYBR Green master mix (Life Technology). GAPDH was used as the internal control. The specific primers were as follows: *Tnfaip8l2* 5'-CTGGCTCTGGCTACACGATT 3' (forward) and 5'- ACCTCACCGAAGCTAAGTGC-3' (reverse); *Il1f6* 5'- CTCTTGAGACGAACAGGGGG-3' (forward) and 5'-ATGTTCCCTTCCCCAAGCTG-3' (reverse); *Nfkb1* 5'- ATTCCGCTATGTGTGTGAAGG-3' (forward) and 5'- GTGACCAACTGAACGATAACC-3' (reverse); *Gapdh* 5'-GGTGAAGGTCGGTGTGAACG -3'(forward) and 5'- CTCGCTCCTGGAAGATGGTG -3'(reverse) and used at 200 nm . The relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$. Experiments were performed in triplicate.

2.9 Immunohistochemistry

Tumor sections were prepared for paraffin blocking following the standard procedure. Paraffin-embedded tissue sections of 3 µm were obtained and then deparaffinized and rehydrated. Sections were incubated with Hydrogen Peroxide Block (Spring Bioscience, Pleasanton, CA, USA) for 10 minutes and then washed in PBS 1 X. Protein Block solution (Spring Bioscience, Pleasanton, CA, USA) was added and sections were incubated for 10 minutes at room temperature and washed with PBS 1X. Then, antigen retrieval was done in Pan Steam at 95°C (Arno, São Paulo, SP, Brazil) with Citrate buffer (pH 6.0) for 30 minutes. Sections were incubated with the primary antibody anti-cleaved Caspase-3 (Ab4051 - Abcam, Cambridge, MA, USA) at 4º C overnight and then with *horseradish peroxidase (*HRP)

conjugate, containing the goat anti-rabbit secondary antibody conjugated to HRP, for 15 minutes at room temperature. Sections were washed in PBS 1X and incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Spring Bioscience, Pleasanton, CA, USA) for 3 minutes and then counterstained with Harris's hematoxylin for 40 seconds. Finally, sections were dehydrate and required and *coverslipped with* Permount mounting medium (Fisher Scientific,Hampton, New Hampshire, USA). Negative control was obtained by omitting the primary antibody, and tonsil was used as internal positive control of the assay.

Cleaved caspase-3 immunostaining was analyzed based on the intensity of the staining by optical density using ImageJ software (NIH, Bethesda MD, USA). Multiple fields from each histological slide were examined using the Nikon Eclipse E 200 microscope (Nikon Instruments, Melville, NY, USA). Then, three areas were photographed at 40X and 20 spots (small circular regions of interest (ROI)) were randomly selected in each photographed area, in particular demarcated areas with distinct brown staining. The intensity was determined from 60 spots marked on each slide. Thus, the intensity was determined from a total of 60 spots of each sample to average the relative intensity of immunoreactivity. The values were obtained as arbitrary units (a.u.), and the mean optical density (M.O.D.) indicated the specific staining intensity in the immunoreactive areas.

3. Statistical analyzes

To analyze the real time PCR and immunohistochemistry data, the results were previously submitted to descriptive analysis to determine the normality. The means of the measurements for each group (melatonin versus control) were compared by Student's t-test. All values were expressed as mean ± *standard deviation (SD)*. Analyses were performed using the GraphPad Prism6 software (Graph-Pad Software, La Jolla, CA, USA) and values less than 0.05 were considered statistically significant.

RESULTS

Melatonin treatment controls tumor growth

Tumor growth was slower in the melatonin-treated group than the control group. There was a significant difference in tumor volume between the groups from day 18 until day 21 ($p<0.05$; **Fig. 1**). The mean tumor volume on day 21 was 18.93 ± 5.39 and 75.68 ± 25.83 for mice treated with melatonin and control, respectively.

DE genes by melatonin treatment are detected only in the TME

First, we aligned each RNA-Seq sample against two reference genomes, human (GRCh37/hg19) and mouse (NCBI37/mm9) separately. Data showed that 57.24% of reads mapped uniquely to human, 29.66% reads in mouse and 11% reads mapped to both human and mouse genomes, which were excluded from further analysis (Supplementary Table 1).

Regarding MDA-MB-231 breast cancer cells, that is, reads aligned to human genome, a total of 31807 genes were detected. Genes were tested for differential expression (**Fig. 2)**, however, only the pseudogene GAPDHP65 was DE after melatonin treatment showing log2 FC -1.07 and padj = 0.01 (Supplementary **Fig. 2)**.

In mouse cells, which characterize the TME, 24719 genes were detected. Of those, 57 genes were DE between melatonin-treated and control mice (adjP < 0.1) (**Fig. 3**), being 14 downregulated (log2 FC ≤ -0.58) and 43 upregulated by melatonin treatment (lod2 FC ≥ 0.58) (**Supplementary Table 2**).

The functional enrichment analyses of GO terms showed that the DE genes of TME were distributed into the categories of biological process, molecular function and cellular component, shown in **Supplementary Fig. 3**. In addition, DE genes involved in the most relevant biological processes enriched for the TME after melatonin treatment are shown in **Table 1**. These genes were mainly involved in immune system process and cell motility (**Table 1**), evidencing the role of melatonin in the immune response. In addition, the

pathways enriched for DE genes in TME after melatonin treatment include the chemokine signaling, pathways in cancer and Wnt signaling, as presented in **Table 2**.

Coexpression analysis between control and melatonin-treated tumor samples

The aim of differential connectivity analysis is to uncover differences in genes behavior in two sub-networks, one generated by expression data from 5 control tumors and another generated by expression data from 5 melatonin-treated tumors. Human and mouse data were analyzed separately.

In tumor cells, we detected 714 differentially co-expressed genes between melatonin-treated and control tumors (K_{diff}>|0.6|). Of those, 331 were highly connected in control tumors, while 383 were highly connected in melatonin-treated tumors (Supplementary Table 3). Functional enrichment analysis of GO terms showed that the highly connected genes in melatonin-treated tumors were enriched to protein-DNA loading ATPase activity and intracellular membrane-bounded organelle (**Supplementary Fig. 4**). For the control tumors, the 331 connected genes were related to cytokine binding and cellular component *major histocompatibility complex* (MHC) protein complex, suggesting an association with the immune system (**Supplementary Fig. 5**). Likewise, interestingly these connected genes of control tumors were also enriched for signaling pathways related with immune response such as "Antigen processing and presentation", "Allograft rejection" and "Acute graft-versus-host disease", as well as the cell adhesion molecules (CAMs) pathway, which also plays a critical role in the immune response (**Supplementary table 4**).

Regarding the coexpression genes from the TME, 13899 genes passed the quality control and were tested for further analysis. We detected 1345 differentially co-expressed genes in the TME between melatonin-treated and control groups. Of those, 1047 were highly connected in control group, while 298 were highly connected in melatonin-treated tumors (Supplementary Table 5).

Highly connected genes in the melatonin-treated group were enriched to processes related with the inhibition of cellular process, such as "negative regulation of cellular

process", "negative regulation of RNA metabolic process", among other (**Supplementary Fig. 6**). Interestingly, the opposite was observed for the 1047 connected genes in the control group. Most of the biological processes enriched for the control group were those negatively regulated in the melatonin-treated group as shown in **Supplementary Fig. 7**. In addition, the "protein processing in endoplasmic reticulum" pathway was also enriched in the control group, suggesting high cell activity consistent with cell proliferation and tumor growth.

Module preservation analysis between control and melatonin-treated groups

Gene members of the same module are supposed to work cooperatively in related pathways or to be under the control of a common set of transcription factors. Thus, we identified 10 gene modules of co-expressed and highly interconnected genes in human tumor cells. Of those, three modules (pink, magenta and black) were discovered to show weak to moderate preservation, which may indicate that melatonin is acting on these gene sets (**Fig. 4A**). The pink module included 48 genes, magenta included 45 genes and black included 62 genes (**Supplementary table 6**). Genes of each individual module were used for functional enrichment. This analysis showed that the black module was enriched to cellular component nuclear matrix, while the magenta module was enriched to intracellular-membrane organelle and 5 genes were involved in the metabolic pathway (**Supplementary Fig. 8**).

In the mouse cells, that is, the TME, 39 modules were found. Because of the high number of modules found, they were grouped using a threshold of 90% correlation between the expression of the modules, resulting in a total of 16 co-expressed gene modules. Of these, three modules (darkolivegreen, yellowgreen and orangered) were strongly related to melatonin treatment in the TME (**Fig. 4B**). These modules included 49, 41 and 30 genes for darkolivegreen, yellowgreen and orangered modules respectively (**Supplementary table 7**).

Genes of darkolivegreen module were enriched to processes related to the posttranscriptional regulation of gene expression (**Supplementary Fig. 9**), while those of yellowgreen module were enriched to "Positive regulation of cell cycle arrest", "Cell cycle

checkpoint" (**Supplementary Fig. 10**) and the TGF-beta signaling pathway, which has a wide spectrum of cellular functions such as apoptosis, cell proliferation, differentiation and migration. Genes of orangered module were related to immune system development, leukocyte differentiation, T cell differentiation, and the signaling pathways of Focal adhesion and Regulation of actin cytoskeleton (**Supplementary Fig. 11**), evidencing again the action of melatonin in the regulation of the immune system in the TME.

Validation of genes modulated by melatonin treatment

Because melatonin does not appear to have its principal activity at the gene level in tumor cells, we evaluated the expression of cleaved caspase-3 at protein levels in the tumor tissue by immunohistochemistry. Our basic assumption was that coexpression and module preservation analyzes have shown processes related to cell proliferation and tumor growth, which are consistent with the reduced tumor growth, and possibly with an increase of apoptosis in melatonin-treated mice. In fact, results showed that caspase-3 expression was higher in the melatonin-treated tumors, indicating increase of apoptosis by melatonin (**Fig. 5**).

For the TME composed of murine cells, results showed that several processes related to immune system were enriched in all analyzes. Thus, we selected two DE genes by melatonin treatment (tnfaip8l2 and il1f6) to perform individual validation by real time PCR. Interleukin 1 family, member 6 (Il1f6), also known as Interleukin-36α (IL-36α) shows an important pro-inflammatory role in chronic immune disorders[27,28] and recently its role in cancer has been described[28,29]. RNA-Seq results showed that il1f6 was overexpressed in the TME of melatonin-treated mice and the PCR results confirmed its increase (**Fig. 6A**).

Tumor necrosis factor-a (TNF-a)-induced protein 8-like-2 (Tnfaip8l2) is a member of the tumor necrosis TNFAIP8 family and an essential negative regulator of both innate and adaptive immunity, showing high expression in immune cells[30]. Its gene was upregulated by melatonin treatment in the TME, and confirmed by individual PCR (**Fig. 6B**).

DISCUSSION

Melatonin regulates a broad spectrum of functions ranging from the regulation of circadian rhythms and antioxidant properties to the control of development and growth of different types of tumors[31,32]. In fact, its anti-proliferative and pro-apoptotic effects have been described in a variety of tumors in both *in vitro* and *in vivo* studies[6,7,33].

As shown in a previous study[8], we also confirmed in this study that melatonin treatment was able to reduce the tumor growth in a xenograft breast cancer model. Consistent with this result, melatonin treatment was able to increase the expression of cleaved caspase-3, which is the main executioner of apoptosis. Similarly, in a mammary carcinoma model induced by 7,12-di-methylbenz(a)anthracene (DMBA), melatonin acted as a protective agent, increasing the apoptosis as observed by the upregulation of TNF-alpha, tissue caspase-3 activity and percentage of DNA fragmentation[33,34]. In addition, we have previously shown the reduction of a cell proliferation marker (Ki-67) by melatonin in a xenograft breast cancer model[8].

It is know that melatonin is a pleiotropic anti-cancer molecule, which controls the development and growth of cancer via multiple mechanisms[35]. Recently, Reiter et al., (2017)[7] described melatonin as a ubiquitously-distributed molecule and suggested that its diverse actions could be merely epiphenomena of an underlying more fundamental melatonin action that remains to be discovered. In our study, RNA-Seq reveals that melatonin treatment did not directly change the gene expression in the tumor cells, but it modulates gene expression especially in the TME, which may have contributed to the tumor growth control. DE and coexpressed genes, as well as the gene modules, were enriched to processes related with different aspects of immune response. Although we used a T-cell deficient mice model (athymic Foxn1nu), which has limitations to studying the response to therapy[36], it is important to note that these mice show an intact humoral adaptive immune system as well as an intact innate immune system[37], which could, to some extent, lead to an immune response.

The connection between cancer and inflammation is widely recognized. The activation of the immune system can induce a potent anti-tumor response[38]. It has been

shown that immune system cells express both membrane and nuclear melatonin receptors[33,39]. In addition, the cells and organs of the immune system, as well as other organs, such as the skin, have the biosynthetic enzymatic machinery to produce melatonin[40], which can act as a paracrine, intracrine or an autocrine agent[33,39,41]. Thus, melatonin influence immune responses in different levels, which may help in the management of tumor growth[42].

Melatonin shows immunoenhancing properties[43], and its exogenous administration enhances both innate and cellular immunity[33], being able to regulate inflammatory cytokines and mediators, expression of transcription factors genes[42], and the haemopoiesis[44]. Melatonin administration also enhance the production of progenitor cells for granulocytes-macrophages, natural killer cells, monocytes and leukocytes[33], as well as the production of cytokines including interleukins (IL-2, IL-6, IL-12), interferon-gamma and TNF-alpha[43,45].

Although many studies have implicated melatonin as a positive regulator of the immune system, other authors have also proposed melatonin as an anti-inflammation agent[40]. In the early phases of inflammation, melatonin can activate pro-inflammatory mediators, such as IL-1 and TNF-alpha, while in the chronic phase, it contributes to the inflammation attenuation by downregulating cytokines, inducing the survival pathway in leukocytes and blocking oxidative stress by its antioxidant properties[33,40,42,46].

In this context, the action of melatonin on the immune system was evidenced in our study, which may modulate the immune response by different mechanisms. Specifically, we validated melatonin action on two genes involved in the immune response and tumor progression. Il1f6 (IL-36α), is a member of the IL-1 family of cytokines, composed also by other two agonists, IL-36-β (IL-1F8) and IL-36γ (IL-1F9) and one antagonist IL-36Ra (IL-1F5)[47,48]. The IL-36 family of cytokines supports the generation of pro-inflammatory immune responses and its possible functions in cancer still under investigation[48]. In 2014, Wang et al. reported that low expression of IL-36α was correlated with larger tumor size and poor prognosis of colorectal cancer patients[49] .

