UFRGS-UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

INTERAÇÃO ENTRE EPIGENÉTICA, MORTE CELULAR, INFLAMAÇÃO, TOXICIDADE SISTÊMICA E A PATOFISIOLOGIA DO TRANSTORNO BIPOLAR

LAURA STERTZ

Porto Alegre, 2014

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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Doutor em Bioquímica.

Porto Alegre, 2014

CIP - Catalogação na Publicação

Stertz, Laura
INTERAÇÃO ENTRE EPIGENÉTICA, MORTE CELULAR, INFLAMAÇÃO, TOXICIDADE SISTÊMICA E A PATOFISIOLOGIA DO TRANSTORNO BIPOLAR / Laura Stertz. -- 2014.
139 f.

Orientadora: Carmem Gottfried.
Coorientador: Flávio Kapczinski.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2014.

1. Transtorno Bipolar. 2. Inflamação. 3. Toxicidade Sistêmica. 4. DAMPs. 5. Epigenética. I. Gottfried, Carmem, orient. II. Kapczinski, Flávio, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

... À minha mãe Therezinha,

por me ensinar que a educação liberta.

"Science is [...] a way of skeptically interrogating the universe with a fine understanding of human fallibility."

Carl Sagan

Charlie Rose: An Interview with Carl Sagan,
May 27, 1996.

Agradecimentos

À minha querida orientadora Profa. Carmem Gottfried, por me ensinar que o carinho e a amizade são essenciais na vida científica e acadêmica.

Ao meu co-orientador Prof. Flávio Kapczinski, por mesmo depois de tantos anos de convívio, continuar me surpreendendo constantemente. Agradeço as oportunidades, os incentivos e os desafios que me ofereceste.

À Profa. Keila, que sempre esteve disponível a me ajudar, muito obrigada pelo carinho.

À Profa Adriane Rosa, pelos conselhos, incentivos e o auxílio nesse momento tão importante.

Ao Gabriel Fries, grande amigo, por me ensinar que mesmo os maiores gênios podem ser extremamente humildes. Obrigado pelo apoio, paciência e amizade nesses anos de convívio.

À Pfaffen, por me ensinar que o perfeccionismo e a paciência aumentam muito a qualidade de qualquer trabalho; à Bi Aguiar, pela dedicação e pela disponibilidade sempre presentes; à Gabi Colpo, por me ensinar que um pouco de bom humor não faz mal a ninguém. Obrigado gurias por toda a ajuda.

À Pâmela Ferrari, pela ajuda incansável na maioria dos experimentos realizados nessa tese.

À "bancada" do Laboratório de Psiquiatria Molecular, juntos sofremos "quebrando galhos", mas também juntos nos divertimos. Obrigada pela paciência com a "mandona".

Aos alunos e profissionais que já passaram ou ainda fazem parte do Laboratório de Psiquiatria Molecular, foi um prazer conviver e aprender com vocês.

Aos colegas do PNG, principalmente Tamara e Gean, por me acolherem sempre que preciso.

Ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da UFRGS.

A CAPES, pela bolsa concedida durante o período do Doutorado, sem a qual não seria possível a realização do mesmo.

À Paula Sukop, por me ensinar a ter mais paciência comigo mesma, obrigada pelo carinho nesses anos de convívio. Esse trabalho não seria possível sem você!

À minha família: meus pais, pelo amor, carinho, dedicação e paciência; minha irmã Suzana, pela amizade; meu cunhado Roberto, pelo bom humor; meu irmão Eduardo, pelo carinho; minha cunhada Karina, pela dedicação; meus sobrinhos Rafael e Mathias; e à minha afilhada Joana, simplesmente por existir!

À minha família de Alegrete, por me acolherem com tanto carinho!

Ao Dinler, meu melhor amigo, meu amor, por além de tudo, nunca deixar de acreditar que é possível.

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INTRODUÇÃO E OBJETIVOS

RESUMO

No transtorno bipolar (TB) diversas evidências apontam que a doença apresenta um curso progressivo, com os pacientes apresentando maiores anormalidades comportamentais, sensibilidade ao estresse e propensão a recaídas com o passar do tempo. A progressão pode ser refletida como uma sensibilização a diversos fatores, entre eles os episódios agudos de humor. Pesquisas indicam que esses episódios são "tóxicos" a múltiplos elementos do organismo humano. O presente estudo tem como objetivo avaliar, através de um estudo pré-clínico (modelo animal de mania induzido por *d*-anfetamina – AMPH) e um estudo clínico (estudo longitudinal aberto de 16 semanas), os mecanismos subjacentes ao episódio agudo de humor e à remissão clínica no TB, focando em vias de epigenética, morte celular, inflamação e toxicidade sistêmica. Nós observamos que a AMPH, um potente psicoestimulante, aumentou significativamente a atividade da enzima histona desacetilase (HDAC) no córtex pré-frontal (CPF) de ratos *wistar*, sem aumentar os níveis de expressão gênica e proteica da neurotrofina BDNF. Mais ainda, lítio, valproato e butirato de sódio atenuaram esse aumento, indicando que a inibição da HDAC é importante no manejamento das estereotipias comumente vistas em modelos animais de mania. Ainda, nós observamos que pacientes com TB não-medicados e durante o episódio agudo de humor apresentam maiores níveis séricos de moléculas associadas ao dano (DAMPs). Após o tratamento farmacológico, apenas pacientes assintomáticos apresentam a normalização dos níveis séricos de DAMPs, além de apresentarem diminuição dos níveis de TNF-α e aumento nos níveis de ácido úrico. Em conclusão, este estudo ressalta que no TB os pacientes podem apresentar diferenças individuais nas respostas ao tratamento em virtude de particularidades a níveis epigenéticos (inibição da HDAC) e bioquímicos (morte celular, DAMPs e inflamação), aumentando assim, a necessidade de farmacoterapias cada vez mais individualizadas.

ABSTRACT

In bipolar disorder (BD) several lines of evidence indicate that the illness has a progressive course, with patients showing greater behavioral abnormalities, sensitization to stress and propensity to relapse over time. This progression may be reflected as a sensitization to several factors, including acute mood episodes. In general, studies support the idea that these episodes are ' toxic ' to multiple parts of the human body. The present study aimed to evaluate, through a pre-clinical study (animal model of mania induced by *d*-amphetamine (AMPH)) and a clinical trial (longitudinal-open study of 16 weeks), the mechanisms underlying the acute mood episode and the clinical remission in BD, focusing in pathways of epigenetic, cell death, inflammation and systemic toxicity. We observed that the AMPH significantly increased the activity of the enzyme histone deacetylase (HDAC) in the prefrontal cortex (PFC) of *Wistar* rats, without increasing the levels of the neurotrophic factor BDNF (gene and protein expression). Further, lithium, valproate and sodium butyrate attenuated the effects induced by AMPH, indicating that HDAC inhibition is important in the management of the stereotypies commonly seen in animal models of mania. In addition, we demonstraded that *drug-free* patients with BD, during acute mood episodes, have higher serum levels of molecules associated with damage (DAMPs). After the initial pharmacological treatment, only asymptomatic patients presented the normalization of serum levels of DAMPs over time, in addition to present reducing in the levels of TNF-α and increasing in the levels of uric acid. In conclusion, our study supports that in BD, patients may exhibit different treatment responses due to particularities in epigenetic pathways (inhibition of HDAC) and biochemical mechanisms (cell death, inflammation and DAMPs), thus increasing the need for more individualized pharmacotherapies.

LISTA DE ABREVIATURAS

AA - ácido araquidônico

AMPH – *d*-anfetamina (sigla do inglês *amphetamine*)

BDNF - Fator Neurotrófico-Derivado do Cérebro (sigla do inglês *Brain-Derivate Neurotrophic Factor*)

COX-2 - ciclooxigenase-2

CPF - córtex pré-frontal

CRH – hormônio liberador de corticotropina (sigla do inglês *Corticotropic releasing hormone*)

ccf DNA - DNA livre circulante (sigla do inglês *circulanting cell-free DNA*)

DAMPs - padrões moleculares associados ao dano (sigla do inglês *damaged-assossiate molecular patterns*)

GSH - glutationa reduzida

HDAC - histona desacetilase

HMGB1 - grupo de proteínas Box 1 de alta mobilidade (sigla do inglês *high mobility group protein B1*)

HPA - Hipotálamo-Hipófise-Adrenal (sigla do inglês *hypothalamic-pituitary-adrenal*)

HSP – proteína de choque térmico (sigla do inglês *heat shock protein*)

IL - interleucina

NAC - *N*-acetilcisteína

NRG - neuroregulinas (sigla do inglês *neuroregulin*),

PCC – conteúdo de carbonil proteico (sigla do inglês *protein carbonil content)*

PGE2 - prostaglandina E2

RE - retículo endoplasmático

SB - butirato de sódio (sigla do inglês *sodium butyrate*)

SNC - Sistema Nervoso Central

TB - Transtorno Bipolar

TLR – receptor do tipo TOLL (sigla do inglês *toll-like repector*)

VPT - valproato de sódio

INTRODUÇÃO[1]

Neurotrofinas, inflamação e estresse oxidativo como biomarcadores da atividade da doença no Transtorno Bipolar

Transtorno Bipolar (TB) é uma doença crônica severa, na qual episódios recorrentes de mania e depressão alternam com períodos de remissão clínica (eutimia). O TB tem sido comumente associado com significativa incapacidade, morbidade e prematura mortalidade (Sanchez-Moreno *et al.,* 2009, Soreca *et al.,* 2009). A recorrência dos episódios agudos e a progressão do transtorno frequentemente se traduzem em piores desfechos em longo prazo, ou seja, altos índices de comorbidades clínicas, prejuízos funcionais e cognitivos, e baixa resposta ao tratamento (Berk *et al.,* 2010, Magalhaes *et al.,* 2012, Rosa *et al.,* 2012, Wingo *et al.,* 2009). Ainda, pacientes com TB possuem maior risco de desenvolver um amplo espectro de condições médicas, incluindo doenças cardiovasculares e cerebrais, distúrbios neurológicos e síndrome metabólica (Fiedorowicz *et al.,* 2008).