Recent studies have demonstrated the use of Il-36 as an immunotherapeutic agent in cancer therapy. Chang et al., (2017) showed that IL-36 gene therapy can inhibit the growth in xenograft model of epithelial ovarian cancer[28]. Moreover, its upregulation was able to regress the tumor masses in fibro sarcoma mouse model and reduce the Ki-67 expression[29]. IL-36γ has described to exert profound antitumor effects in a melanoma mice model, transforming the TME in favor of tumor eradication[48].

An interesting review by Weinstein and Storkus (2015) describes IL-36 as an orchestrator of Tertiary Lymphoid Structures (TLS), an ectopic lymphoid formation, in the TME. Authors suggest that IL-36 delivery or its production in the TME have potential protective and therapeutic roles in cancer-bearing host, by acting in both humoral and cellular immunity[50]. In this context, given the important role of Il1f6 on immune response, its increase in TME by melatonin is an important alteration that may be contributing to its capacity to control of tumor growth.

Another gene with involved in the immune system and upregulated by melatonin in the TME was the Tnfaip8l2. Despite of its role in maintaining immune homeostasis, Tnfaip8l2 is also involved in the development and progression of several tumors[51,52]. It has been shown that its expression is reduced or absent in some cancers, such as gastric cancer, lung cancer, hepatocellular carcinoma [51,53] and breast cancer[30]. Recently, Wang et al., (2017) showed that the overexpression of Tnfaip8l2 inhibited the tumor growth in a xenograft MDA-MB-231 breast cancer model. In addition, Tnfaip8l2 prevented the epithelial-to-mesenchymal transition (EMT) phenotype, by inhibiting the expression of β-catenin, cyclin D1 and c-Myc in MDA-MB-231 and MCF-7 breast cancer cells[54]. Similar results were also reported in a xenograft model of gastric cancer. Adenovirus-mediated Tnfaip8l2 overexpression suppressed the tumor growth by increasing apoptosis and reduced cell migration, invasion and metastasis via reversal of EMT[55].

Zhang et al., (2017) using a syngeneic model of breast cancer (Balb/c mice implanted with 4T1 cells) showed that the overexpression of Tnfaip8l2 inhibited the proliferation of 4T1 cells *in vitro* and *in vivo*. Interestingly, the treatment with Tnfaip8l2 gene delivery *in vivo*

promoted CD8+T and NK cell-mediated anti-tumor immune responses in the TME. In addition, Tnfaip8l2 inhibited the expansion and recruitment of myeloid-derived suppressor cells (MDSCs), which exert immune-suppressive effects and promote tumor progression[56]. Previously, the same authors showed that the overexpression of Tnfaip8l2 in MDA-MB-231 cells lead to a reduction of proliferation, migration and invasion *in vitro* and inhibited the tumorigenesis of breast cancer *in vivo*[30].

Taken together, studies indicate the importance of Tnfaip8l2, as well as the Il1f6, on the immune response. Thus, the capacity of melatonin to increase these genes in the TME may characterize new and additional actions of melatonin that contribute to the control of tumor growth. In conclusion, our study has shown that melatonin acts by regulating gene expression mainly in the TME, and suggest new actions to its molecule on modulation of immune response and tumor growth control in a MDA-MB-231 breast cancer xenograft mouse model.

Author Contributions

BVJP conducted the study, contributing with concept/design, acquisition of data, data analysis/interpretation and drafting of the manuscript. LLC contributed with acquisition of data and analysis. NMS and RPJ contributed with concept/design and acquisition of data. PAA, ORJ and RPJ contributed with data analysis/interpretation and drafting of the manuscript. RC contributed with concept/design, data interpretation and critical revision of the manuscript. HF contributed to data analysis/interpretation and critical revision of the manuscript. DAPCZ contributed with concept/design, data interpretation and critical revision of the manuscript.

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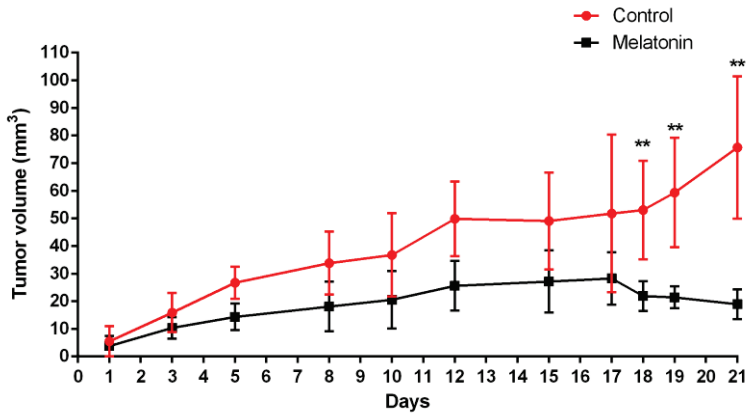


Figure 1. Melatonin treatment reduced tumor growth in MDA-MB-231 xenograft model. Tumor growth was monitored by caliper during 21 days. **p<0.005; Statistical significant difference between melatonin-treated and control groups using Student's t-test.

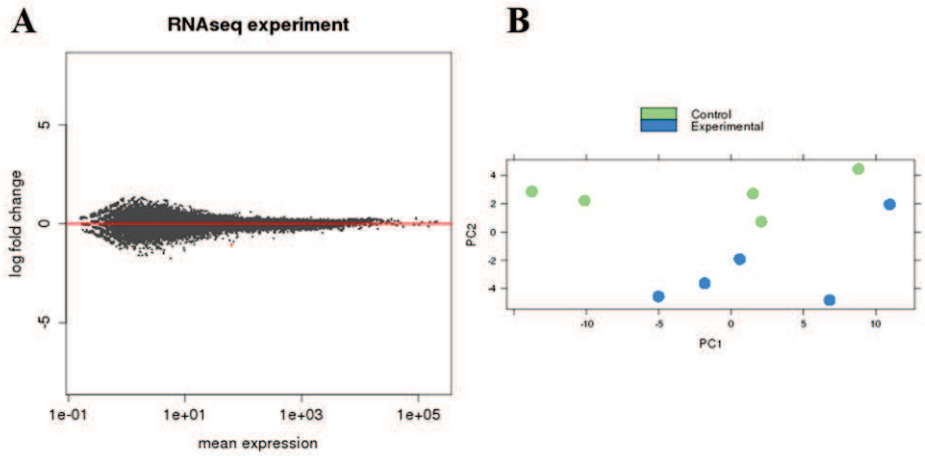


Figure 2. Differentially expressed (DE) genes between melatonin-treated and control groups in MDA-MB-231 human breast cancer cells. (**A)** Correlation analysis of gene expression between melatonin-treated samples vs. control samples. Red line shows the line of identity and each point represents a gene. (**B)** Principal component (PC) analysis was performed for the samples using the gene expression values. The percentage variability captured by the first three principal components is displayed across PC1 and 2 represented on X and Y axes. Scatter plot model of differential gene expression ratios in melatonin-treated breast cancer (blue dots) vs. control (green dots) shows a reasoned discrimination where the distance between dots is a dimensional measure for the similarity of the expression profiles.

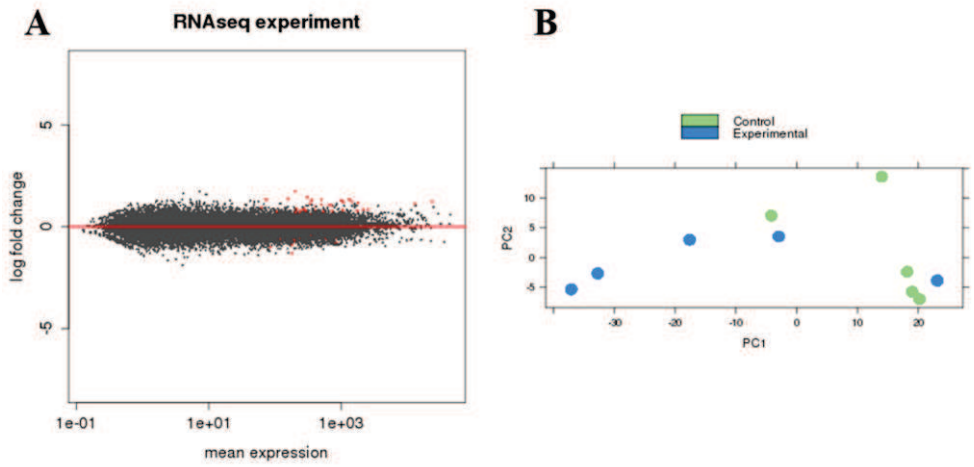


Figure 3. Differentially expressed (DE) genes between melatonin-treated and control groups in mouse cells, characterized as the TME. (A) Correlation analysis of gene expression between melatonin-treated samples vs. control samples. Red line shows the line of identity and each point represents a gene. (**B)** Principal component (PC) analysis was performed for the samples using the gene expression values. The percentage variability captured by the first three principal components is displayed across PC1 and 2 represented on X and Y axes. Scatter plot model of differential gene expression ratios in melatonin-treated samples (blue dots) vs. control (green dots) shows a reasoned discrimination where the distance between dots is a dimensional measure for the similarity of the expression profiles.

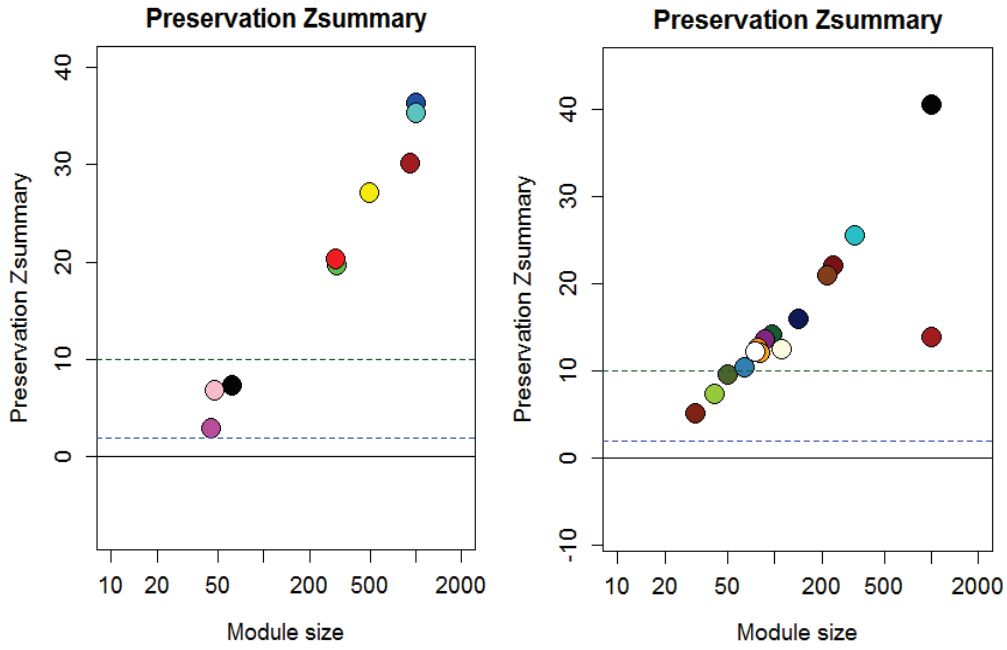


Figure 4. Weighted gene coexpression network analysis (WGCNA) identified modules of co-expressed genes. Values of Zsummary above 10 represent modules of genes highly preserved. Values between 2 and 10 represent poor to moderate preservation, which can

indicate sets of genes influenced by melatonin treatment. **(A)** Three modules of genes (pink, magenta and black) were identified to MDA-MB-231 human breast cancer cells; **(B)** Three modules of genes (darkolivegreen, yellowgreen and orangered) were identified to the tumor microenvironment (murine cells).

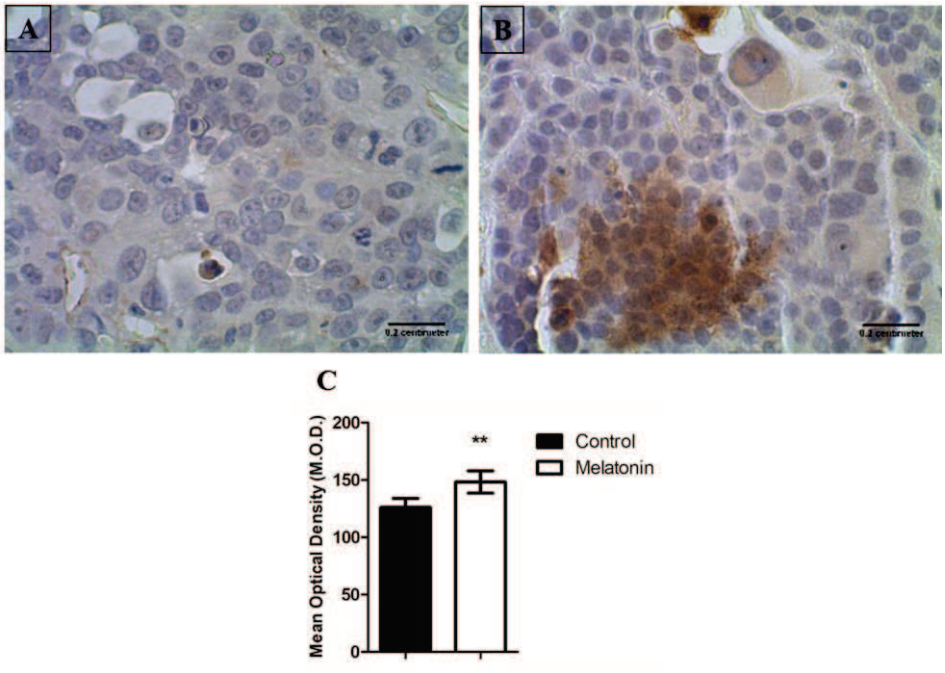


Figure 5. Immunohistochemistry staining with Cleaved Caspase-3 in breast cancer cells. **(A)** Representative image of control and **(B)** melatonin-treated tumor. Images were taken with 40X magnification. Scale bar = 0.2 cm. **(C)** A significant increase was observed in melatonin-treated tumors compared with control tumors (**p=0.0078).

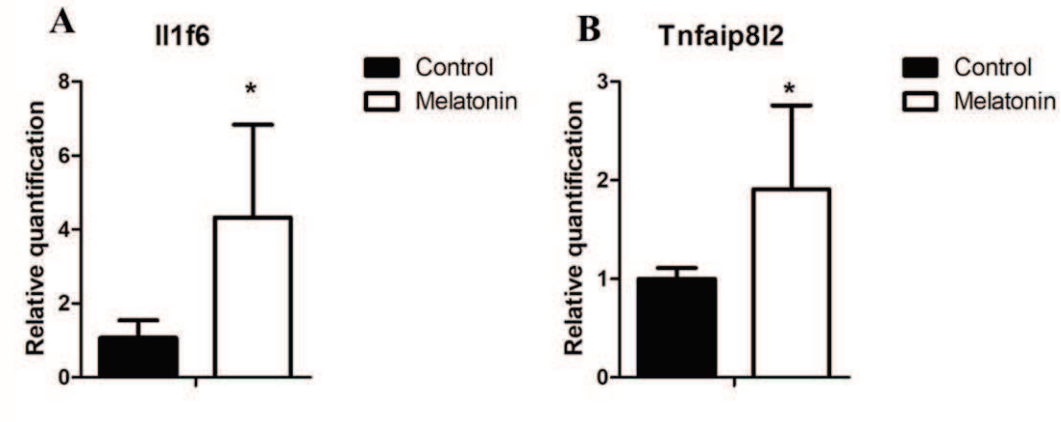


Figure 6. Analysis of gene expression by Real time PCR. (A) Il1f6; (B) Tnfaip8l2. Genes were normalized to GAPDH. Values are expressed as mean ± standard deviation (SD) of experiments performed in triplicate. $*p < 0.05$ melatonin-treated tumor vs. control tumors by Student t-test.

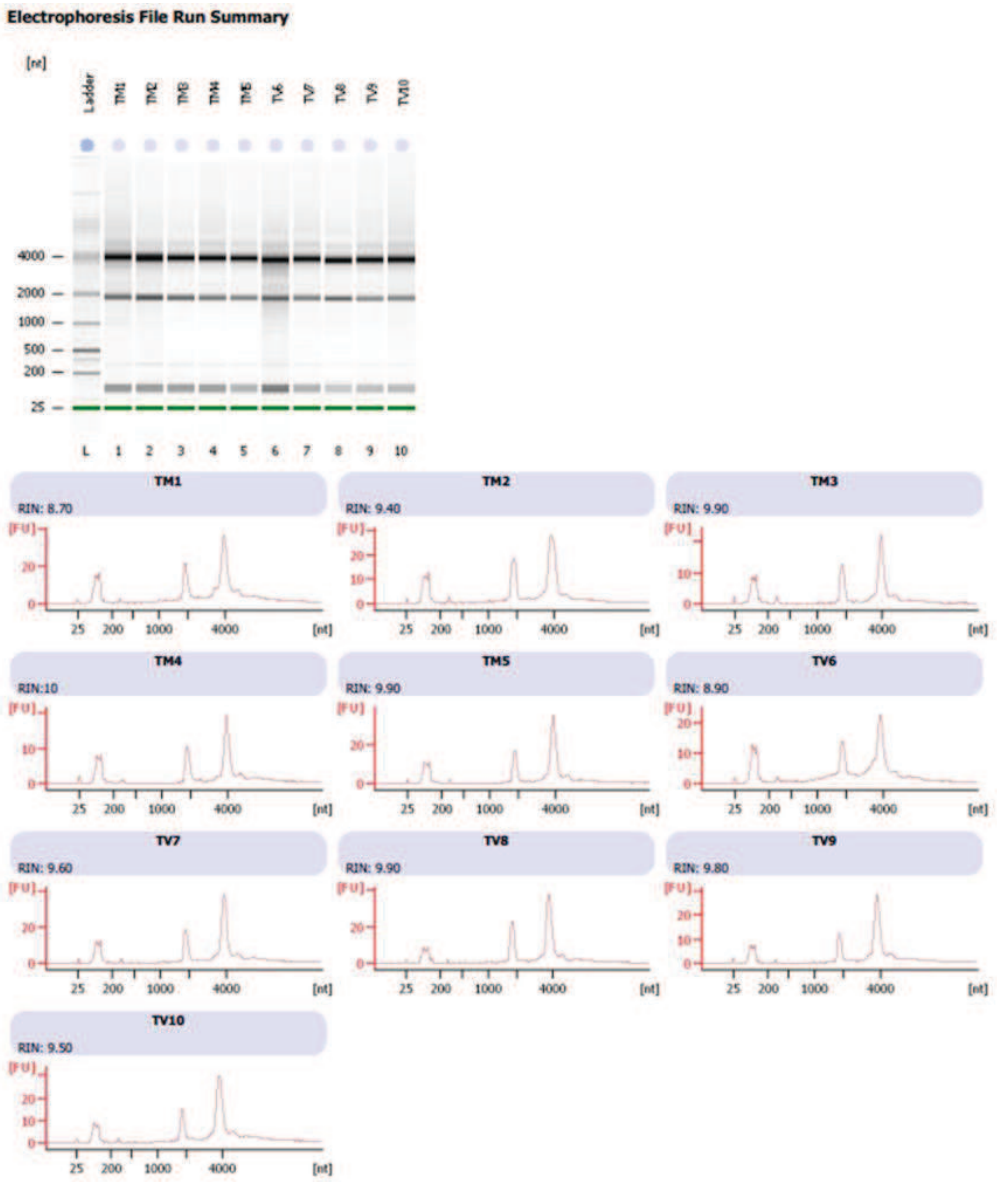
Table 1. DE genes associated with the most relevant biological processes enriched for the TME (mouse tissue) after melatonin treatment. Last column show the biological processes associated with each gene, represented by number and detailed bellow.

Gene	Log2FC	Gene name	Entrez Gene	Numbers corresponding to biological processes
F2rl1	-0.75	coagulation factor II (thrombin) receptor-like 1	14063	1-2-3-4-5-6-7-9-10-11-13-14
Dsg2	-0.95	desmoglein 2	13511	1-16
Egfl7	0.68	EGF-like domain 7	353156	1-3-4-5
Aif1	0.76	allograft inflammatory factor 1	11629	1-2-3-4-5-6-7-8-9-10-16
Ccl12	0.83	chemokine (C-C motif) ligand 12	20293	1-2-6-7-8-9-12-13-14
Mmp3	0.80	matrix metallopeptidase 3	17392	1-3-4-5-16
Apod	0.63	apolipoprotein D	11815	1-2-3-4-5-6-11-12-13-14-15
Prkca	-0.66	protein kinase C, alpha	18750	1-2-3-4-5-6-7-9-10-12-13-14-15
Mdk	0.92	Midkine	17242	1-3-4-5-14
Tnfaip8l2	0.79	tumor necrosis factor, α-induced protein 8-like 2	69769	2-12-13-14
Ctla2a	0.72	cytotoxic T lymphocyte-associated protein 2 α	13024	2-13-14
Ifit3	0.76	interferon-induced protein with tetratricopeptide repeats 3	15959	2-14
Ereg	0.78	Epiregulin	13874	2-14
Krt1	1.23	keratin 1	16678	2-14
Il1f6	1.74	interleukin 1 family, member 6	54448	13-14
Penk	-0.59	Preproenkephalin	18619	14
Defb14	1.33	defensin beta 14	244332	14
Gpx3	0.82	glutathione peroxidase 3	14778	15

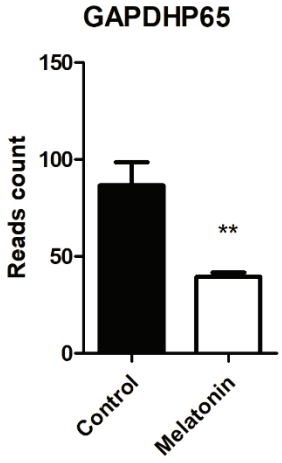
Table 2. Pathways enriched for differently expressed genes in tumor microenvironment (mouse tissue) after melatonin treatment.

PathwayName	Genes	Statistics
Chemokine signaling pathway	Ccl12, Gng11, Gngt2	C=185;O=3;E=0.18;R=17.06;rawP=0.0007;adjP=0.0042
Phosphatidylinositol signaling system	Prkca, Calm4	C=78;O=2;E=0.07;R=26.97;rawP=0.0025;adjP=0.0058
Glioma	Prkca, Calm4	C=66;O=2;E=0.06;R=31.88;rawP=0.0018;adjP=0.0058
ErbB signaling pathway	Prkca, Ereg	C=87;O=2;E=0.08;R=24.18;rawP=0.0032;adjP=0.0067
Pathways in cancer	Fzd1, Prkca, Col4a5	C=325;O=3;E=0.31;R=9.71;rawP=0.0037;adjP=0.0071
GnRH signaling pathway	Prkca, Calm4	C=99;O=2;E=0.09;R=21.25;rawP=0.0041;adjP=0.0072
Leukocyte transendothelial migration	Prkca, Cldn5	C=120;O=2;E=0.11;R=17.53;rawP=0.0059;adjP=0.0087
Vascular smooth muscle contraction	Prkca,Calm4	C=123;O=2;E=0.12;R=17.11;rawP=0.0062;adjP=0.0087
Tight junction	Prkca, Cldn5	C=137;O=2;E=0.13;R=15.36;rawP=0.0076;adjP=0.0094
Insulin signaling pathway	Ppp1r3b, Calm4	C=137;O=2;E=0.13;R=15.36;rawP=0.0076;adjP=0.0094
Cell adhesion molecules (CAMs)	Nectin3, Cldn5	C=149;O=2;E=0.14;R=14.12;rawP=0.0089;adjP=0.0104
Wnt signaling pathway	Fzd1, Prkca	C=154;O=2;E=0.15;R=13.66;rawP=0.0095;adjP=0.0105
Calcium signaling pathway	Prkca, Calm4	C=178;O=2;E=0.17;R=11.82;rawP=0.0126;adjP=0.0132
Focal adhesion	Prkca, Col4a5	C=200;O=2;E=0.19;R=10.52;rawP=0.0157;adjP=0.0157

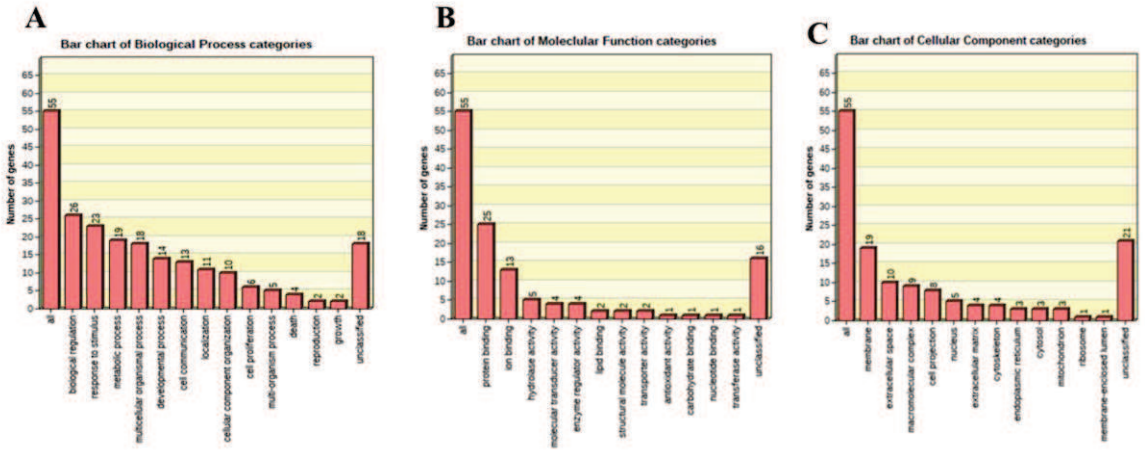
Supplementary



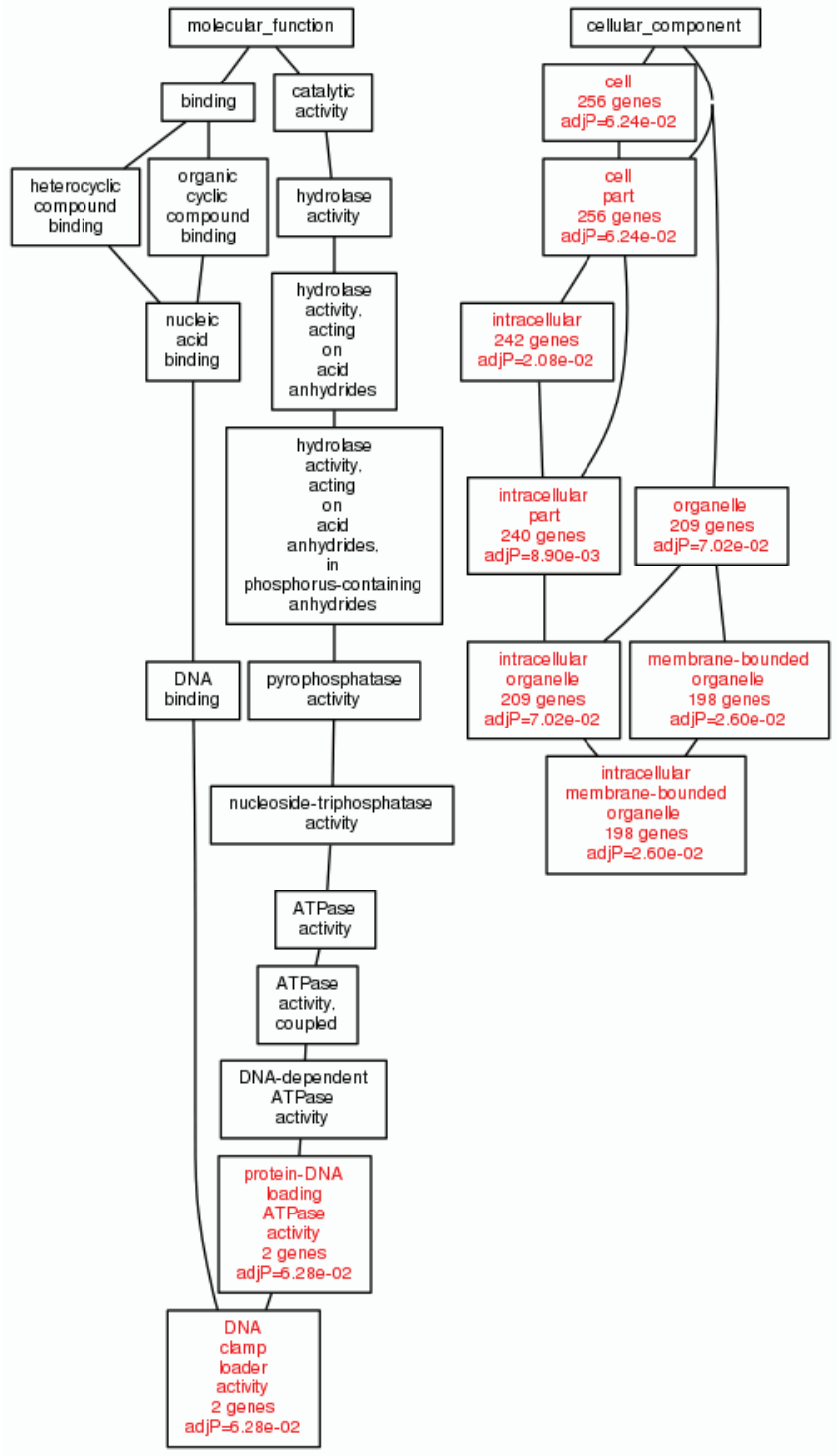
Supplementary figure 1. **Bioanalyzer electropherograms.** RNA quality for breast cancer samples. TM indicate the melatonin-treated samples and TV indicated vehicle-treated samples (control). All samples showed the RNA Integrity Number (RIN) higher than 8.7, confirming the RNA integrity and quality.