Uma das hipóteses propostas para explicar os mecanismos subjacentes à pesada carga médica e ao dano cumulativo relacionado ao TB é a teoria da carga alostática (Grande *et al.,* 2012, Kapczinski *et al.,* 2008b, Vieta *et al.,* 2013). De acordo com essa teoria, a ativação crônica de mecanismos para restaurar a homeostase após condições estressoras levam a um desgaste no corpo e no cérebro que tem sido chamado de carga alostática (Kapczinski *et al.,* 2008b, McEwen *et al.,* 2003). Esses eventos são funções adaptativas vitais, mas eles também podem promover efeitos mal adaptados na plasticidade cerebral, assim como nas patofisiologias metabólica, imune e cardiovascular, todas as vezes que esses mediadores forem excessivos em número ou permanecerem ativos (McEwen *et al.,* 2011). Recentemente, o paradigma da carga alostática tem sido incorporado em um novo conceito no

[1] Introduçaõ parcialmente baseada no artigo de revisão originalmente publicado em *Expert Review of Neurotherapeutics* (ANEXO I): Pfaffenseller B, Fries GR, Wollenhaupt-Aguiar B, Colpo GD, **Stertz L**, Panizzutti B, et al. Neurotrophins, inflammation and oxidative stress as illness activity biomarkers in bipolar disorder. Expert review of neurotherapeutics. 2013 Jul;13(7):827-42. PubMed PMID: 23898853).

TB, o de neuroprogressão, que é descrito como um processo patológico de reprogramação cerebral que acontece quando a deterioração clínica e cognitiva é observada como resultado da progressão do transtorno (Berk *et al.,* 2011). Nesse sentido, existe um crescente interesse em entender os mecanismos sistêmicos patológicos que contribuem para a disfunção resultante de múltiplos episódios de humor no TB e, especialmente, em identificar os caminhos associados aos mediadores da alostase envolvidos em neuroproteção, estresse oxidativo e inflamação.

Neste âmbito, vários estudos têm sido realizados na tentativa de detectar biomarcadores periféricos que possam ser usados como indicadores de prejuízo celular e de toxicidade em pacientes com TB (Kapczinski *et al.,* 2010, Kapczinski *et al.,* 2011). Diferentes biomarcadores podem ser associados com a atividade da doença (indicando se a doença está ativa ou em remissão), com a neuroprogressão da doença ou com ambas. Digno de nota, marcadores sistêmicos já foram propostos no TB como mediadores de alostase (Berk *et al.,* 2011, Juster *et al.,* 2010, Kapczinski *et al.,* 2008b). Esses estudos podem ser importantes em aperfeiçoar nosso entendimento da atividade da doença e sua progressão, e também no fornecimento de informações para novas abordagens de tratamento e novos biomarcadores.

Evidências atuais disponíveis sobre possíveis biomarcadores de atividade da doença no TB são indicadas a seguir, destacando o papel de neurotrofinas, da inflamação e do estresse oxidativo, assim como possíveis mecanismos pelos quais essas rotas são ativadas em episódios agudos de humor e oportunidades terapêuticas emergentes no campo do TB.

Biomarcadores periféricos no Transtorno Bipolar

Existe um considerável interesse em incorporar biomarcadores na psiquiatria (Singh *et al.,* 2009), usando-os como indicadores biológicos para avaliar com maior objetividade as condições psiquiátricas. Um marcador biológico ou biomarcador é uma característica que pode ser objetivamente mensurada e avaliada como um indicador de processos biológicos normais, de processos patológicos ou de respostas farmacológicas a uma intervenção terapêutica (National

Institutes of Health [NIH] Definition Working Group, 2001). Biomarcadores podem ser genes, proteínas ou outras moléculas, assim como características morfológicas identificadas com base em mecanismos fisiológicos ou biológicos.

Mais do que aperfeiçoar o diagnóstico, biomarcadores poderiam auxiliar na previsão do prognóstico das doenças e no risco potencial de se desenvolver um transtorno, com aplicações válidas no monitoramento do estágio da doença e na resposta a uma intervenção terapêutica ou a uma estratégia de manejamento (Puntmann*,* 2009). Ainda mais, biomarcadores poderiam contribuir na descoberta dos mecanismos da patofisiologia em transtornos psiquiátricos complexos (Schwarz *et al.,* 2008). Particularmente no TB, biomarcadores podem tornar-se ferramentas úteis na detecção da atividade da doença associada a diferentes estados de humor (um marcador de estado) ou na identificação de características específicas do curso da doença em longo prazo (marcador de traço) (Frey *et al.,* 2013).

Apenas marcadores validados podem ser utilizados no contexto clínico. Em outras palavras, a fim de ser usado clinicamente, um biomarcador deve provar ser preciso, ter alta sensibilidade e especificadade para o desfecho esperado, ser altamente reprodutível, possuir alto custo-benefício, ser um ensaio rápido, minimamente invasivo e facilmente aceito pelo paciente, além de produzir um resultado clínico relevante, com informações facilmente interpretáveis (Puntmann*,* 2009). Entre as estratégias adotadas para o descobrimento e o uso de biomarcadores no TB, um grande interesse em biomarcadores periféricos é observado: certas proteínas encontradas no sangue periférico podem ser transportadas através da barreira hemato-encefálica e entrar no Sistema Nervoso Central (SNC) (Abbott *et al.,* 2010). Por exemplo, proteínas da família das neuroregulinas (NRG, do inglês *Neuroregulin*), que poderiam aumentar a mielinização de neuritos, e o Fator Neurotrófico-Derivado do Cérebro (BDNF, do inglês *Brain-Derivate Neurotrophic Factor*) poderiam entrar na medula espinhal e no cérebro através de um mecanismo saturável mediado por receptores (Kastin *et al.,* 2004, Pan *et al.,* 1998). Um estudo recente encontrou uma correlação entre níveis de BDNF no fluído

cerebroespinhal e no plasma de sujeitos *drug-naïve* [2] em primeiro episódio psicótico (Pillai *et al.,* 2010).

Essa abordagem, usando biomarcadores periféricos, tem diversas vantagens, incluindo fácil coleta, baixo custo, ampla disponibilidade e viabilidade para estudos em larga escala. Diversos marcadores periféricos têm sido estudados como mediadores de alostase no TB (Berk *et al.,* 2011, Kapczinski *et al.,* 2008b). Os estudos tem focado principalmente em mecanismos biológicos relacionados à neuroplasticidade no TB, incluindo o papel de neurotrofinas, inflamação, estresse oxidativo e processos subjacentes.

Neurotrofinas

Fatores neurotróficos são pequenas proteínas secretadas que atuam em uma gama de funções biológicas relacionadas à interação com diferentes receptores, sua distribuição local e transporte para o SNC (Chao *et al.,* 2006). O Fator de Crescimento Neural (NGF do inglês *Nerve growth fator*) foi a primeira neurotrofina a ser identificada, por Levi-Montalcini em 1966. Depois disso, diversos estudos revelaram outras neurotrofinas, como BDNF, Fator de Crescimento Derivado da Glia (GDNF do inglês *glial cell-line derived neurotrophic fator*), Neurotrofina 3 (NT-3) e Neurotrofina 4/5 (NT-4/5), todas desempenhando papéis fundamentais na plasticidade sináptica, arborização dendrítica e na conectividade neuronal. Além disso, todas tem sido demonstradas alteradas no TB (Berk*,* 2009, Kapczinski *et al.,* 2008b).

O BDNF é a neurotrofina mais abundante e mais amplamente distribuída no SNC, e também a mais estudada; estudos atuais mostram que a expressão alterada de BDNF contribui para diversos transtornos, incluindo o TB. Além disso, foi sugerida a existência de uma correlação entre níveis séricos de BDNF e outros marcadores de dano do SNC (Lang *et al.,* 2007, Schmidt *et al.,* 2010).

[2] Estado fisiológico de não-habituação a um fármaco específico ou a um conjunto de fármacos. Nessa revisão usaremos o termo "pacientes *drug-naïve*" quando nos referirmos àqueles que nunca fizeram uso de medicações psiquiátricas

Um crescente grupo de evidências aponta para a relação entre níveis periféricos de BDNF e atividade da doença no TB.

Níveis séricos de BDNF têm sido encontrados reduzidos no TB durante episódios maníacos e depressivos quando comparado a pacientes eutímicos e controles saudáveis, até mesmo em pacientes *drug-free* [3] (Cunha *et al.,* 2006, de Oliveira *et al.,* 2009, Goldstein *et al.,* 2011, Machado-Vieira *et al.,* 2007). Em crianças e adolescentes *drug-free* em episódios agudos de mania, uma diminuição nos níveis de BDNF (tanto em mRNA derivado de linfócitos como em níveis proteicos derivados de plaquetas) foi encontrada em comparação a controles saudáveis (Pandey *et al.,* 2008). Devido a algumas discrepâncias entre os estudos (Barbosa *et al.,* 2013, Dias *et al.,* 2009, Huang *et al.,* 2012, Kapczinski *et al.,* 2010, Monteleone *et al.,* 2008), metanálises foram conduzidas para medir o tamanho de efeito das diferenças entre os níveis de BDNF em pacientes em diferentes estados de humor e controles (Fernandes *et al.,* 2011, Regenold *et al.,* 2009). A última dessas metanálises demonstrou que níveis periféricos de BDNF diminuem significativamente durante episódios de mania e de depressão, e aqueles pacientes que sofreram mais episódios apresentam menores níveis de BDNF.