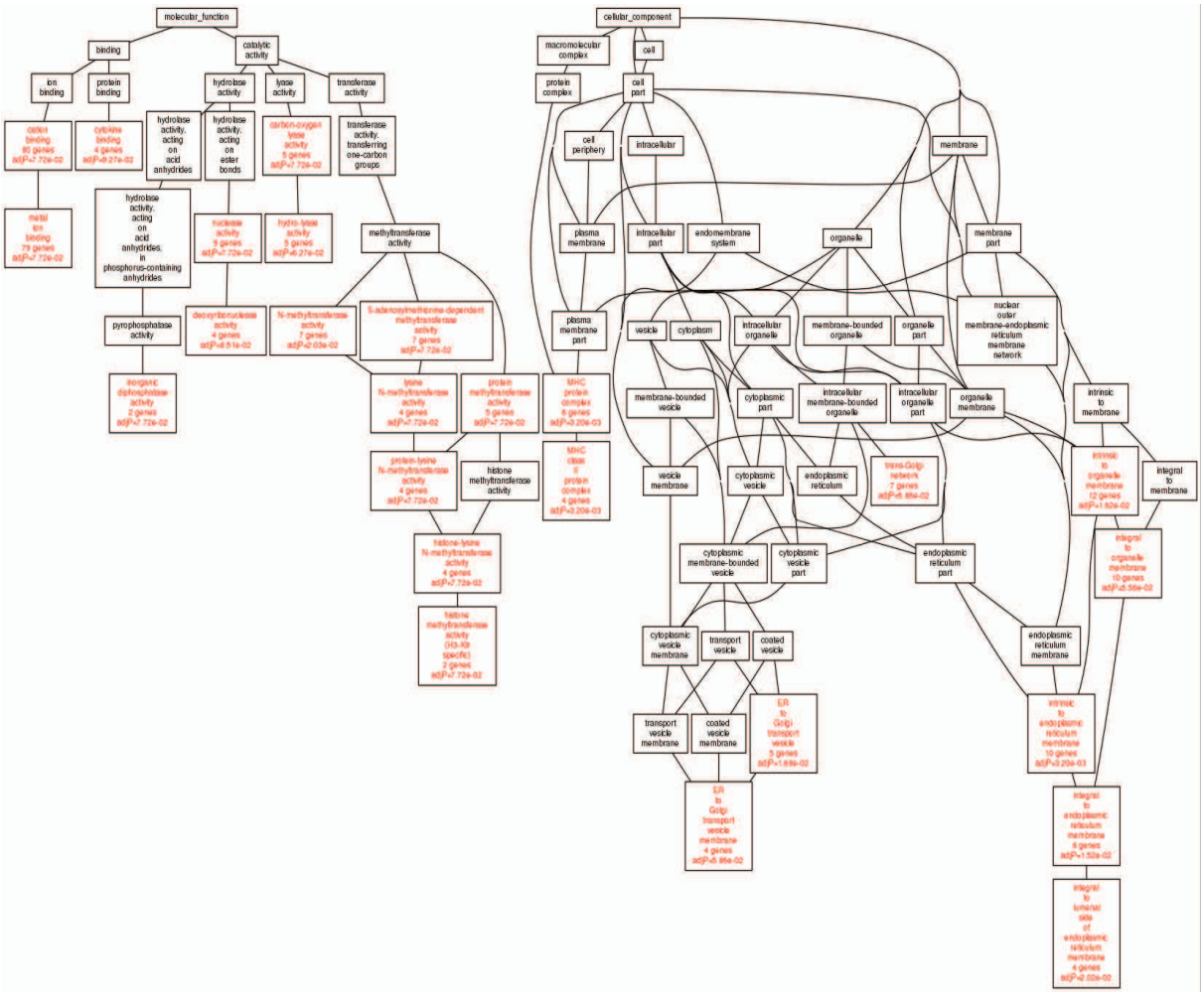
Supplementary figure 2. **Expression of the pseudogene GAPDHP65**, which was differentially expressed in MDA-MB-231 breast cancer cells after melatonin treatment. Expression is represented as a mean of reads counts of melatonin-treated tumors (n=5) and control tumors (n=5). ** p = 0.01.



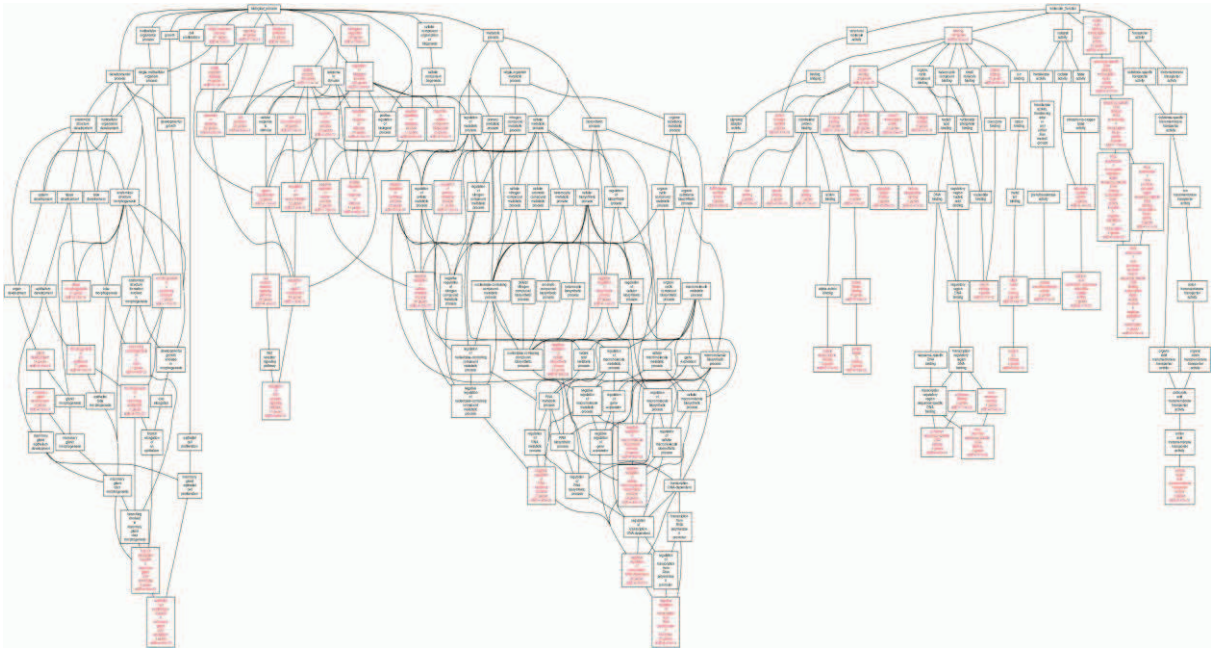
Supplementary figure 3. **Distribution into the categories of biological process**, molecular function and cellular component of differentially expressed (DE) genes in the tumor microenvironment by melatonin treatment. Figure was was generated by the web application WebGestalt.



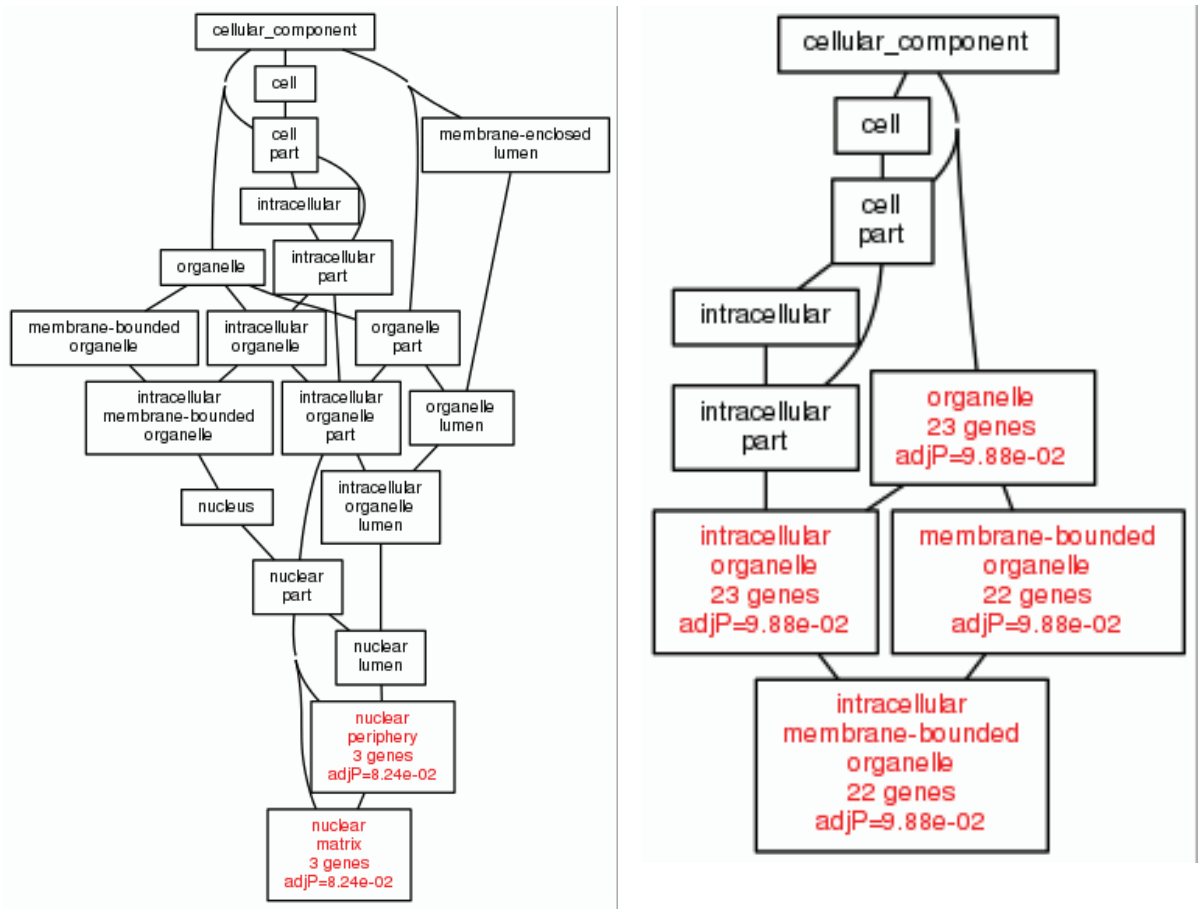
Supplementary figure 4. **Gene ontology (GO) analysis of connected genes in the MDA-MB-21 human breast cancer cells of the melatonin-treated group**. GO analysis was performed using Gene Set Analysis Toolkit software (www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are shown in red (hypergeometric; P < 0.1; red) and their nonenriched parents are shown in black.



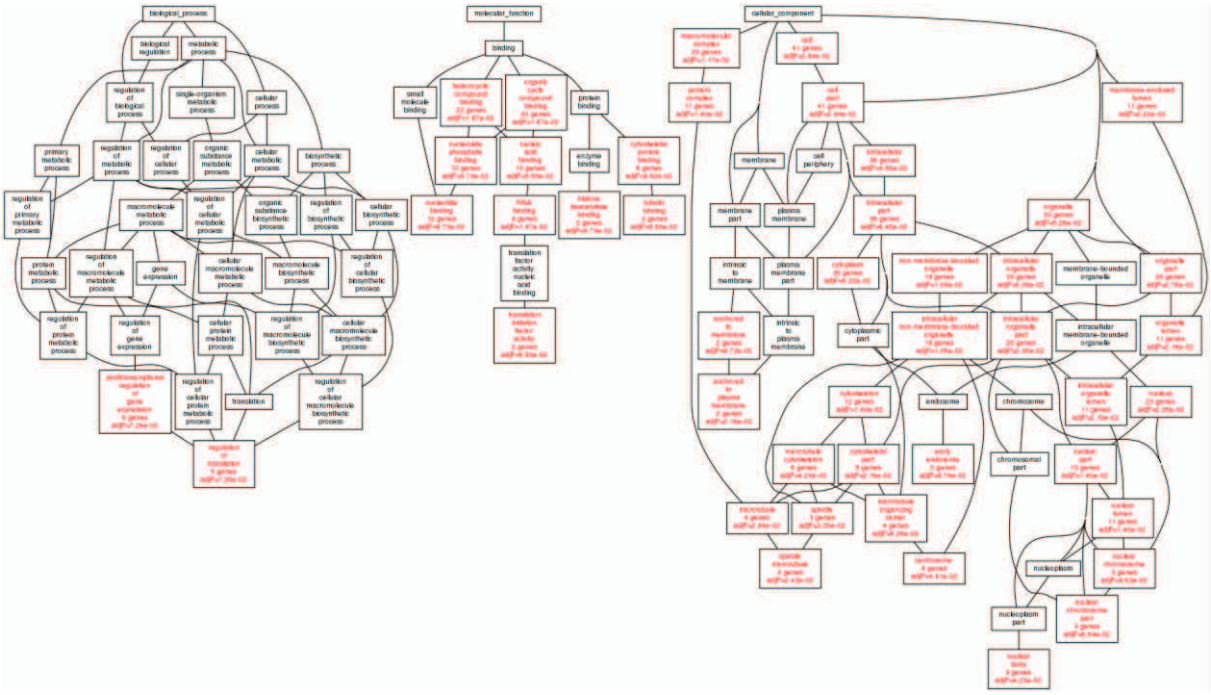
Supplementary figure 5. **Gene ontology (GO) analysis of connected genes in the MDA-MB-21 human breast cancer cells of the control group**. GO analysis was performed using Gene Set Analysis Toolkit software (www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are in red (hypergeometric; P < 0.1; red) and their nonenriched parents are shown in black.



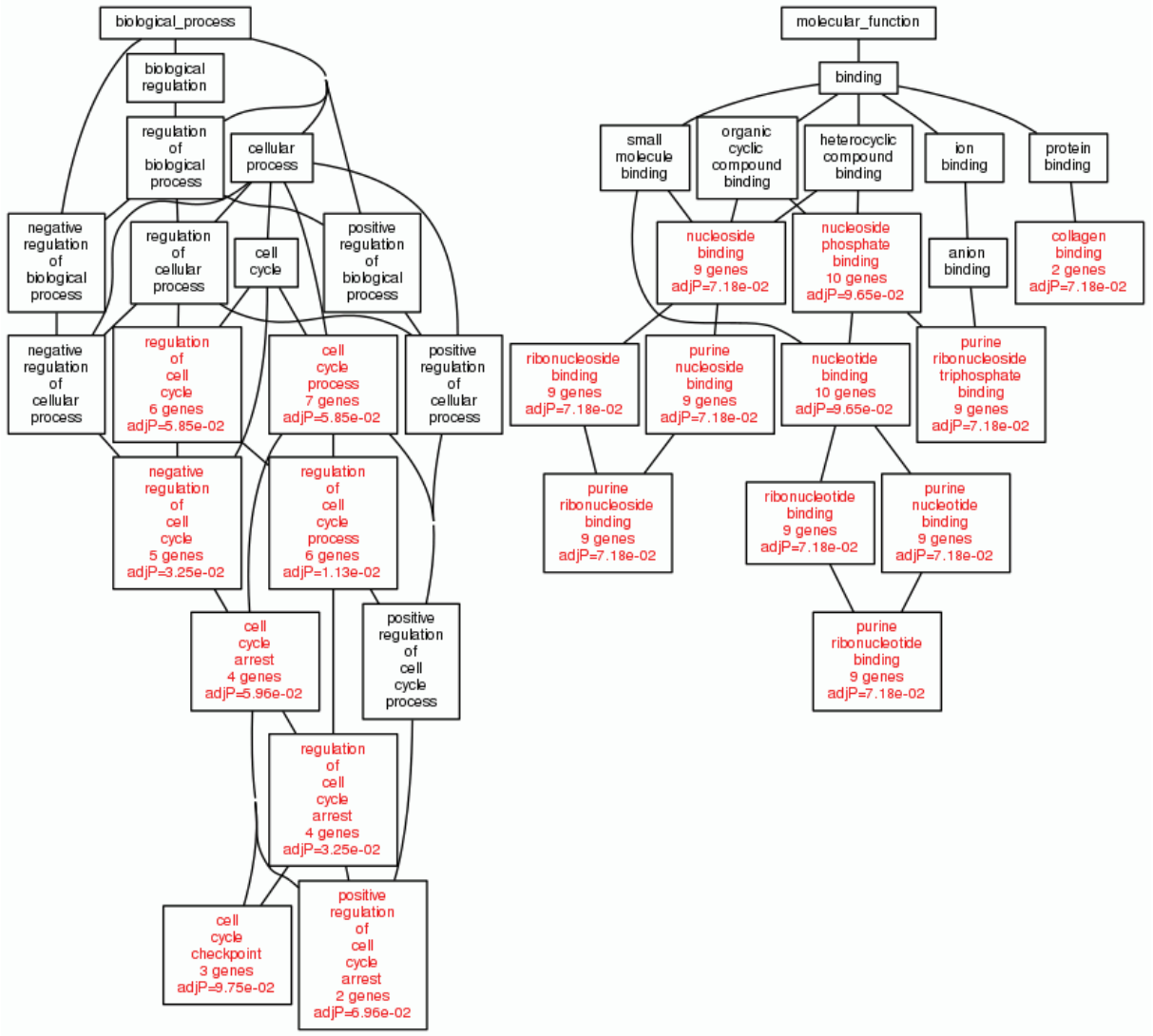
Supplementary figure 7. **Gene ontology (GO) analysis of connected genes in murine cells, which represent the tumor microenvironment (TME) of the control group**. GO analysis was performed using Gene Set Analysis Toolkit software www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are shown in red (hypergeometric; P < 0.1; red) and their nonenriched parents are shown in black.



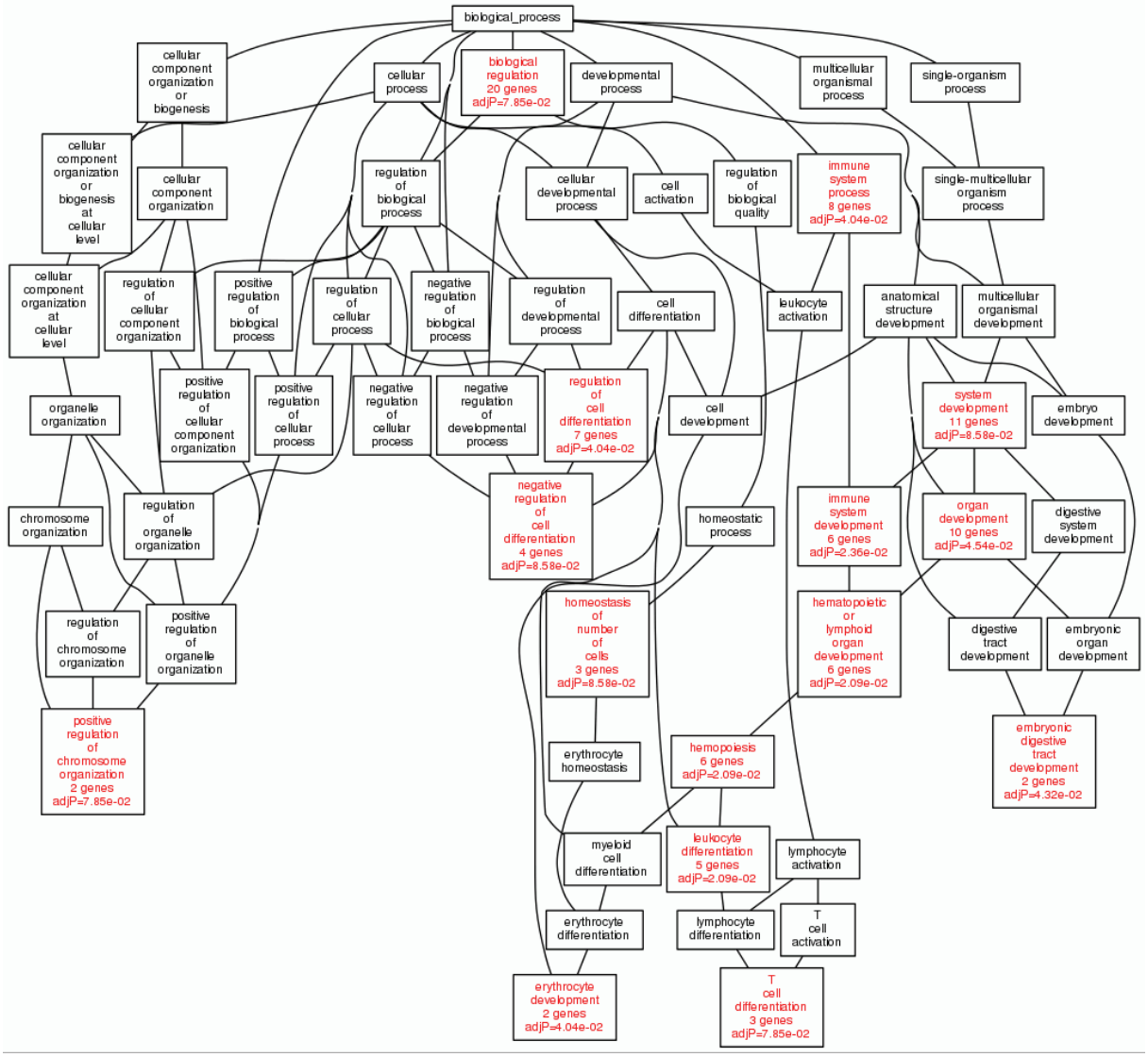
Supplementary Figure 8. **Gene ontology (GO) analysis of co-expressed genes modules altered by melatonin treatment in human tumors cells**. (A) Black module (B) Magenta module. Pink module did not show enrichment. GO analysis was performed using Gene Set Analysis Toolkit software (www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are shown in red (hypergeometric; P < 0.1; red) and their nonenriched parents are shown in black.



Supplementary figure 9. **Gene ontology (GO) analysis of darkoliver co-expressed gene module altered by melatonin treatment in the murine cells,** which represent the tumor microenvironment (TME). GO analysis was performed using Gene Set Analysis Toolkit software (www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are shown in red (hypergeometric; P < 0.1; red) and their nonenriched parents are shown in black.



Supplementary figure 10. **Gene ontology (GO) analysis of yellowgreen co-expressed gene module altered by melatonin treatment in the murine cells**, which represent the tumor microenvironment (TME). GO analysis was performed using Gene Set Analysis Toolkit software (www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are shown in red (hypergeometric; $P < 0.1$; red) and their nonenriched parents are shown in black.



Supplementary figure 11. **Gene ontology (GO) analysis of orangered co-expressed gene module altered by melatonin treatment in the murine cells**, which represent the tumor microenvironment (TME). GO analysis was performed using Gene Set Analysis Toolkit software (www.bioinfo.vanderbilt.edu/webgestalt). The enriched GO categories are shown in red (hypergeometric; P < 0.1; red) and their nonenriched parents are shown in black.

ARTIGO CIENTÍFICO III

MODIFICATIONS OF THE DIURNAL PROFILE OF PLASMA AMINO ACIDS IN MICE BEARING THE HUMAN BREAST CANCER XENOGRAFT MDA-MB-231

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Abstract

Breast cancer is the most incident type in women, among which, Triple Negative Breast Cancer (TNBC) is the most severe and almost always have a rare aggressive metabolic phenotype. Metabolic dysregulation and reprogramming is a common characteristic in neoplastic developments. Studies in animals and human have shown significant daily variations of the metabolism with oscillation of the plasma metabolite levels. This diurnal rhythm is under control of exogenous factors such as food and exercise and endogenous rhythms controlled by biological clocks In this study were compare the rhythms of plasma metabolites in normal mice and mice harbouring a human breast cancer xenograft MDA-MB-231. Samples collected every 3 hours were evaluated by LC-MS analysis for circulating amino acids. Multivariate analysis showed separation of the amino acids profile through the eight time points and based on the tumor presence. Of 20 amino acids quantified, 10 were observed having similar behavior across one diurnal variation between tumor and control. A significant increase in arginine, ornithine, citrulline, proline, serine, asparagine, lysine, leucine methionine and phenylalanine was observed In the day time (Please check) and a significant decrease during the night time. Even though this study examines the plasma metabolomics of an animal bearing a human tumor, this suggests that care should be taken in metabolomics studies using human tumor xenografts. Should this study translate to syngeneic tumor bearing animals or humans, it suggests that extreme care must be taken when examining exometabolomic changes at single time points.

Key words: Breast Neoplasms, Plasma, Tumor Xenograft, Metabolism, Amino acids.

Worldwide, breast cancer is the most common type in women and the fifth leading cause of cancer-related death (1, 2). It represents a highly heterogeneous group of tumors at both the clinical and molecular levels (3-5). Triple Negative Breast Cancer (TNBC) that represents approximately 15-20% of all breast cancer cases is generally considered one of the subgroups of breast cancer with worst prognosis (6, 7). This subgroup's patients do not express estrogen receptor (ER), progesterone receptor (PR) as well as Human epidermal growth factor receptor-2 (Her2) expression, and possess a rare aggressive metabolic phenotype. Complementary to viewing cancer as a genetic disease, cancer actually has been known as a metabolic disease. This standpoint has a long history starting with Warburg's seminal work (Warburg, 1956) and continues to raise interest in recent years unravelling the complex face of the tumor metabolome beyond aerobic glycolysis (8-12).

Metabolic dysregulation and reprogramming is a common characteristic implicated in neoplasm development and it is characterized as a hallmark of cancer (7, 10, 13, 14). The transforming process from normal to malignant cells is associated with a disturbance in the activity of a number of metabolic pathways. Cancer cells require large quantities of different energy to adapt their metabolism in order to accelerate the growing and proliferation (13, 15, 16). Increasingly, evidences have suggested that alterations in cancer metabolism, especially amino acids may provide potential targets for breast cancer analysis (17, 18). Traditional techniques have focused on protein and nucleic acids, however the analysis of amino acids and small molecules became crucial to detect significant alterations in specific cancer metabolic pathways (19, 20). Studies regarding cancer cell metabolism have revealed a number of common characteristics regarding amino acids including: (i) elevated consumption of amino acids and upregulation of corresponding transporters; (ii) demand for specific nonessential amino acids that exceeds intracellular supply, leading to dependence on exogenous sources; and (iii) altered levels of enzymes that catalyze amino acid synthesis and/or catabolism (21). Therefore, strategies to target specific nodes of cancer cell amino acid profiling have progressed from preclinical studies to clinical trials, and are showing efficacy as a method of discovering biomarkers for early diagnosis of cancer (21, 22).

Like many other physiologic aspects, the metabolism exhibits diurnal rhythm variations, which possess multifactorial features including exogenous factors such as food and exercise and endogenous rhythms controlled by biological clocks (23-26). Although the intricate connection between the internal clocks and metabolism remains poorly understood, previous pathway-specific studies have suggested that several aspects of the human amino acids metabolism are under rhythm control (25-29). Meanwhile, one recent temporal study by metabolite profiling exploration on clock regulation of mouse liver has shown half of metabolites exhibiting rhythmic variation. Serum analysis in both, animal and human, have identified a broad range of plasma metabolites such as sugars, fatty acids, phospholipids, nucleotides and amino acids showing clock-dependent oscillation (25, 26, 30-32). Metabolic disturbances might affect plasma free amino acids concentrations and this abnormal profile is often observed in patients with various diseases such as Alzheimer, obesity, diabetes, chronic kidney disease, acute and chronic liver disease, metabolic syndrome and various types of cancer (31, 33-37). Thus, diurnal metabolome profile can provide a convenient way to analyze clock involvement for detecting and predicting the risk for certain diseases (38-41).

Nowadays, complex diseases such as cancer have been widely explored through analysis called "omics" (genomics, transcriptomics and proteomics) in order to identify new biomarkers as well as complement the usual diagnostic methods and therapy response assessment (42-44). So far, approaches based on cancer metabolism have been used in a large variety of applications, including early disease detection, drug response, toxicity and nutritional studies, and basic systems biology (45-50). Then a new “omic” (metabolomics) was in this group to provide an overview of the metabolic processes (15, 51). Thereby amino acids are of increasing interest in the field of metabolomics, which aims to establish the metabolic responses of living systems to external or internal perturbations. Metabolomics studies have revealed that the variation abundance of many amino acids in several types of cancer is higher than in the corresponding normal tissue, suggesting that the tumors have increased biosynthetic needs for amino acids (34, 51-53).