Com relação ao tratamento farmacológico, estabilizadores de humor tem demonstrado capacidade de aumentar os níveis de BDNF (Fernandes *et al.,* 2011). Por exemplo, a recuperação de pacientes a partir de um episódio maníaco após tratamento com lítio tem sido associada com um aumento dos níveis séricos de BDNF (Tramontina *et al.,* 2009). Na mesma linha, Rybakowski e Suwalska descobriram pacientes que respondem bem ao lítio apresentam maiores níveis plasmáticos de BDNF quando comparados aqueles que não respondem, e níveis similares quando comparados ao grupo controle (Rybakowski *et al.,* 2010). Outros dados têm demonstrado um aumento significativo nos níveis de BDNF após monoterapia de lítio para o manejamento de episódios maníacos, sugerindo um papel direto dos efeitos regulatórios do lítio nos níveis de BDNF na mania (de Sousa *et al.,* 2011).

[3] Nessa tese usaremos o termo "pacientes *drug-free*" ou "não-medicados" quando nos referirmos àqueles que não fizeram uso de medicações psiquiátricas nas últimas duas semanas.

Um recente estudo longitudinal aberto em pacientes previamente não-medicados quantificou os níveis séricos de BDNF sequencialmente por 16 semanas. Relevantemente, os níveis de BDNF tenderam a aumentar com o tratamento, mas apenas em pacientes que estavam agudamente depressivos na primeira semana. Por sua vez, aqueles em episódio maníaco ou misto, mostraram uma diminuição dos níveis de BDNF nas primeiras semanas de tratamento. Esses resultados sugerem que episódios maníacos ou mistos podem ser particularmente tóxicos quando comparados aos episódios depressivos, talvez necessitando mais tempo de tratamento para retornar aos seus níveis basais (Grande *et al.,* 2012). Todos estes estudos que descrevem um papel importante do BDNF na fisiopatologia do TB levaram a uma crescente investigação sobre novos alvos desta neurotrofina, além da já conhecida ação terapêutica dos estabilizadores de humor.

Mudanças em outros fatores neurotróficos também têm sido descritas em pacientes com TB, como por exemplo, o aumento nos níveis séricos de NT-3 durante episódios maníacos e depressivos quando comparados a pacientes eutímicos ou controles (Fernandes *et al.,* 2010, Walz *et al.,* 2007); ou o aumento nos níveis séricos de NT-4/5 em pacientes *vs.* controles saudáveis, independente do estado sintomático (Walz *et al.,* 2009). Um estudo recente encontrou níveis plasmáticos de GDNF aumentados em pacientes eutímicos quando comparados a pacientes maníacos e a controles (Barbosa *et al.,* 2011), embora outro estudo tenha observado níveis aumentados de GDNF em pacientes maníacos e depressivos, mas não em pacientes eutímicos quando comparados ao grupo controle (Rosa *et al.,* 2006). Além disso, um estudo prévio encontrou níveis séricos de GDNF diminuídos em pacientes durante a mania e a depressão, e níveis aumentados após a remissão dos sintomas (Zhang, X. *et al.,* 2010). Ao contrário, outro estudo mostrou níveis diminuidos de GDNF em pacientes com TB em remissão (Takebayashi *et al.,* 2006). Juntos, esses achados reforçam as implicações do GDNF na patofisiologia do TB, entretanto com um papel ainda obscuro. Evidências adicionais são necessárias para avaliar se os níveis periféricos de GDNF são correlacionados com os níveis no SNC desse neurotrofina (Lundborg *et al.,* 2010).

Inflamação

Nos últimos anos, o número de publicações que focaram em anormalidades imunológicas envolvidas na patofisiologia do TB cresceu substancialmente. Distúrbios imunes têm sido relacionados com a severidade e a recorrência dos episódios de humor (Kauer-Sant'Anna *et al.,* 2009), a progressão da doença (Barbosa *et al.,* 2012, Leboyer *et al.,* 2012), os altos índices de comorbidades (Leboyer *et al.,* 2012) e os efeitos farmacológicos (Dean *et al.,* 2011, Nery *et al.,* 2008).

Acima de tudo, os episódios de humor têm sido caracterizados como sendo estados pró-inflamatórios (Frey *et al.,* 2013), baseando-se em estudos que mostram aumento nos níveis periféricos de citocinas pró-inflamatórias, como interleucina (IL)-6 e fator de necrose tumoral alfa (TNF-α, do inglês *tumor necrosis factor alpha*) durante episódios de depressão, e IL-2, IL-4, IL-6 e TNF-α durante episódios de mania, quando comparado a pacientes eutímicos e controles saudáveis (Brietzke *et al.,* 2009, Kim, Y. K. *et al.,* 2007b, Ortiz-Dominguez *et al.,* 2007). Uma metanálise recente mostrou que pacientes durante o episódio de mania possuem altos níveis de TNF-α, do receptor solúvel do tipo 1 do fator de necrose tumoral (sTNF-R1, do inglês *soluble tumor necrosis factor receptor type 1*), e do receptor solúvel da IL-2 (sIL-2R, do inglês *soluble inlerleukin-2 receptor*) quando comparado a controles, e altos níveis de sTNF-R1 e TNF-α quando comparado a pacientes eutímicos (Munkholm *et al.,* 2012). Outra metanálise recente encontrou que pacientes maníacos apresentam níveis aumentados de TNF-α e sIL-2R e uma tendência a níveis aumentados de sTNF-R1 quando comparados a pacientes eutímicos, além de altos níveis do antagonista do receptor de IL-1 (IL-1RA, do inglês *IL-1 receptor antagonist*) e uma tendência a maiores níveis de IL-6 quando comparado a controles saudáveis. Níveis aumentados de IL-10 também foram encontrados em pacientes em depressão *vs.* controles, entretanto sem alcançarem diferenças significativas entre as fases agudas. Por fim, algumas citocinas, como IL-1RA, têm demonstrado níveis alterados em eutimia quando comparado a controles (Modabbernia *et al.,* 2013).

Um estudo feito com monócitos de pacientes com TB demonstrou nestas células, expressão gênica inflamatória com características alteradas, algumas envolvendo citocinas comumente correlacionadas com TB, principalmente, *TNF-α e IL-6* (Padmos *et al.,* 2008). Nesse estudo, a expressão do *ligante de quimiocina 2* (*CCL2*, do inglês *Chemokine (C-C motif) ligand 2)* e da *proteína cinase ativada por mitógeno 6* (*MAPK6,* do inglês *mitogen-activated protein kinase 6)* estava significativamente maior nos monócitos durante episódios de mania e depressão. Além disso, a superexpressão de *IL6*, *proteína relacionada a pentaxina 3* (*PTX3,* do inglês *pentaxin-related protein 3*) e genes de sinalização para sobrevivência/apoptose celular [*proteína epitelial de membrana 1* (*EMP1,* do inglês *epithelial membrane protein 1)* e *proteína relacionada a leucemia/linfoma de células B A1* (*BCL2A1,* do inglês *B-cell leukemia/lymphoma 2–related protein A1*)] foi detectada durante a fase depressiva quando comparado a pacientes eutímicos, sugerindo uma ativação diferencial do sistema da resposta inflamatória. O estudo mencionado anteriormente demonstrou que o estado inflamatório dos monócitos de pacientes com TB é familiar, o que significa que resultados similares foram encontrados nos monócitos dos descendentes desses pacientes. Mesmo assim, o estudo falhou em responder se essa assinatura era resultante de fatores genéticos ou ambientais. Então, num estudo de sequência, mostra que em gêmeos monozigóticos e dizigóticos a ativação monocitária pró-inflamatória é provavelmente devida a fatores ambientais compartilhados (Padmos *et al.,* 2009). Seguindo essa mesma direção, o grupo demonstrou que pacientes com esquizofrenia também apresentam uma ativação monocitátia inflamatória. A "assinatura" dessa ativação é parecida com a encontrada em pacientes com TB, com aumento da regulação do *fator de ativação da transcrição 3* (*ATF3*, do inglês *Activating transcription factor 3*), da *proteína fosfatase de dupla especificidade 2* (*DUSP2*, do inglês *Dual specificity protein phosphatase 2*), da *proteína de reposta precoce ao crescimento 3* (*EGR3*, do inglês *Early growth response protein 3*) e *da proteína de dimerização MAX 1* (*MXD1*, do inglês *MAX Dimerization Protein 1*) e diferindo de pacientes com TB com relação à *proteína fosfatase tirosina do tipo não-receptor 7* (*PTPN7*, do inglês *Protein tyrosine phosphatase non-receptor type 7*) e da *proteína de ligação ao NGFI-A 2* (NAB2, do inglês *NGFI-A-binding protein 2*) que

apresentam aumento da regulação no TB *vs.* diminuição da regulação em pacientes esquizofrênicos, sendo ambos os fatores de regulação da transcrição da MAPK (Drexhage *et al.,* 2010).

Entretanto, essa assinatura inflamatória aumentada em pacientes com TB no nível de transcrição gênica ainda não foi demonstrada ao nível proteico. Além disso, outro estudo identificou alterações na expressão de sete proteínas pró-inflamatórias e cinco pro-/antiinflamatórias no soro de pacientes em eutimia (Herberth *et al.,* 2011). Esse soro foi utilizado posteriormente para tratar células periféricas mononucleares sanguíneas, observando-se uma diminuição na viabilidade celular dessas células. Esses resultados apontam para um aumento na resposta inflamatória e, provavelmente, para a morte de células do sistema imune de pacientes com TB.

Em concordância com as anormalidades anteriormente mencionadas, adolescentes com TB também apresentam um determinado tipo de distúrbio imunológico. Achados preliminares encontrados em uma amostra de adolescentes com TB indicaram uma associação entre severidade de sintomas maníacos e proteína C reativa de alta sensibilidade (hsCRP, do inglês *high-sensitivity C-reactive protein*), assim como uma associação negativa com os níveis séricos de IL-6 e BDNF (Goldstein *et al.,* 2011). Na mesma linha, demonstrou-se que. descendentes de pacientes com TB também apresentam alteração na expressão de genes relacionados à inflamação (Padmos *et al.,* 2008). As alterações mencionadas acima servem como fonte de informações a respeito das bases biológicas do TB. Entretanto, essas alterações nem sempre são encontradas e nem sempre a mesma citocina acaba sendo implicada nos resultados. Portanto, mesmo sendo ainda limitado o uso de marcadores inflamatórios como biomarcadores para a predição do prognóstico no TB, os achados recentes podem apontar para novos alvos para o tratamento e monitoramento desses pacientes, com o objetivo de melhorar sua qualidade de vida.

A origem do desbalanço imunológico visto no TB é ainda desconhecida. Entretanto, alguns trabalham apontam para a necessidade de estudos futuros com foco em fatores como sono e alterações no ritmo circadiano, estresse, ativação imune por infecção por retrovírus ou disfunção

imune (Rutten *et al.,* 2009), estilo de vida não saudável e longos períodos de exposição a drogas (Goldstein *et al.,* 2009, Murray *et al.,* 2009).

Pacientes com TB são conhecidos por terem alto risco de desenvolver comorbidades médicas, incluindo doenças cardiovasculares, síndrome metabólica e diabetes (Goldstein *et al.,* 2011, McIntyre *et al.,* 2010, Weiner *et al.,* 2011). A principal conexão entre essas doenças parece ser a presença de inflamação sistêmica crônica ou os altos níveis de marcadores inflamatórios mencionados anteriormente. De fato, é precisamente devido ao crescente conjunto de evidências que sugerem uma inflamação crônica leve na periferia e no cérebro de pacientes com TB (Berk *et al.,* 2009, Brietzke *et al.,* 2009, Hamdani *et al.,* 2012) que alguns autores tem se referido ao transtorno como sendo uma doença inflamatória multisistêmica (Leboyer *et al.,* 2012, Stertz *et al.,* 2013).

Um grande fator de confusão que está presente em quase todos os estudos elaborados para investigar a presença da inflamação no TB é a exposição dos pacientes a fármacos. Alguns estudos propuseram que o lítio pode restaurar o desbalanço inflamatório observado no TB (Knijff *et al.,* 2007). Guloksuz e cols. (Guloksuz *et al.,* 2012) encontraram uma correlação entre a baixa resposta ao lítio e altos níveis de TNF-α. Mais estudos são necessários para elucidar a relação entre marcadores inflamatórios, tratamento e o desenvolvimento de comorbidades clínicas no TB.

Estresse Oxidativo

Um conjunto crescente de evidências tem demonstrado que o estresse oxidativo desempenha um papel importante na patofisiologia do TB (Andreazza *et al.,* 2008, Dean *et al.,* 2009, Zhang, X. Y. *et al.,* 2013). O estresse oxidativo é definido como um desequilíbrio entre agentes oxidantes e antioxidantes, com potencial para causar dano celular. Níveis reduzidos de antioxidantes, ou uma produção aumentada de pró-oxidantes, resultará em um estado de estresse, acarretando finalmente danos a macromoléculas como lipídios, proteínas (receptores e enzimas), carboidratos e DNA (Halliwell, B. & Guteride, 2007).

O SNC é particularmente vulnerável ao dano oxidativo, devido ao elevado consumo de oxigênio e a consequente geração de radicais livres, além desta estrutura apresentar uma capacidade antioxidante relativamente baixa (Olmez *et al.,* 2012). Níveis oxidativos aumentados em neurônios podem ter efeitos deletérios sobre a transdução de sinal, plasticidade sináptica e resiliência[4] celular (Khairova *et al.,* 2012). O sistema antioxidante é a principal linha de defesa contra o estresse oxidativo e pode ser dividido em sistema enzimático, compreendendo as enzimas chave superóxido dismutase (SOD, do inglês *superoxide dismutase*), catalase e glutationa peroxidase (Gpx, do inglês *glutathione peroxidase*), e o sistema não enzimático (Halliwell, B*.,* 2011). O mais importante antioxidante e regulador REDOX celular não-enzimático é a glutationa (GSH), o antioxidante dominante no encéfalo (Wood *et al.,* 2009).

Em condições fisiológicas, as mitocôndrias são uma importante fonte de radicais livres (oxidantes), produzidos nos complexos da cadeia transportadora de elétrons (Drose *et al.,* 2012). No TB, a hipótese dominante é a de que uma maior carga de estresse oxidativo é gerada como resultado de uma função mitocondrial comprometida (Berk *et al.,* 2011). Esta hipótese foi corroborada por estudos *post mortem* reportando alterações na atividade do complexo I mitocondrial (Andreazza *et al.,* 2010) e níveis reduzidos de GSH (Gawryluk *et al.,* 2011) no córtex pré-frontal (CPF) de pacientes. A disfunção mitocondrial no TB será revisada em outra seção.

Estudos clínicos demonstraram alterações sistêmicas em diversos parâmetros de estresse oxidativo e enzimas antioxidantes em pacientes com TB. Algumas destas mudanças foram relacionadas com episódios de humor. Por exemplo, Andreazza e cols. (Andreazza *et al.,* 2007a) relataram um aumento na atividade de SOD durante episódios maníacos e depressivos, mas não em eutimia. Estes achados foram confirmados por Machado-Vieira e cols. (Machado-Vieira *et al.,* 2007),

[4] Termo do latim *resilio*, que significa voltar atrás, voltar de um salto. Na física é utilizado para definir o nível de resistência que um material pode sofrer frente às pressões sofridas e sua capacidade de retornar ao estado original sem a ocorrência de dano ou ruptura. Posteriormente, foi incorporado à psicologia, para indicar como as pessoas respondem às frustrações diárias, em todos os níveis, e sua capacidade de recuperação emocional. Ao logo dessa tese, usaremos o termo *resiliência celular* para indicar um conjunto de repostas celulares que acontecem frente as mudanças e estímulos que afetam a homeostase celular.

o qual demonstrou uma atividade aumentada de SOD em pacientes maníacos não-medicados, bem como por Kunz e cols. (Kunz *et al.,* 2008), que também relatou um aumento da atividade de SOD em episódios agudos de TB, mas não em eutimia. No entanto, outros trabalhos apresentaram redução da atividade de SOD em episódios agudos do TB e em eutimia (Gergerlioglu *et al.,* 2007, Ranjekar *et al.,* 2003, Selek *et al.,* 2008). Adicionalmente, Raffa e cols (Raffa *et al.,* 2012) não encontraram diferenças nos níveis de SOD em pacientes com TB quando comparados a controles saudáveis. A atividade da catalase também foi encontrada reduzida em pacientes eutímicos (Andreazza *et al.,* 2007a, Raffa *et al.,* 2012, Ranjekar *et al.,* 2003), mas aumentada em pacientes não-medicados durante a mania (Machado-Vieira *et al.,* 2007). Estes resultados sugerem que alterações em enzimas antioxidantes podem ocorrer em função do tratamento e dos episódios da doença. Uma frequência aumentada de dano ao DNA, possivelmente causado por estresse oxidativo, foi demonstrada em pacientes com TB e foi correlacionada com a severidade dos sintomas maníacos e depressivos (Andreazza *et al.,* 2007b). Reciprocamente, uma metanálise investigando marcadores de estresse oxidativo em TB demonstrou que as substâncias reativas ao ácido tiobarbitúrico (TBARS, do inglês *thiobarbituric acid reactive substances*), um marcador da peroxidação lipídica, e o óxido nítrico (NO, do inglês *nitric oxide*), uma espécie reativa de nitrogênio, estavam significativamente aumentados em todas as fases do TB (Andreazza *et al.,* 2008), sugerindo um papel relevante destes parâmetros como possíveis biomarcadores de traço da doença.

Cabe também salientar que evidências de estudos pré-clínicos, clínicos e epidemiológicos sugerem um benefício do uso adjuvante de componentes antioxidantes no TB (Pillai*,* 2008). A N-acetilcisteína (NAC), por exemplo, se demonstrou segura como adjuvante para estabilizadores de humor em dois ensaios randomizados (Berk *et al.,* 2008, Berk *et al.,* 2012, Magalhaes *et al.,* 2011a). Dados preliminares também sugerem efeitos clínicos de componentes antioxidantes na mania e na depressão, bem como um efeito particularmente forte em pacientes com comorbidades (Magalhaes *et al.,* 2011a, b).

Mecanismos subjacentes à toxicidade sistêmica e alterações celulares no transtorno bipolar

Os mecanismos responsáveis pela diminuição à resiliência frente a condições estressantes, associada a episódios agudos no TB, envolve, provavelmente, organelas e rotas de sinalização tipicamente responsáveis pela manutenção da homeostase celular, como mitocôndria e retículo endoplasmático (RE), podendo ainda afetar células da periferia e do SNC, como neurônios e glia. A pesquisa básica contribuiu de maneira significativa para a compreensão destes mecanismos, especialmente no que tange a toxicidade relacionada à recorrência de episódios de humor no TB. Nesta seção, tentaremos resumir alguns dos mecanismos responsáveis pela toxicidade no TB.