Although few studies have focused on plasma daily rhythms, the profiling of cancer plasma samples using metabolomics analysis has been reported. In this study we aimed to evaluate associations of circulating levels of several metabolites in a TNBC breast cancer xenograft model according with the diurnal environmental changes in order to verify specific plasmatic amino acids alterations. To verify the association of metabolic alterations between tumor and non-tumor bearing animals, we performed plasma analysis using LC-MS screening platform.

RESULTS

Separation of the amino acids profile. We compared the plasma amino acids profile between Breast tumor-bearing and non-tumor-bearing animals. For this, plasma samples were collected in eight time points across 24 hours. All the spectra obtained from the plasma samples were of high quality without any obvious evidence of haemolysis. Thus, by using a targeted high-throughput LC/MS metabolomics platform we have extracted metabolites features for each sample. Firstly, unbiased analysis of the amino acids data by Principal Component Analysis (PCA) was performed in order to visualize the structure of the samples and examine the intrinsic variation within the groups. Each point represents an individual sample in the scores plot and the first two primary components contain 95% original amino acids data information of serum samples as shown in Figure 1A which is based on the time of day variation meanwhile the Figure 1B is based on the groups separation. As the blood plasma is a complex biological matrix, OPLS models were chosen in order to improve the metabolic differences by maximizing separation according the time points. Secondly, the OPLS-DA model of the amino acids profile according of the time of day variation (Figure 1C) showed again a reasonable fitting of the data (R2X1 = 0,491; R2X2 = 0,0884) while the model based on the tumor presence displayed clearest discrimination on the data (R2X1 = 0,563; R2X2 = 0,0924) (Figure 1D).

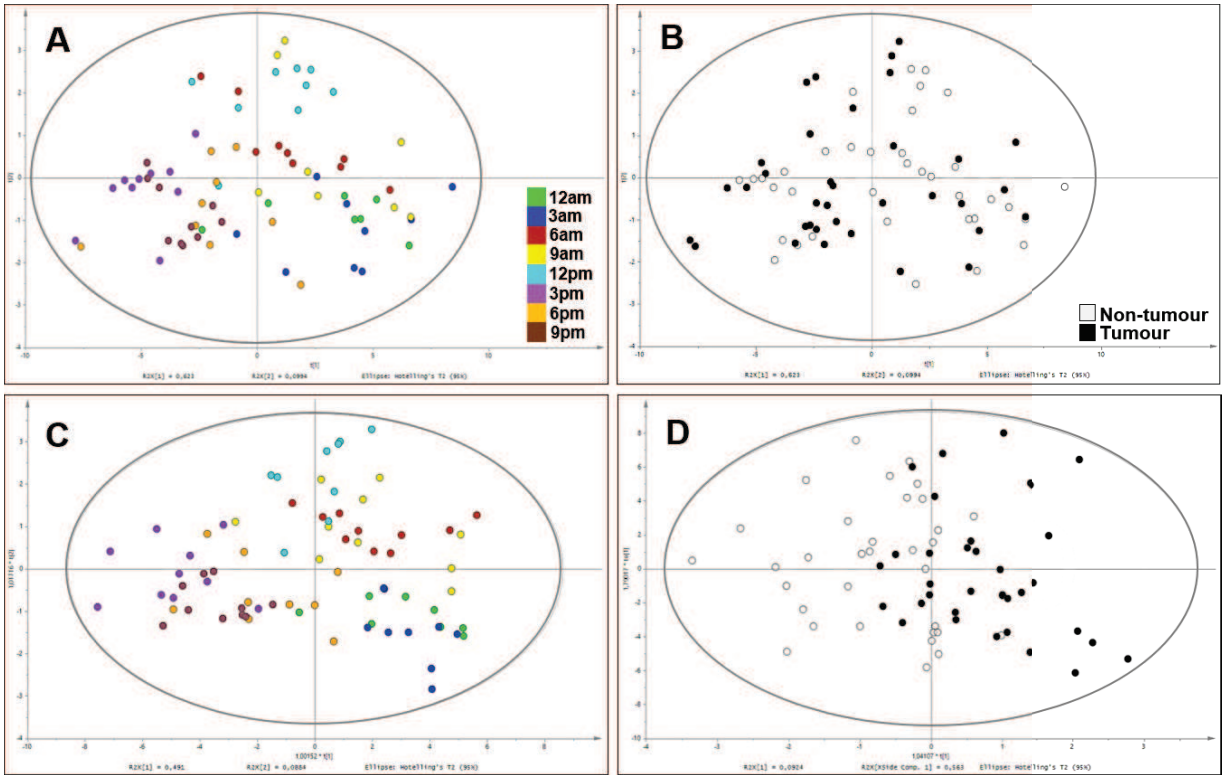

Figure 1. Multivariate modelling of the Breast Xenograft Tumour amino acids profile. [A] Scores plot of PCA displaying amino acids profile discrimination of all samples by time of day effect where each dots colours represent each time point (n = 10 per time); and **[B]** between Non-Tumour in white dots and Tumour-bearing in black dots (n = 40 per group). **[C]** Scores plot of OPLS displaying the amino acids profile discrimination by Time of day variation, where each dots colours represent each time point (n = 10 per time). **[D]** Scores plot of OPLS displaying the amino acids profile discrimination between Non-tumour in white dots and Tumour in black dots (n = 40 per group). The PCA model was obtained with the 2 Principal Components Time and Tumour while the OPLS-DA was with Time **[A]** or Tumour **[B]** as the first component. The OPLS-DA PCA model was obtained with Time effect **[C]** or Tumour presence **[D]** as the first component. The ellipses indicate the 95% confidence limit of each model.

The OPLS scores plot revealed that Breast tumor-bearing animals displayed different metabolic profiles from the Non-tumor-bearing, which implies possible alterations in the biological and pathological mechanisms. By looking for specific class of metabolites we found that the amino acids profile was influenced by the diurnal variation as well as the presence of breast tumor. Therefore, to assess the classification potential of the amino acids approach, we performed specifics OPLS-DA in which Tumor and Non-tumor samples were separated through the day variation. For each model we plotted plasma amino acids

concentrations in each time point. Distinct separation trends of the samples were achieved between Non-tumor and Tumor amino acids profiles most time of the day (Figure 2).

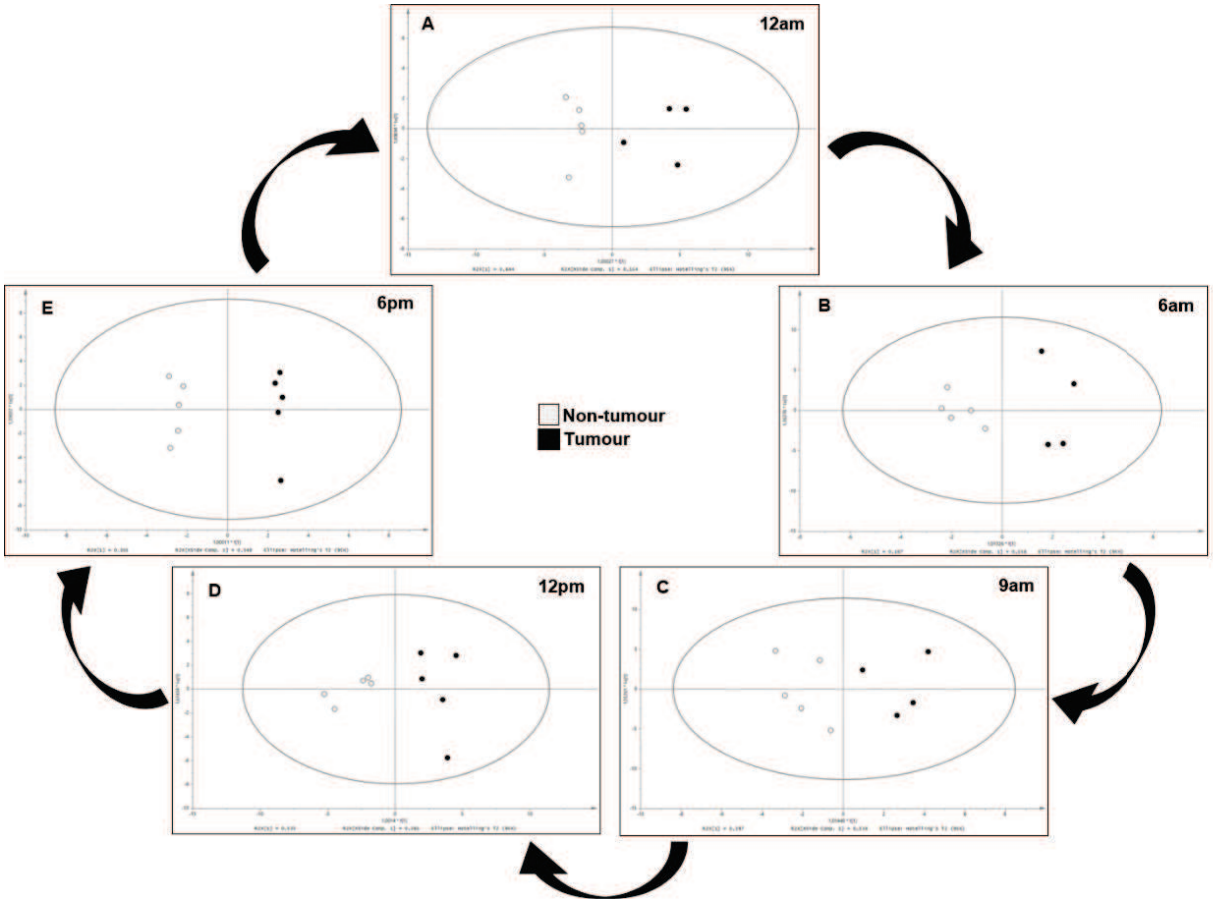

Figure 2. Multivariate modeling of the amino acids profile in each time point. Scores plot of OPLS-DA displaying amino acids discrimination between Non-tumour (white dots) and Tumour (black dots) bearing animals samples in the most time points of the day (n = 5 per group): **[A]** 0am, **[B]** 6am, **[C]** 9am, **[D]** 12pm and **[E]** 6pm.

Amino acids profile shifting in early morning. Given that there is an interaction between the tumor presence and time of day variation on the metabolic profile by Two Way ANOVA analysis where: tumor presence has no significance, time variation and interaction between both has shown significance ($p < 0,001$). We saw out of the 20 amino acids detected that 10 have exhibited similar behaviour and significant differences between normal and tumor-bearing mice. We found semi, non-essentials and essentials amino acids with time of day variation respectively including: Arginine (Arg), Ornithine (Orn), Citrulline (Cit), Proline (Pro), Serine (Ser), Asparagine (Asn), Lysine (Lys), Leucine (Leu), Phenylalanine (Phe) and Methionine (Met). Data presented on Figure 3 and 4 display the cyclic variation of

the plasma amino acids concentrations across 24 hours within the groups and show an inversion between Tumor and Non-tumor AAs profile at the early morning when the light phase begins.

In non-tumor mice, maximum levels of Arg, Orn, Cit and Pro were observed at 12am that slightly decline until 3 am, when Ser and Asn shown high levels, and remained decreasing until the light phase come up in 6 am but Asn differently increased in 9 am. After that, a rise of the AAs, except Orn, was observed in 12 pm and almost all the amino acids have plunged their plasma levels in 3pm. The levels of those semi and non-essential have increased again at the dark phase in 6 pm but back to decrease until 9 pm. In contrast Tumor-bearing mice showed lower levels of these amino acids at midnight and most of the AAs levels rise up until 3 am except Pro. In the beginning of the light phase in 6 am the plasma AA in tumor samples have increased except Asn. After that Arg, Orn, Ser and Asn rised until 9am but Cit and Pro have declined. Actually, in those time points at early morning we saw the amino acids profile having a shift when tumor-bearing animals have shown higher plasma concentrations compared with the normal mice. Posteriorly there was a decreasing in all AA until 12pm when they recovered the lower levels compared with normal mice. In 3pm both mice groups have achieved similar levels, but after that the Tumor samples have kept lower levels than Non-tumor until the dark phase at 6pm but in 9pm the amino acids profile has achieved similar concentrations again between the groups (Figure 3).

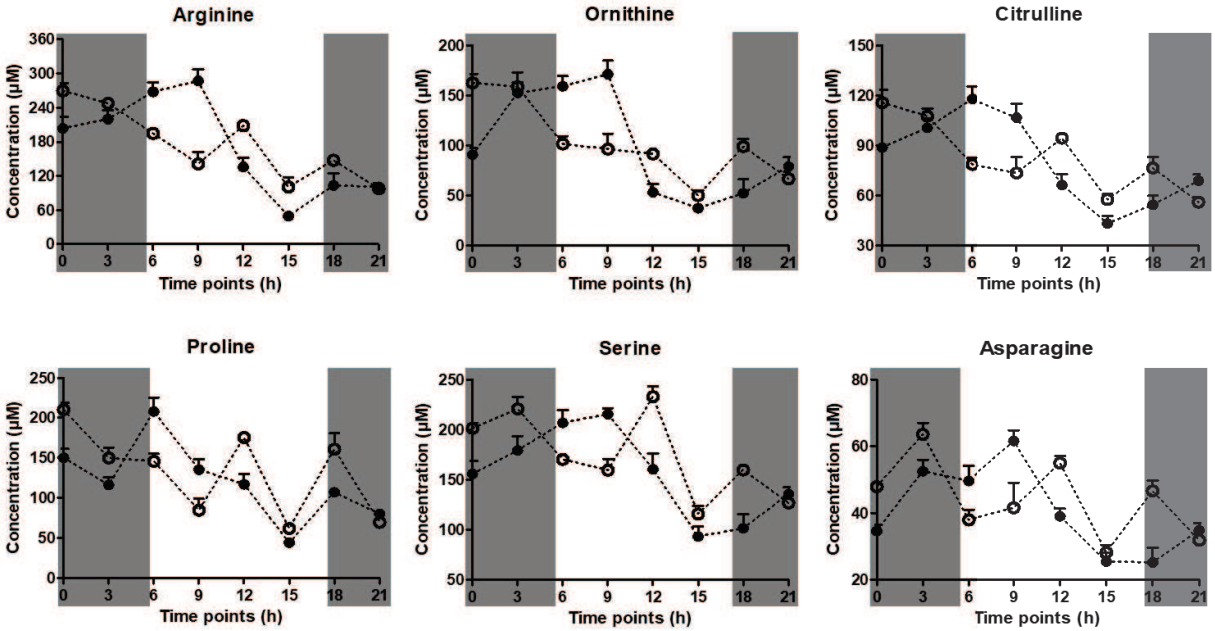

Figure 3. Plasma semi-essential and non-essential amino acids concentrations across one diurnal variation exhibiting shift at 6am and 9am. Six amino acids from the samples have shown similar behaviour between the groups (white circle for Non-tumor and black circle for tumor-bearing) across the time of day including the semi-essential Arg, and its precursors Orn and Cit plus Pro, Ser and Asn which are non-essentials. On the horizontal axis the numbers indicate the eight time points across a normal diurnal variation with 12 hours in light phase and 12 hours in dark phase, starting at 0am (0) finishing at 9pm (21) (24 hou rs).