Disfunção mitocondrial e o papel do estresse crônico

Um conjunto crescente de evidências tem sugerido um papel central da disfunção mitocondrial no TB (Manji *et al.,* 2012). Metabolismo energético debilitado, alterações nos complexos da cadeia respiratória, níveis alterados de cálcio citoplasmático e regulação negativa de genes relacionados à mitocôndria integram algumas das anormalidades descritas (Manji *et al.,* 2012). Adicionalmente, vários estudos genéticos, de imagem e *post mortem* indicaram uma associação entre disfunção mitocondrial e TB (Cataldo *et al.,* 2010). Concentrações médias de lactato no fluído cerebroespinhal são significativamente maiores em pacientes quando comparados a controles, indicando aumento do metabolismo anaeróbico e extra-mitocondrial da glicose, sendo consistente com um quadro de metabolismo mitocondrial debilitado no TB (Regenold *et al.,* 2009). Recentemente, foi relatada uma redução no ancoramento da hexoquinase 1 à membrana mitocondrial externa em tecidos *post mortem* do córtex pariental de cérebros de indivíduos com TB, sendo essa redução relacionada ao aumento da atividade de uma rota anaeróbica alternativa do metabolismo da glicose (Regenold *et al.,* 2012). Do mesmo modo, alterações no formato e na distribuição das mitocôndrias poderiam ser uma das causas subjacentes a disfunção energética no TB, conforme demonstrado no córtex pré-frontal de cérebros *post mortem* e em células periféricas de pacientes com TB (Cataldo *et al.,* 2010). O papel da disfunção mitocondrial no TB é adicionalmente endossada por estudos

reportando que estabilizadores de humor e antidepressivos podem aumentar a atividade mitocondrial (Bachmann *et al.,* 2009, Manji *et al.,* 2012, Valvassori *et al.,* 2010). Por exemplo, foi demonstrado que o lítio estimula a atividade das enzimas da cadeia respiratória mitocondrial em concentrações clinicamente relevantes (Maurer *et al.,* 2009).

Em uma escala mais abrangente, anormalidades poderiam estar associadas com as consequências da exposição crônica ao estresse, o qual parece desempenhar um papel na patofisiologia do TB (Post, R. M. *et al.,* 2012). O eixo hormonal do estresse, mais comumente conhecido como eixo Hipotálamo-Hipófise-Adrenal (HPA, do inglês *hypothalamic-pituitary-adrenal*), está claramente alterado em distúrbios do humor, conforme sugerido pelo alto número de pacientes com TB que apresentam deficiência na supressão da liberação de cortisol no teste de supressão com dexametasona (Daban *et al.,* 2005). Esta deficiência do eixo HPA resulta em uma anteroalimentação na produção de cortisol em resposta ao estresse, bem como em uma redução da habilidade de retornar aos níveis fisiológicos após a interrupção da exposição ao estresse (Tatro *et al.,* 2009). Consequentemente, pacientes com TB apresentam níveis semelhantes de cortisol nos três episódios do distúrbio, superiores aqueles observados em controles (Cervantes *et al.,* 2001). Estes níveis aumentados de cortisol podem, em longo prazo, acarretar importantes consequências aos pacientes. Por exemplo, estudos *in vitro* e com modelos animais demostraram que o estresse crônico e a exposição crônica aos glicocorticoides podem induzir disfunção mitocondrial, causando redução no consumo de oxigênio, no potencial da membrana mitocondrial e na capacidade de retenção de cálcio, levando finalmente a apoptose (Du *et al.,* 2009, Gong *et al.,* 2011). Glicocorticoides podem ainda agravar a inflamação e induzir toxicidade no SNC, reduzindo a capacidade dos neurônios de remover glutamato da sinapse e sequestrar radicais livres (Sorrells *et al.,* 2009). Adicionalmente, toxicidade e dano neuronal poderiam ser gerados por um aumento sinérgico de inflamação, estresse oxidativo e disfunção mitocondrial (Yamamoto *et al.,* 2008).

Em conjunto, nós postulamos que algumas das disfunções mitocondriais nos pacientes com TB são induzidas e agravadas pelo estresse crônico. Consequentemente, mitocôndrias debilitadas

podem prejudicar a resiliência celular aos estímulos ambientais, finalmente induzindo a ativação de caspases e apoptose. Após sua morte, estas células podem acabar liberando moléculas imunoestimulatórias e, deste modo, induzindo alterações em marcadores inflamatórios. Por sua vez, estas alterações sistêmicas podem ser responsáveis por efeitos prejudiciais sobre células periféricas, possivelmente induzindo apoptose e assim completando um ciclo vicioso de toxicidade periférica e redução da resiliência celular.

Estresse do retículo endoplasmático

O RE desempenha um papel central no armazenamento e sinalização do cálcio (Ca), bem como na síntese, dobramento e controle de qualidade de proteínas de membrana e proteínas secretadas (Rutkowski *et al.,* 2004). Alterações no ambiente luminal do RE, tais como alterações no estado REDOX e na homeostase do Ca, privação nutricional, ou defeitos nas modificações pós-traducionais de proteínas, podem afetar a função desta organela e subsequentemente resultar no acúmulo de proteínas mal enoveladas. Esta condição é conhecida como estresse do RE e a resposta celular para esta condição é referida como resposta a proteínas mal enoveladas (UPR, do inglês *unfolded protein response*), um processo adaptativo fisiológico no qual as células ativam mecanismos de proteção para restaurar a homeostase no RE. Estresse prolongado do RE (como em situações onde a UPR não é suficiente para restaurar o balanço) leva a morte celular (Kimata *et al.,* 2011, Walter *et al.,* 2011).

Alguns estudos sugeriram um envolvimento da disfunção da UPR na patofisiologia do TB. Por exemplo, um resposta reduzida de XBP1 (um fator de transcrição que induz a expressão de chaperonas do RE) e CHOP (um fator de transcrição envolvido na apoptose induzida por estresse do RE) foi encontrada em células linfoblastóides de pacientes expostas a dois indutores de estresse do RE (So *et al.,* 2007). Outros achados confirmaram estes resultados, relatando a redução no *splicing* de *XBP1* induzido por estresse e na expressão de *GRP94* (outra chaperonas do RE) em pacientes com TB (Hayashi *et al.,* 2009). Além disso, evidências farmacológicas sugerem que o estabilizador de humor valproato modula a resposta ao estresse do RE (Kakiuchi *et al.,* 2009, Kim, B. Kim CY, Lee MJ, Joo YH. ,

2009, Zhang, Z. *et al.,* 2011). Em um estudo recente, linfócitos de pacientes com TB, em contraste com controles saudáveis, falharam na indução de proteínas relacionadas a UPR e apresentaram níveis aumentados de morte celular em resposta ao estresse do RE induzido *in vitro*, sugerindo que esta resposta debilitada ao estresse do RE possa refletir uma suscetibilidade celular aumentada (Pfaffenseller*,* 2012).

Em conjunto, estes achados sugerem que pacientes com TB apresentam uma resposta debilitada ao estresse do RE, inadequada e insuficiente para manter a homeostase. Esta resposta debilitada ao estresse do RE pode estar relacionada a várias disfunções neuronais relatadas para estes pacientes, considerando que componentes da UPR também estão envolvidos no desenvolvimento e plasticidade neural, maturação e transporte de vários receptores, bem como na sinalização por cálcio (Vandenberghe *et al.,* 2005, Verkhratsky*,* 2002, Weng *et al.,* 2011).

O RE está proximamente ligado com as mitocondrias, tanto em termos morfológicos quanto funcionais; intercâmbio de Ca é provavelmente a principal forma de comunicação entre estas organelas (Giorgi *et al.,* 2009). Sinais de Ca derivados do RE modulam a bioenergética mitocondrial. Como resultado, alterações nas interações entre RE e mitocôndrias, tais como alterações nos níveis celulares de Ca, influenciam a regulação do metabolismo celular e podem causar disfunção mitocondrial, desbalanço metabólico e finalmente desencadear a morte celular (Wang *et al.,* 2011). É importante salientar que a alteração nos níveis intracelulares de cálcio é um achado consistente no TB (Uemura *et al.,* 2011).

Uma relação prejudicial entre estas organelas também parece estar envolvida com o dano oxidativo (Csordas *et al.,* 2009). Levando em consideração o prolongado estresse do RE e disfunção mitocondrial observada no TB, a ruptura das interações RE-mitocondrias pode potencialmente ser responsável pelas alterações metabólicas e pela toxicidade periférica associada com o distúrbio. O estresse do RE pode também estar relacionado com rotas de neurotrofinas (Chen, G. *et al.,* 2007, Hashimoto*,* 2013), as quais podem contribuir com a manutenção do dano oxidativo e da inflamação

sistêmica no TB, uma vez que estes processos estão intimamente relacionados (Adolph *et al.,* 2012, Malhotra *et al.,* 2008).

Alterações gliais

Em 1858, Rudolf Virchow descreveu as células gliais como um tecido conectivo que mantém unidos os elementos neuronais (Parpura *et al.,* 2012). Conforme o conhecimento atual, o papel destas células vai muito além: células gliais são componentes funcionais do sistema nervoso. Por vezes referidas como neuroglia, suas funções incluem manutenção da homeostase (astrócitos), formação da mielina (oligodendrócitos), além de suporte e proteção dos neurônios no encéfalo (microglia). Células gliais são capazes de responder a alterações no ambiente celular e extracelular e apresenta capacidades de comunicação que complementam as dos neurônios, possivelmente através de uma rede glial (Parpura *et al.,* 2012). Tendo em vista o importante papel desempenhado por estas células no SNC, é natural considerar-se que elas também desempenham um papel importante no estabelecimento e no desenvolvimento de distúrbios neurológicos. De fato, diversos estudos demonstraram alterações em células gliais em distúrbios psiquiátricos, incluindo uma reduzida densidade glial na amídala de pacientes com depressão maior (Altshuler *et al.,* 2010) e regulação positiva de proteínas da matriz extracelular em astrócitos da amígdala e do córtex entorrinal de pacientes esquizofrênicos (Pantazopoulos *et al.,* 2010). Mais especificamente no TB, os resultados nos últimos cinco anos são escassos.

Observações históricas e estudos de imagem corroboram achados de anormalidades na mielina e alterações gliais no TB (Herring *et al.,* 2011) Oligodendrócitos expressam transferrina (TF), uma proteína carreadora de ferro que atua como um fator trófico e de sobrevivência para neurônios e astrócitos, apontando para outra importante função dos oligodendrócitos além da mielinização (Silvestroff *et al.,* 2012). Um estudo *post mortem* demonstrou que a TF está subexpressa na capsula interna de pacientes com TB; em contraste, dois genes associados aos astrócitos (*GFAP* and *ALDH1L1*) apresentaram valores médios aumentados em todas as regiões do cérebro (Barley *et al.,* 2009). Estes

resultados podem indicar uma função deficiente dos oligodendrócitos e algum nível de astrocitose (aumento de marcadores de astrócito). Outro estudo relatou que os marcadores astrogliais e microgliais (proteína glial fibrilar ácida, óxido nítrico sintetase induzida, c-fos e CD11b) estavam regulados positivamente, de modo significativo, no córtex frontal *post mortem* de pacientes com TB, particularmente a cascata do IL-1R envolvido na ativação microglial (Rao *et al.,* 2010). A microglia representa os macrófagos residentes do cérebro, os quais se tornam ativados em resposta a dano tecidual ou infecções no cérebro (Harry *et al.,* 2012). Adicionalmente, a localização da *NRG*, um gene envolvido com o desenvolvimento de oligodendrócitos e mielinização no SNC, em um dos *loci* genéticos para TB (Taveggia *et al.,* 2008) representa outro indício de que alterações gliais merecem mais atenção. Na verdade, é possível que disfunções gliais no TB possam resultar em interações neurônio-glia anormais, conforme previamente relatado para mania (Ongur *et al.,* 2008).

Nós especulamos que as anormalidades acima descritas possam estar inter-relacionadas, afetando resiliência e função celular, tanto na periferia quanto no cérebro de pacientes com TB. Indo ao encontro de hipóteses anteriores (Kapczinski *et al.,* 2008b), existe provavelmente um conjunto de processos complexos e inter-relacionados ocorrendo no TB, os quais podem levar ao comprometimento celular e estar relacionados com a toxicidade encontrada em pacientes durante episódios agudos (Kapczinski *et al.,* 2010). Mais estudos focados na associação entre estes processos e os estados de humor são necessários para uma melhor compreensão dos mecanismos responsáveis pela toxicidade sistêmica no TB.

Epigenética

Dentre os diversos mecanismos que podem estar relacionados com a neuroprogressão, mecanismos epigenéticos são fortes candidatos. Epigenética refere-se a modificações relativamente estáveis na função gênica (podendo se manter após várias divisões celulares) que ocorrem no DNA e/ou na cromatina e que não envolvem alterações na sequência do gene. Resumidamente, a expressão gênica é, parcialmente, o resultado do controle de acesso aos genes. Estudos indicam que essas alterações, que acontecem tanto em células que se dividem como em células que não se

dividem, podem ser transmitidas entre gerações (Weaver *et al.,* 2004). Portanto, a estabilidade das modificações epigenéticas a tornam foco de interesse no desenvolvimento de novos tratamentos.

Entre os mecanismos epigenéticos mais conhecidos, estão as alterações do estado de acetilação e metilação de genes e histonas. As histonas compõem a unidade do genoma, o nucleossomo, e sua hiperacetilação normalmente resulta em relaxamento da cromatina e aumento da atividade gênica. A acetilação das histonas, que é controlada por enzimas como aceil-transferase (HAT) e histona desacetilase (HDAC), confere uma espécie de "código", que acaba definindo quais partes do genoma se tornam acessíveis a transcrição em um determinado tecido em um dado momento (Jenuwein *et al.,* 2001). O papel da metilação das histonas não está completamente definido e pode indicar aumento ou repressão da transcrição (Tsankova *et al.,* 2007). Estabilizadores de humor (como VPT) assim como drogas de abuso (álcool e cocaína) demonstram capacidade de modular o estado da cromatina através da modificação da atividade da enzima Histona Desacetilase (HDAC). O Li perece compartilhar alguns mecanismos com os inibidores de HDAC, mas sua capacidade de inibir a enzima diretamente ainda não está estabelecido (Bordonaro *et al.,* 2007). Até agora, pouco se sabe sobre a relação entre a atividade da HDAC e os efeitos comportamentais induzidos pelos estabilizadores de humor. Estudos apontam para a capacidade do butirato de sódio (SB), um inibidor direto da enzima, em aumentar os níveis de BDNF *in vitro*, além de apresentar propriedades antidepressivas (Schroeder *et al.,* 2007, Yasuda *et al.,* 2007).

A metilação do DNA, por sua vez, ocorre através da transferência de um grupo metila de uma *S*-adenosil metionina (SAM, sigla do inglês *S-adenosyl methionine*) pela DNA metiltransferase (DNMT, sigla do inglês *DNA methyltransferase*). A metilação da região promotora dos genes é normalmente associada com a inibição da transcrição gênica. Importantes relações entre modificações ambientais e alterações na metilação do genes tem sido demonstrados. Por exemplo, em um estudo com macacos *rhesus*, filhotes trocados de mães não-abusivas para mães abusivas demonstraram comportamentos de abuso na vida adulta, similares as suas mães adotivas, sugerindo transmissão de comportamentos via mecanismos epigenéticos (Maestripieri*,* 2005). Outro estudo mostrou que alterações na metilação

do DNA no gene do receptor de glicocorticoide (*GR*) está associada com cuidado materno e vulnerabilidade ao estresse (Weaver *et al.,* 2004). No TB, estudos revelaram que a metilação do DNA difere em gêmeos monozigóticos discordantes para o transtorno. Gêmeos afetados demonstram aumento da metilação do gene *SMS* (do inglês *spermine synthase*) e diminuição da metilação do gene *PPIEL* (do inglês *petidylprolyl isomerase E-like*) (Kuratomi *et al.,* 2008).

Em suma, estresse, abuso de substâncias e comportamentos do tipo depressivo podem induzir alterações epigenéticas relativamente estáveis (Tsankova *et al.,* 2007), podendo representar uma "memória" da vulnerabilidade ao episódio de humor, culminando em anormalidades comportamentais e neurobiológicas. Além disso, regulações transcricionais e alterações epigenéticas induzidas por mecanismos de sensibilização podem estar direcionando algumas alterações neurológicas vistas clinicamente, incluindo baixos níveis de BDNF e aumentos persistentes de CRH (do inglês *Corticotropic releasing hormone*) e de glicocorticoides (Post, R. M., 2010a, Post, R.M. *et al.,* 2010b).

Novas terapias para o transtorno bipolar

Tendo em vista as rotas com implicações conhecidas sobre a atividade da doença, novas terapias podem ser propostas para um melhor manejo do TB. Pensando em um futuro mais imediato, alternativas interessantes podem envolver terapias adjuvantes que atuem sobre as rotas mencionadas nesta revisão (Kapczinski *et al.,* 2010, Kapczinski *et al.,* 2011). Alguns destes agentes, com efeitos antioxidantes, anti-inflamatórios e neuroprotetores, serão descritos em maiores detalhes abaixo.

Foi demonstrado em estudos básicos e clínicos que a NAC, um precursor da GSH, atenua o estresse oxidativo, modula a inflamação, atua na neurogênese e atua nas rotas glutamatérgica e dopaminérgica (Berk *et al.,* 2008, Dean *et al.,* 2011). A suplementação do tratamento convencional para TB com substâncias que atuam sobre o estresse oxidativo já foi investigada em estudos clínicos.

O tratamento com NAC em conjunto com a medicação usual para o TB, durante a fase de manutenção, melhorou significativamente os sintomas depressivos, a qualidade de vida e a funcionalidade em um estudo randomizado, duplo-cego, controlado por placebo, com grande tamanho de efeito (Berk *et al.,* 2008). Uma análise exploratória secundária revelou que o uso adjuvante de NAC apresentou eficácia promissora para pacientes com diagnóstico de depressão bipolar (Magalhaes *et al.,* 2011a). Recentemente, um ensaio randomizado, duplo-cego, controlado por placebo, investigando os efeitos de manutenção da NAC não conseguiu encontrar diferenças significativas nos sintomas ou recorrência durante a fase de manutenção (Berk *et al.,* 2012). Mais ensaios randomizados verificando o uso adjuvante de NAC são necessários para verificar de maneira confiável o tamanho efetivo dessa abordagem terapêutica.