Regarding the essential amino acids, we saw in Figure 4 that healthy mice showed highest levels of Leu, Phe and Met in midnight and slightly decrease until 3 am, except Lys that slightly increase in this time point. When the light phase begins, we observed a decreasing in all essential in 6am that keep reducing until 9am. After that, there was a rising of those AAs levels in 12pm but they plunged in 3am and the levels increased again at the dark phase in 6pm, but decrease in 9pm. For the other hand, in tumor-bearing animals group there was lower levels of those essential AAs in 12am that rise until 3am except Met. In contrast to normal mice, in tumor-bearing animals the plasma levels of the essential AAs increased at the light phase in 6am and 9am and they drastic decreased until 12am and 3pm. When the dark phase begin the essentials AAs slightly rised until 6pm and 9pm.

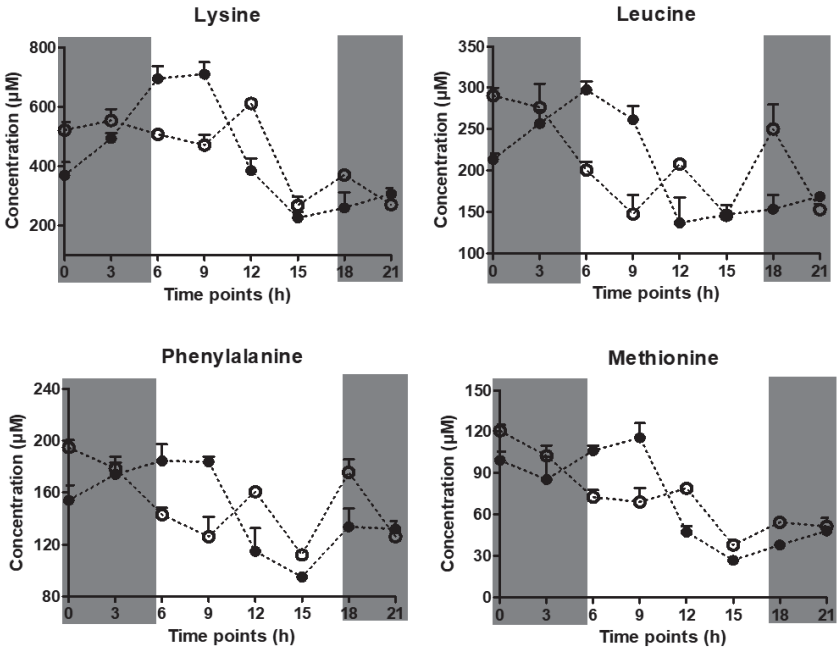

Figure 4. **Plasma essential amino acids concentrations across one diurnal variation exhibiting shift at 6am and 9am.** Four amino acids from the samples have shown similar behaviour between the groups (white circle for Non-tumor and black circle for tumor-bearing) across the time of day including the essentials Lys, Leu, Phe and Met. On the horizontal axis the numbers indicate the eight time points across a normal diurnal variation with 12 hours in light phase and 12 hours in dark phase, starting at 0am (0) finishing at 9pm (21) (24 hours).

Table 1. Differences of the plasma mean concentrations

Amino acids	Non-tumour	Tumour	Time Point	p value
Arginine	264 µM	196 µM	12 am	p < 0.05
	199.5 µM	**263 µM**	**6 am**	**p < 0.01**
	134 µM	**272.5 µM**	**9 am**	**p < 0.001**
	209 µM	144 µM	12 pm	p < 0.01
	143 µM	114 µM	6pm	N/S
Ornithine	158 µM	92.7 µM	12 am	p < 0.001
	98.2 µM	**166 µM**	**6 am**	**p < 0.01**
	85.1 µM	**165 µM**	**9 am**	**p < 0.001**
	91.5 µM	60.3 µM	12 pm	p < 0.05
	94.7 µM	66 µM	6pm	p < 0.05
Citrulline	109 µM	88.8 µM	12 am	N/S
	81.5 µM	**122 µM**	**6 am**	**p < 0.001**
	75.1 µM	**108.9 µM**	**9 am**	**p < 0.01**
	96 µM	69.4 µM	12 pm	p < 0.01
	77.8 µM	58.2 µM	6 pm	p < 0.05
Proline	211 µM	161 µM	12 am	p < 0.01
	146.5 µM	**223 µM**	**6 am**	**p < 0.01**
	88.3 µM	**134.5 µM**	**9 am**	**p < 0.05**
	181 µM	117 µM	12 pm	p < 0.001
	188 µM	107 µM	6pm	p < 0.01
Serine	201 µM	156 µM	12 am	p < 0.05
	173 µM	**212 µM**	**6 am**	**N/S**
	159.5 µM	**223 µM**	**9 am**	**p < 0.01**
	224 µM	134 µM	12 pm	p < 0.001
	156.5 µM	105µM	6pm	p < 0.01
Asparagine	47.7 µM	33.8 µM	12 am	p < 0.05
	38.5 µM	**49.4 µM**	**6 am**	**N/S**
	41.5 µM	**61.5 µM**	**9 am**	**p < 0.01**
	54.3 µM	39.7 µM	12 pm	p < 0.001
	45.7 µM	29.4 µM	6pm	p < 0.01
Lysine	524 µM	359 µM	12 am	p < 0.01
	505.5 µM	**725 µM**	**6 am**	**p < 0.01**
	471 µM	**716 µM**	**9 am**	**p < 0.001**
	630 µM	369 µM	12 pm	p < 0.001
	357 µM	224 µM	6 pm	N/S
Methionine	121 µM	94 µM	12 am	N/S
	74 µM	**106 µM**	**6 am**	**p < 0.05**
	70 µM	**113 µM**	**9 am**	**p < 0.001**
	75.1 µM	46.7 µM	12 pm	p < 0.05
	56 µM	38.6 µM	6pm	N/S
Leucine	286 µM	210 µM	12 am	p < 0.05
	192 µM	**291.5 µM**	**6 am**	**p < 0.01**
	156 µM	**277 µM**	**9 am**	**p < 0.001**
	208 µM	165 µM	12 pm	N/S
	261.5µM	170 µM	6 pm	p < 0.01
Phenylalanine	195 µM	162 µM	12 am	p < 0.05
	141 µM	**193 µM**	**6 am**	**p < 0.05**
	112 µM	**185 µM**	**9 am**	**p < 0.01**
	161 µM	124 µM	12 pm	p < 0.5
	185 µM	147.5 µM	6pm	p < 0.01

DISCUSSION

In this work we explored he plasma amino acids profile across an entire diurnal rhythm of a xenograft triple negative breast cancer model with eight time points within 24-h. We detected lower levels of circulating amino acids in the animals with breast cancer when compared to healthy mice, suggesting high tumor demand for this metabolites. Besides this amino acids plasma profile is under the host diurnal rhythmic control and showed an inversion in the levels between the groups with peaks at early morning. For the purposes of this study, the term diurnal variation is used throughout to refer generally to all the sources of systematic variation within individuals during the usual events (fasting, feeding, activity, inactivity, circadian influences) of a typical day. Targeted amino acids platform was employed to discover novel 24-h rhythmic profile in mice plasma. It is stressed that rigorous conditions of sample collection were performed, as the plasma metabolites is known to be exquisitely sensitive to a broad range of intrinsic and extrinsic factors (54, 55). As the metabolomics data typically contain a large number of variables that are interrelated, a multivariate statistical analysis such as PCA, OPLS-DA and ANOVA 2 WAY were used.

Our findings showed a well significantly separation of the amino acids profile according the eight time points across 24-h. Analysis comparing the groups have also shown clear separation between Non-tumor and Tumor-bearing animals. We identified 10 amino acids including arginine, ornithine, citrulline, proline, serine, asparagine, lysine, leucine and phenylalanine displaying similar behaviour across the day with significant differences between the groups. We observed that in most time points of the day there were predominant increased levels of these amino acids in tumor-bearing animals compared with non-tumor mice. However, we have surprisingly faced a shifting in the plasma profile of these amino acids between tumor and non-tumor mice, with peak observed in early morning time points, 6am and 9am. In humans the metabolic profiling have been reported during sleep and acute total sleep deprivation conditions characterizing rhythmic changes in circulating metabolites. Most of the amino acids detected showed no significant variation with time of day, which may be caused by the timed meals on these amino acid profiles (56, 57).

However, there was daily rhythms on glutamate, ornithine, proline, and two of the BCAAs, isoleucine and valine with levels peaking at night.

Significant difference between the metabolism of normal and malignant cells has been vastly reported (58, 59). For instance, bio-energetic requirements for homeostasis in normal cells are fulfilled by catabolic metabolism. On the other hand, the majority of the tumor cells alter their metabolic program ('metabolic remodeling') and consume additional nutrients in order to maintain a balance between elevated macromolecular biosynthesis (59, 60) and adequate levels of ATP for survival (59, 61, 62). In particular, several tumor types are auxotrophic for one or more amino acids owing to deficiencies in a corresponding endogenous biosynthesis (21, 60, 63). The endogenous supply of nutrients becomes inadequate during intense growth and consequently these cells have a particular reliance on the import of a given amino acids from the extracellular serum pool (60). Thus, tumor cells become auxotrophic for exogenous nutrients in their microenvironment to fulfill the elevated energy requirements (59, 64).

In most time points of the day less levels of Arginine, Ornithine and Citrulline in the tumor-bearing animals suggests that this metabolism network might be altered in our breast cancer animal model. This includes the urea cycle where these amino acids are intermediates intimately connected, thus the lower levels of these suggest high demand in Tumor-bearing mice. Owing to the involvement of arginine in a plethora of cellular pathways, arginine dependence of tumor cells has rapidly emerged as a potential target for cancer (59, 65). Classic examples of arginine auxotrophic tumors include hepatocellular carcinoma, malignant melanoma, malignant pleural mesothelioma, prostate cancer and renal cancer (66). Under certain physiological and pathophysiological conditions, such as growth or stress, arginine can be de novo synthesized intracellularly via selected steps and enzymes of the urea cycle (63, 66, 67). Arginine is a conditionally essential amino acid and its metabolism is an integral step in urea cycle which the production of arginine in cells is self-maintained by the convertion of citrulline to arginine by argininosuccinate synthetase 1 (ASS1) and ornithine produced from arginine hydrolization is further converted to citrulline by

ornithine transcarbamylase (OTC) to re-start the cycle (32). Tumor types with a loss of ASS1 may lose the ability to synthesise arginine and have to rely on external arginine sources to support rapid growth with minimal energy expenditure (66). Our findings showed that the levels of arginine, ornithine and citrulline have shifted for high plasma levels in Tumor-bearing animals suggesting that one possible cyclic event in early morning appears affecting these metabolites. In this scenario, recent findings have shown in human cells and mouse liver that metabolic enzymes, including ASS1 and OTC have CLOCK-dependent activity via direct gene-acetylation in a rhythmic manner, which contributes to the temporal plasma variation and biosynthesis of these amino acids (32, 68, 69).

In general, cancer cells might transiently or permanently become auxotrophic for essential, semi-essential and non-essential amino acids. Evidentially they appear to very efficiently utilize all physiological exogenous sources to cover their enhanced demand for proteinogenic and glucogenic amino acids and even the absence of non-essential AAs can disturb the delicate (abnormal) balance in tumor metabolism (63). Our analysis also showed in tumor-bearing animals lower plasma levels of non-essential and essential amino acids such as proline, serine, asparagine; lysine, methionine, leucine and phenylalanine in the most time points of the day. It is known that redistribution of plasma circulating amino acids can be an essential feature of protein metabolic abnormalities in cancer patients (37, 70-72). Studies evolving plasma free amino acid profiles reported similar decreases in circulating amino acid levels in pancreatic and breast cancer patients, which were interpreted as a result of the enhanced consume of amino acids in tumors (37). In the previous studies, the alterations in amino acids plasma profiles in cancer patients sometimes seem inconsistent (73-76). Prior investigations have particularly focused on establishing breast cancer biomarkers using metabolomics approach and numerous amino acids including glycine, histidine, tyrosine and proline showed increased flux in breast cancer cell lines (77, 78). It is noted though, that uptake of various, even non-essential amino acids correlates with cancer cell proliferation, and that reduced exogenous supply can critically affect proliferative activity, survival or tumorigenic potential (63). Miyag et al. in 2011 showed these alterations in amino

acids profiles even in patients with early-stage cancer, most of whom had no apparent symptoms.

We found that many studies have investigated the association between amino acid and cancer showing that non-essential amino acids are promising metabolites in order to detect cancer metabolism deregulation. They can be synthesized by normal cells and thus an extracellular source is not required for their health, whereas many tumor cells require an external supply of nonessential amino acids (79). Besides the Warburg effect, metabolism of non-essential amino acids, i.e. glutamine, serine, aspartic acid and proline, has been shown to contribute to tumor metabolic reprogramming (79-82). De novo proline synthesis has been shown to be critical in breast cancer metastatic cells (83). In tumor tissue, proline may be channeled into functions at the expense of protein synthesis (34, 84, 85). Proline is interconvertible with glutamate, in which glutamic-c-semialdehyde (GSA) are utilized as intermediate, which is derived from glutamate or proline that can be converted into ornithine, which serves as a precursor for arginine synthesis in the Urea cycle (84). A recent study has discovered proline catabolism involvement in vivo metastasis formation through high expression of proline dehydrogenase (PRODH). They particularly found that PRODH expression and proline catabolism is increased in metastases compared to primary breast cancers of patients and mice (86). Recently, researches showed that not necessarily due to disruption of protein synthesis, the inhibition of proline synthesis may interrupts cell proliferation (84, 87). Unbiased metabolomic profiling using LC/GC-MS identified proline, which increased in tumor samples during progression when compared with benign prostate tissue adjacent to the tumor (88). Moreover, it has been shown that proline biosynthesis can contribute to sustaining intracellular nucleotide levels (82, 86).