Além de influenciar o estado REDOX, as propriedades neuroprotetoras da NAC podem estar associadas com sua habilidade de induzir neurogênese, a qual está provavelmente relacionada com mecanismos de proteção mitocondriais (Fries *et al.,* 2011); Adicionalmente, os efeitos de modulação da NAC sobre a inflamação (Ferreira *et al.,* 2012) podem ser fundamentais para sua eficácia como um agente estabilizador de humor, considerando a já descrita relevância da inflamação sistêmica na patofisiologia do TB (Kapczinski *et al.,* 2010). Portanto, embora poucos estudos tenham investigado o uso de agentes anti-inflamatórios como terapia adjuvante para o TB, rotas inflamatórias constituem outro grupo de novos alvos terapêuticos potenciais para o desenvolvimento de tratamentos mais eficientes para o TB. Foi demonstrado que estabilizadores de humor convencionais apresentam efeito sobre citocinas pró- e anti-inflamatórias (Guloksuz *et al.,* 2010, Guloksuz *et al.,* 2012). Entre as drogas anti-inflamatórias, o inibidor de ciclooxigenase-2 (COX-2) celecoxib foi estudado em um ensaio randomizado, duplo-cego, controlado por placebo, como um adjuvante no tratamento de pacientes com TB durante episódios depressivos ou mistos. O tratamento com celecoxib foi associado com uma rápida melhora dos sintomas depressivos após uma semana, comparado com placebo, mas a diferença foi estatisticamente significativa apenas para pacientes que completaram o tratamento de seis semanas. Estes achados sugerem um potencial efeito antidepressivo dos inibidores da COX (Nery

et al., 2008). Neste contexto, alguns estudos demonstraram que o tratamento com estabilizadores de humor aprovados para o tratamento do TB reduzem a expressão de marcadores da cascata metabólica do ácido araquidônico (AA) no cérebro de roedores, além de reduzirem a excitoxicidade e a regulação positiva destes marcadores induzida por neuroinflamação (Rao *et al.,* 2009). Artigos recentes demonstrando neuroinflamação, excitoxicidade (Rao *et al.,* 2010) e regulação positiva do metabolismo do AA (Kim, H. W. *et al.,* 2011) em cérebros *post mortem* de pacientes com TB corroboram a hipótese de alteração na cascata do AA no TB.

Outro componente sendo investigado atualmente é a minociclina, um antibiótico tetracíclico que atravessa a barreira hematoencefálica e apresenta efeitos antioxidantes, anti-inflamatórios e neuroproterores (Dean *et al.,* 2012). Uma vez que estas rotas se sobrepõem aos mecanismos patofisiológicos observados para o TB, o uso de minociclina foi apontado como um potencial tratamento adjuvante. Mais especificamente, a minociclina inibe a liberação dependente de microglia das citocinas pró-inflamatórias IL-1β, TNF-α, IL-6, e p38, e promove a liberação da citocina anti-inflamatória IL-10 (Hailer*,* 2008). Também é um eficiente sequestrador de espécies reativas de oxigênio e protege contra a excitotoxicidade glutamatérgica (Pae *et al.,* 2008). Relatos de caso de indivíduos com distúrbios psiquiátricos demonstraram benefícios do tratamento com minociclina, quanto à severidade dos sintomas. Atualmente, um estudo clínico está testando a eficácia da minoclina e/ou aspirina no tratamento da depressão bipolar e avaliando os efeitos anti-inflamatórios destes compostos (Savitz *et al.,* 2012).

A suplementação com ácidos graxos poli-insaturados ômega-3 (ω-3 PUFAs, do inglês *omega-3 polyunsaturated fatty acids*) também foi considerada um potencial novo tratamento para o TB, uma vez que estas gorduras apresentaram capacidade neuroprotetora e antioxidante em modelos animais (Wu *et al.,* 2004). Uma recente revisão de ensaios clínicos utilizando nutracêuticos em combinação com o tratamento padrão para TB demonstrou que os ω-3 PUFAs melhoraram os sintomas da depressão bipolar (Sarris *et al.,* 2012). A rota de sinalização do BDNF é um dos possíveis mecanismos de ação através do qual os ω-3 PUFAs mediam a regulação do humor em pacientes com TB (Balanza-

Martinez *et al.,* 2011). Novos ensaios clínicos randomizados, duplo-cegos, controlados por placebo com longos períodos de acompanhamento (*follow-up*) e tamanhos de efeito e poder adequados são necessários para que tenhamos uma melhor compreensão desta relação e do papel terapêutico dos ω-3 PUFAs no TB.

Fatores neutróficos estão emergindo como alvos terapêuticos promissores na TB. Foi demonstrado que o Lítio, o estabilizador de humor clássico, é eficiente na restauração dos níveis periféricos de BDNF em pacientes com TB (de Sousa *et al.,* 2011, Fernandes *et al.,* 2011). Neste sentido, estudos que tentam prevenir, tratar e reverter desbalanços moleculares representam interessantes avenidas para terapias novas e aprimoradas no TB (Soeiro-de-Souza *et al.,* 2012). Em particular, a liberação de fatores neurotróficos por biomateriais em *scaffolds* parece ser uma promissora área de pesquisa para o tratamento de diversos distúrbios afetando o SNC. Este sistema de liberação de drogas permite controlar o sítio e o período da liberação do agente terapêeutico, garantindo que agentes biologicamente ativos, como fatores neurotróficos, sejam transportados para a localização desejada para auxiliar no tratamento de um dado distúrbio (Hosseinkhani *et al.,* 2009). Em uma revisão recente, foram avaliadas as vantagens e desafios associados com diferentes sistemas de liberação de droga, tendo sido levantada a possibilidade de se combinar sistemas de liberação de droga com terapia gênica, sugerindo que o dispositivo de liberação de droga poderia fornecer uma liberação controlado de fatores neurotróficos (Mohtaram *et al.,* 2013).

Com relação ao mecanismo de ação de estabilizadores de humor tradicionalmente utilizados no tratamento do TB (lítio e ácido valpróico), hipóteses envolvendo as rotas discutidas nesta revisão são frequentemente discutidas. Um trabalho recente revisou achados pré-clínicos demonstrando que estas drogas, adicionalmente a outras funções, regulam a transcrição e a expressão de fatores envolvidos em efeitos neuroprotetores, neurotróficos e anti-inflamatórios. Além disso, rotas de estresse oxidativo e cascatas de sinalização de sobrevivência celular podem ser futuramente implicadas nas ações benéficas destes tratamentos consagrados (Chiu *et al.,* 2013).

Em resumo, a identificação de alvos terapêuticos específicos, normalmente modulados por estas drogas, pode revelar novas avenidas para o uso eficiente de terapias adjuvantes, com o objetivo primário de tratar episódios agudos de humor e prevenir sua recorrência.

Biomarcadores periféricos e atividade da doença no transtorno bipolar

Conforme discutido acima, um conjunto crescente de evidências aponta para mudanças na neuroplasticidade, no estresse oxidativo e nas rotas de inflamação no TB, sobretudo durante episódios de humor. No entanto, estes biomarcadores periféricos têm sido usualmente investigados de maneira individual, contrariando a ideia de que biomarcadores isolados são provavelmente insuficientes para identificar distúrbios complexos. Em vez disso, a pesquisa deveria ser orientada para a avaliação de conjuntos de biomarcadores, refletindo diferentes processos implicados em uma dada condição (Puntmann*,* 2009, Singh *et al.,* 2009).

Para avaliar estes biomarcadores simultaneamente, Kapczinski e cols. conduziram uma avaliação *en bloc* de um conjunto de alvos relacionados ao estresse oxidativo, neurotrofinas e inflamação, todos previamente descritos como biomarcadores individuais de episódios de humor no TB. Os resultados demonstraram correlações significativas entre a maioria dos biomarcadores, os quais foram então utilizados para extrair um índice de toxicidade sistêmica. Pacientes em episódios maníacos e depressivos apresentaram toxicidade sistêmica aumentada quando comparados a pacientes eutímicos e controles, mas inferior à toxicidade apresentada por pacientes com sepse (grupo de controle "positivo" para doença periférica extrema) (Figura 1 e Figura 2) (Kapczinski *et al.,* 2010, Kapczinski *et al.,* 2011).

Os achados acima, associando episódios agudos e significativa toxicidade sistêmica no TB, corroboram com a ideia de que o TB possa ser visto como uma doença multisistêmica, na qual a patofisiologia periférica é um componente principal (Soreca *et al.,* 2009). No entanto, estes dados são incapazes de explicar por si só como mudanças periféricas se correlacionam com alterações cerebrais.

O cérebro coordena todos os processos fisiológicos, sendo assim sensível ao dano sistêmico (McEwen *et al.,* 2011).

É importante observar que a patofisiologia central e periférica poderia estar conectada com estados pró-oxidativos (Gigante *et al.,* 2010), possivelmente através de mudanças na permeabilidade da barreira hematoencefálica. Foi demonstrado que a toxicidade periférica altera significativamente o estresse oxidativo no cérebro (Chaudhary *et al.,* 2010). De fato, conforme mencionado acima, pode haver uma conexão entre inflamação, estresse oxidativo e rotas de neuroplasticidade no TB. Por exemplo, foi demonstrado que a inflamação causa estresse oxidativo através da ativação de proteínas dependentes de cálcio e inibição direta da cadeia transportadora de elétrons mitocondrial (de Gonzalo-Calvo *et al.,* 2010). Mudanças no estresse oxidativo, por sua vez, podem ser associadas com níveis reduzidos de BDNF observados em pacientes durante episódios agudos de humor (Kapczinski *et al.,* 2008a).

Em resumo, foi consistentemente demonstrado que biomarcadores periféricos diferenciam entre pacientes com TB em episódios maníacos ou depressivos e indivíduos eutímicos. Enquanto que alterações em um único marcador usualmente apresentam pequeno tamanho de efeito, a avaliação de múltiplos biomarcadores, especialmente mediadores primários, poderia ser uma abordagem prática para aprimorar estratégias diagnósticas e promover intervenções precoces (Juster *et al.,* 2010, Singh *et al.,* 2009).

Relevâncias do estudo

As informações descritas acima destacam a toxicidade sistêmica relacionada aos episódios agudos de humor no TB e discute possíveis mecanismos subjacentes a esse processo. Pesquisas clínicas e pré-clínicas suportam de maneira geral a idéia de episódios da doença como tóxicos a múltiplos elementos do corpo. Com relação ao uso de biomarcadores periféricos como meio de acessar a atividade da doença, podemos considerar candidatos promissores divididos em três grandes áreas: estresse ovidativo, inflamação e neurotrofinas, particularmente o BDNF.