There are promising evidences that nonessential amino acids, as for serine metabolism, become essential in many tumor cells, including breast cancer cells (79). Studies from the late 1980s to more recent years, strongly suggest that cancer cells have an increased capacity for de novo serine synthesis via the phosphoglycerate dehydrogenase (PHGDH) pathway (89). Recent reports have implicated serine metabolism as being

important for breast cancer and melanoma were the gene for PHGDH, which encodes the enzyme that catalyzes the first committed step in serine biosynthesis has been found to be amplified (61, 90, 91). The enzyme PHGDH along with other enzymes in the serine biosynthetic pathway were upregulated in highly metastatic breast cancer, a finding associated with overall poor patient survival (89, 92). Bennegård et al. (1984), examining 18 cancer patients with more than 7% weight loss, he found a significant decrease in proline and serine levels. Watanabe et al, (1984?) have also investigated PFAA concentrations in 14 cirrhotic patients with hepatocellular carcinoma (HCC). They found significant decreases in the levels of serine yet it is one of the most rapidly consumed nutrients after glucose by many cultured cancer cell lines (21, 93, 94). Serine is a critical nutrient for many cancer cells, which rely on both de novo synthesis and extracellular sources of serine (61, 90, 95). This high demand for serine in proliferating cancer cells is consistent with its many roles in biosynthesis. As an amino acid, serine is needed for protein synthesis, but it is also a precursor for numerous other metabolites that contribute to cell biomass (96).

Cells also have the feature to synthesize asparagine at the expense of macromolecule synthesis and cellular energy, specially, through the asparagine synthetase (ASNS). It is reported that the expression of ASNS is an independent factor affecting the survival of hepatocellular carcinoma (HCC) patients, and low ASNS expression in HCC was correlated with worse surgical outcomes (97, 98). Moreover, high expression of ASNS has also been associated with biological aggressiveness of gliomas (99). ASNS plays an important role in the cell cycle arrest of cancer cells and coordinates with L-asparaginase activity in leukemia and ovarian cancer (98, 100, 101). Recent data showed that the deprivation of endogenous ASNS expression led to inhibited cell proliferation, impaired colony formation and blocked cell cycle progression (98). These findings advance our understanding of the basic biological mechanism of ASNS in human breast cancer cells in vitro and revealed the critical role of asparagine and ASNS in managing cell growth and cell cycle progression. For the other hand, the importance of asparagine for tumor growth has also been demonstrated by the effectiveness of extracellular asparaginase enzymes in

treating low-ASNS-expressing leukaemia (102, 103)). The most notable example of this strategy in a therapeutic setting is certainly the success of L-Asparaginase in the treatment of acute lymphoblastic leukemia (ALL) (104). Asparaginase enzymes work by breaking down the amino acid asparagine without which the cancer cells cannot make DNA. Whereas normal cells can endogenously synthesize the non-essential amino acid L-asparagine (LAsn) through the catalytic action of asparagine synthetase (ASNS), certain ALL lymphoblasts lack or express very low levels of ASNS and therefore require uptake of serum L-Asn (60, 105-107). Although asparaginase is effective as a therapeutic for cancers that obtain the majority of their asparagine from the environment, cancers that are capable of synthesizing asparagine de novo via ASNS are less responsive to asparaginase therapy (100).

Beyond those non-essential amino acids, tumors also preferentially uptake for the essential amino acids (89, 108). The metabolism of the essential amino acid methionine is appreciated to be closely linked to growth-dependending because it evolves DNA synthesis, epigenetics, single carbon metabolism, histone methylation and animal embryogenesis (109-111). Through the action of methionine adenosyltransferase (MAT), methionine is converted to SAM, the major cellular methyl donor for DNA and histone methyltransferases (112-114). It was identified in human, mouse, and rat malignant cells that an absolute methionine-dependency by growth of the cells in methionine-depleted and in homocysteine (the immediate precursor of methionine)-supplemented media (Met- Hcy+). In contrast, normal cells were not affected by the Met- Hcy+ media (115). Recently, the effects of methionine deprivation on migration, invasion and cell viability came to the surface through two studies in vitro and in vivo. They showed that methionine-deprived diet decreased proliferation and migration, increased apoptosis and suppressed metastasis in mice with breast cancer (116, 117). In addition to tumor behaviour, methionine dependence may also indicate an overall imbalance in transmethylation (118). Researches have shown that the main reason for the effect of methionine strategy in cancer is based on the prevention of this altered methionine/transmethylation metabolism (118-120). Methionine is seen to provoke alteration and excessive transmethylation in cancer cell through a “methyl-sink”, whereby methyl

groups are diverted from DNA (119). However, Dash et al. (2016) have reported that decreased levels of methionine and its metabolites may decrease cellular function in multiple organs at a systemic level. The effectiveness of methionine restriction for treating cancer is dependent upon many factors, such as age, innate immunity, type and severity of cancer, intestinal health, diet, and nutritional requirements.

Branched-chain amino acids (BCAAs) as leucine, isoleucine, and valine are essential amino acids that can be used for protein synthesis or oxidized for energy purposes by tumors (108, 121). Many animal model researches have demonstrated an increase in BCAA demand that is not met by BCAA supply in tumor-bearing state, as well as other catabolic states (122). In recent years, it has become evident that the enzymes catalyzing the first step in BCAA degradation are overexpressed in many cancers (123-125). Our results corroborate (126), which showed no difference in total BCAAs in patients with breast cancer however only concentrations of leucine were significantly lower in breast cancer patients when compared with control groups. This is likely because BCAAs are required for muscle protein synthesis, in particular during the late-stage of cancer with cachexia occurring (71, 127). This difference lacking in leucine levels between the groups suggests the lower leucine concentrations as a manifestation of early stages of breast cancer (126). For the other hand researches displayed increased levels in breast cancer patients of three metabolites belonging to the branched chain amino acid (BCAA) metabolism (2-hydroxy-3- methylbutiric acid, 2-hydroxy-3-methylpentanoic acid, and 3-methylglutaric acid) (128).

Most of the amino acids found in our xenograft model were and can follow either pathway, glucogenic or ketogenic, may be depending on the need of the tumor and the body at the time of day. The amino acids through the glucogenic route they are easily converted to glucose in the liver in few steps. In contrast we found exclusively ketogenic as leucine and lysine which are readily broken down into either acetoacetate, a ketone body or acetylCoA which can be a direct participant in the Kreb cycle (129). Recently it has been displayed on neuroblastoma (NB) xenografts the effect of ketogenic dietary (KD) intervention on the amino acid levels. They used as the KD, 50% less protein compared to the control diet and found

showed significantly lower levels of lysine, valine, leucine, isoleucine, threonine and phenylalanine in the KD groups compared to the control group. Similar changes of these amino acids were detected in the tissue samples, with the exception that phenylalanine levels were found to be similar in all dietary groups. Among the non-essential amino acids, significantly higher levels of glutamine, serine, and glycine were found in the plasma of the KD groups compared to the CTRL group (130). Significant decreases in the levels of several amino acids including lysine and phenylalanine were also observed in pancreatic cancer patients (131). However, other study demonstrated a decrease of phenylalanine whereas an increase of lysine in patients with breast cancer (76).

In our model, the data show that the human breast cancer cells previously in vivo can have their own biological clocks, which are peripheral and may be unsynchronized when they are in culture growing. For the other hand, in vivo, the mice have their circulating metabolites under their central oscillator rhythmic control. Once they are together in the xenograft model the inoculated cells in the animal matrix might have resynchronized their biological clock within the new the central oscillator rhythm (132). Actually we observed the metabolites from a human tumor in a mouse background as a reflex of the altered metabolism in these animals. The human triple negative cells may have, direct or indirectly, determined the metabolic changes which are visualized through the circulating metabolites levels. In this work we also have seen that depending on the sample time point we may see an increase or a decreased levels, which may be a general tumor associated with the diurnal effect but we cannot check as there is no syngeneic model. Meanwhile the amino acids shift profile in tumor-bearing animals suggest that a possible cyclic event from the host metabolism in early morning appears affecting the plasmatic amino acids levels.

In summary this study has shown that circulating amino acids detection and quantification can be helpful for cancer screening and also that the metabolic amino acids profile is under diurnal rhythm coordination in our breast cancer xenograft model. Despite few studies reporting on plasma or serum levels of amino acids, with contradictory results, we observed lower levels of plasma amino acids in the tumor-bearing animals in most time

points of the day comparing with control. The low plasma levels of these amino acids in animals with tumor, implies high tumor uptake of these amino acids. There also was a shifting in the amino acid profile at initial phase of the morning, when the tumor samples have higher amino acids comparing with the controls. Recently it has been shown that differently in humans, amino acids serum peaks appear at night. This inversion of the profile during the morning requires more investigation because it indicates that a cyclic event from the host may be affecting the tumor metabolism or only the serum levels of these molecules. Particularly, specific variations in each time point have to be cautiously screened in order to reliably verify the cyclic alterations in different time of the day which might be affecting the plasmatic levels of metabolites. The results in this report showed that single or few time-points data is not capable to visualize the tumor behaviour through circulating metabolites according the diurnal rhythm. And we confirm that in a xenograft model, the cells from different species can resynchronize their internal biological clocks within the new central oscillator.

Matherials and Methods

In vitro

Triple negative human breast cancer cells (MDA-MB-231) (ATCC, Manassas, VA, USA) were grown in 75 cm^2 culture flasks with Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (GIBCO, Grand Island, NY, USA), penicillin (100 IU/mL) and streptomycin (100 mg/mL) (GIBCO, Grand Island, NY, USA) until they reach 80–90% confluent.

In vivo

Ethical Committee

The study has carried out following the national and international standards for ethics in animal experimentation. The project was approved by the Ethics Committee on the Use of Animals of the Faculdade de Medicina de Sao Jose do Rio Preto (Prot. 001-003336 / 2014).

Tumor developing

Female nude mice, 7–8 weeks of age and 20g in weight (Charles River Laboratory, Inc.) were kept in pathogen-free conditions, at 21 to 25 °C and exposure to a normal diurnal variation with 12 hours of light and 12 hours of dark. MDA-MB-231 cell line has grown, harvested and re-suspended with serum free media at a concentration of $6x10^7$ cells/ml. The animals received subcutaneous injection of 100 µl of cell suspension (3 million cells) in the right mammary gland. The mice were randomly divided into two groups with five animals each: non-tumor bearing (n = 5), tumor bearing (n = 5). The control group received 100 uL of vehicle solution to submit the animals to the same stress handling.

Sample collection

On the 22nd day all the animals underwent cardiac puncture and were bleeding in eight time points across one entire day variation using a heparinized syringe. This time of bearing model was based on our previous study regarding breast cancer and angiogenesis but with different implantation site (132). We supposed that the tumor implantation near the mammary gland would give us a more reliable metabolic changes caused by the cells. It is necessary to emphasize that during the dark time points the sample collection have been done under stressed conditions with minimum light at room using red light. The blood was collected using a heparinized syringe and the plasma was subsequently extracted by centrifugation at 11000 rpm for 2 min at 4°C and stored - 80 ºC for analysis.

Target metabolomics analysis

In order to identify and quantify metabolite concentrations in plasma samples we have measured through a targeted metabolite profiling standard using the AbsoluteIDQ p180 targeted metabolomics kit (Biocrates Life Sciences AG, Innsbruck, Austria). The kit provided absolute concentrations for amino acids among others classes of metabolites. Plasma samples were prepared following the manufacturer's instructions and to correct for variation

between batches in the targeted analysis, a correction factor was applied to each metabolite concentration value.

Data *analysis*

To derive the metabolite concentrations, analytical process was performed using MassLynx™ (Waters corporation Manchester UK) and the MetIDQ software package (Biocrates Life Sciences, Insbruck, Austria). The featuring of the metabolites was differentially expressed between the animals from two groups. The data matrix obtained was subsequently subjected to multivariate analysis using SIMCA software (MKS Umetrics AB, Sweden). Heat maps were generated using the concentration changes relative to vehicle control animals at each time point. For unsupervised multivariate analysis, Principal Component Analysis (PCA) and Orthogonal Partial Last Squares (OPLS) were undertaken using SIMCA v.13 software (Umetrics). In addition the GraphPad Prism program (v.6, California, USA) was used for statistical analyses by Two Way ANOVA followed by Bonferroni posttests, across the eight time points of day between Non-tumor vs Tumor. All p-values presented are two-sided and values <0.05 were considered statistically significant and the outliers where exclude when necessary.

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3. CONCLUSÕES

A partir dos resultados obtidos nos estudos investigados, concluímos que:

Artigo I:

A melatonina diminui a expressão gênica e proteica do NF-kB *in vivo* nos tumores do modelo xenográfico de câncer de mama.

Interessantemente, *in vivo,* a melatonina aumenta apenas a expressão proteica do NF-kB em linhagem celular de câncer hepático.

Artigo II:

O tratamento com a melatonina age por regulação de expressão gênica principalmente no microambiente tumoral, possivelmente aumentando a expressão dos genes Tnfaip8l2 e Il1f6.

Os genes Tnfaip8l2 e Il1f6 são importantes na modulação da resposta imune local durante a carcinogênese.

Artigo III:

Animais com tumor apresentam níveis reduzidos de aminoácidos no plasma na maior parte do dia comparados com grupo controle.

As células tumorais humanas causam efeito direto ou indireto no perfil metabólico dos animais, vizualizado pelos níveis dos aminoácidos circulantes.

Existe uma inversão no perfil desses aminoácidos com picos plasmáticos nos primeiros horários da manhã, quando animais com tumor têm níveis aumentados comparados com os controles, mostranso estar sob influência do ritmo do hospedeiro.

Essas conclusões associadas mostram que o tratamento com a melatonina parece cada vez mais eficaz em reduzir o tamanho tumoral. Esse hormônio age principalmente por meio de modulação gênica e proteica da resposta imune no microambiente tumoral durante a carcinogênese. No entanto, a melatonina parece desempenhar uma função dupla na expressão do NF-kB dependendo do tipo celular. O efeito da melatonina sobre o NF-kB no câncer de mama está melhor definido, porém no cancer hepático seu papel permanence

controverso. As alterações metabólicas causadas pelo tumor também influenciam nos níveis plasmáticos de vários metabólitos. Aminoácidos são pequenas moléculas fundamentais para o crescimento tumoral, porém seus níveis séricos são parcialmente controlados pelo ritmo diurno do hospedeiro, mostrando que o metabolismo do tumor também pode estar associado com a variação rítmica diária. Desse modo, os nossos resultados apontam que durante a carcinogênese existe uma forte associação entre a resposta imune e a cronobiologia do tumor que exigem futuras investigações, pois esse campo ainda permanece pouco conhecido.

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