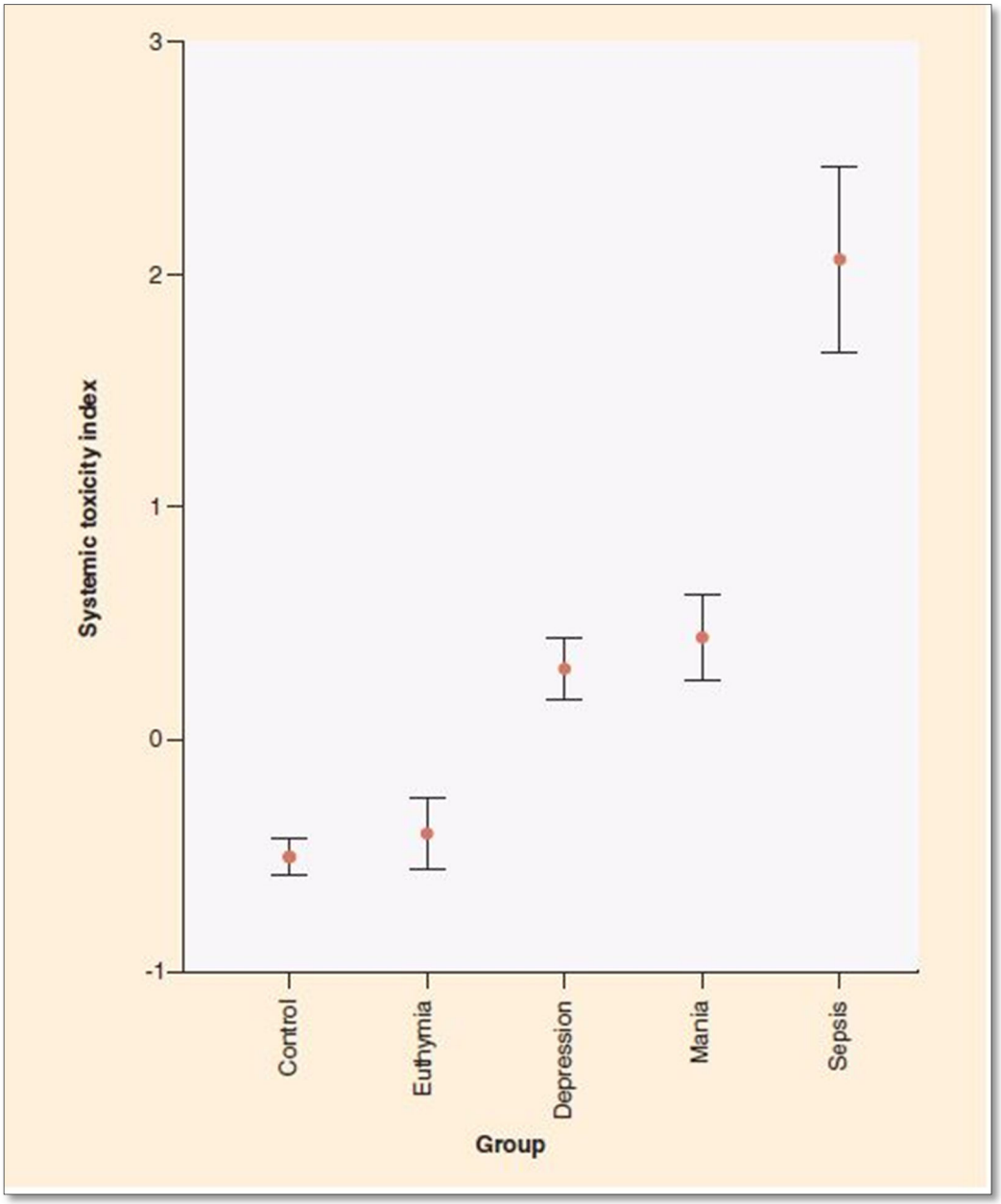

Figura 1. Índice de toxicidade sistêmica para avaliar mudanças periféricas nos episódios de humor.
Para avaliar a toxicidade sistêmica associada com a atividade da doença no TB, Kapczinski e cols. conduziram uma avaliação *en bloc* de um grupo de alvos relacionados a estresse oxidativo, neurotrofinas e inflamação, todos previamente descritos individualmente como biomarcadores dos episódios de humor. Esses marcadores periféricos foram medidos em diferentes estados agudos de humor bem como em indivíduos saudáveis. Ainda, eles também foram avaliados em pacientes com sepse (controle 'positivo' para doença periférica extrema) com o objetivo de destacar a relevância de diferenças potencias entre os grupos. Mais especificamente, os biomarcadores medidos foram neurotrofinas (BDNF, do inglês *brain-derivaded neurotrophic fator* e NT-3, do inglês neurotrophin – 3), marcadores de estresse oxidativo (PCC, do inglês *protein carbonil content*, TBARS do inglês *thiobarbituric acid reactive substances* e TRAP, do inglês *total reactive antioxidante potential*) e marcadores inflamatórios (IL-6, IL-10 e TNF-α). Os resultados demonstraram significativas correlações entre a maioria dos biomarcadores que foram utilizados para extrair um índice de toxicidade sistêmica. Pacientes em episódio maníaco e depressivo mostraram maior toxicidade sistêmica do que pacientes em eutimia e controles saudáveis; entretanto, foram menores do que pacientes com sepse. Figura reproduzida com a permissão de Kapczinski e cols., 2010. (Kapczinski *et al.,* 2010)

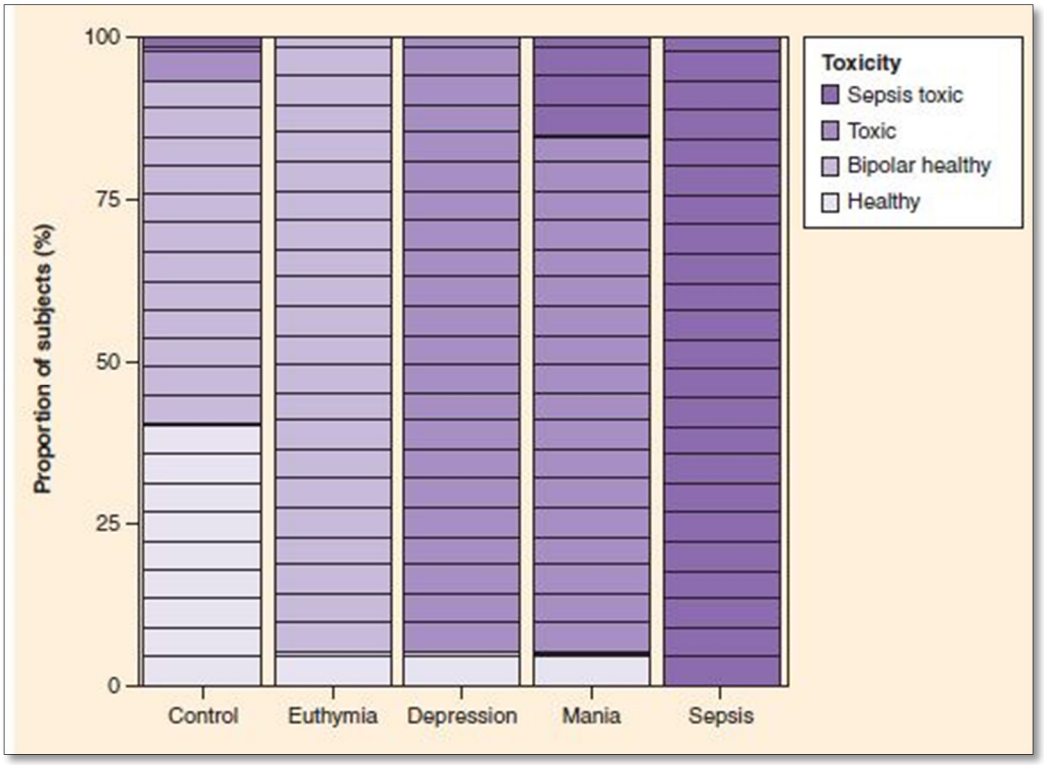

Figura 2. Biomarcadores periféricos e atividade da doença no transtorno bipolar. O gráfico mostra a proporção de sujeitos no estudo que foram classificados em diferentes categorias: a categoria mais tóxica, incluindo pacientes com sepse, foi chamada de '*sepsis toxic*'; a categoria menos tóxica, contendo a maioria dos controles saudáveis, foi chamada de '*healthy*'; a categoria que apresentava os níveis dos biomarcadores periféricos na direção tóxica e incluía pacientes em estado agudo de humor foi chamada de '*toxic*'; a última categoria era intermediária em termos de biomarcadores periféricos e continha principalmente pacientes em eutimia, portanto foi denominada '*bipolar healthy*'. Figura reproduzida com a permissão de Kapczinski e cols., 2011 (Kapczinski *et al.,* 2011).

Esses três grupos ainda não preenchem as categorias empíricas de um biomarcador tradicional; entretanto, eles são relevantes por fornecerem informações a respeito da patofisiologia e da atividade da doença no TB. A avaliação da toxicidade sistêmica através de um grupo de biomarcadores periféricos pode facilitar o entendimento de danos ao corpo e ao cérebro relacionados com a recorrência dos episódios de humor e como isso interfere no manejamento da doença.

Talvez o efeito mais importante de termos biomarcadores da atividade da doença validados seja a identificação de características biológicas que destaquem o surgimento de um episódio agudo de humor antes que apareçam sintomas específicos ou a persistência da atividade da doença, apesar da aparente resposta. Outra aplicabilidade potencial pode ser a detecção de uma resposta precoce,

antes da resolução dos sintomas. Alterações em biomarcadores periféricos em um episódio agudo de humor podem seguir três diferentes padrões, sendo que qualquer um deles poderia ser uma poderosa ferramenta no direcionamento das terapias (Figura 3).

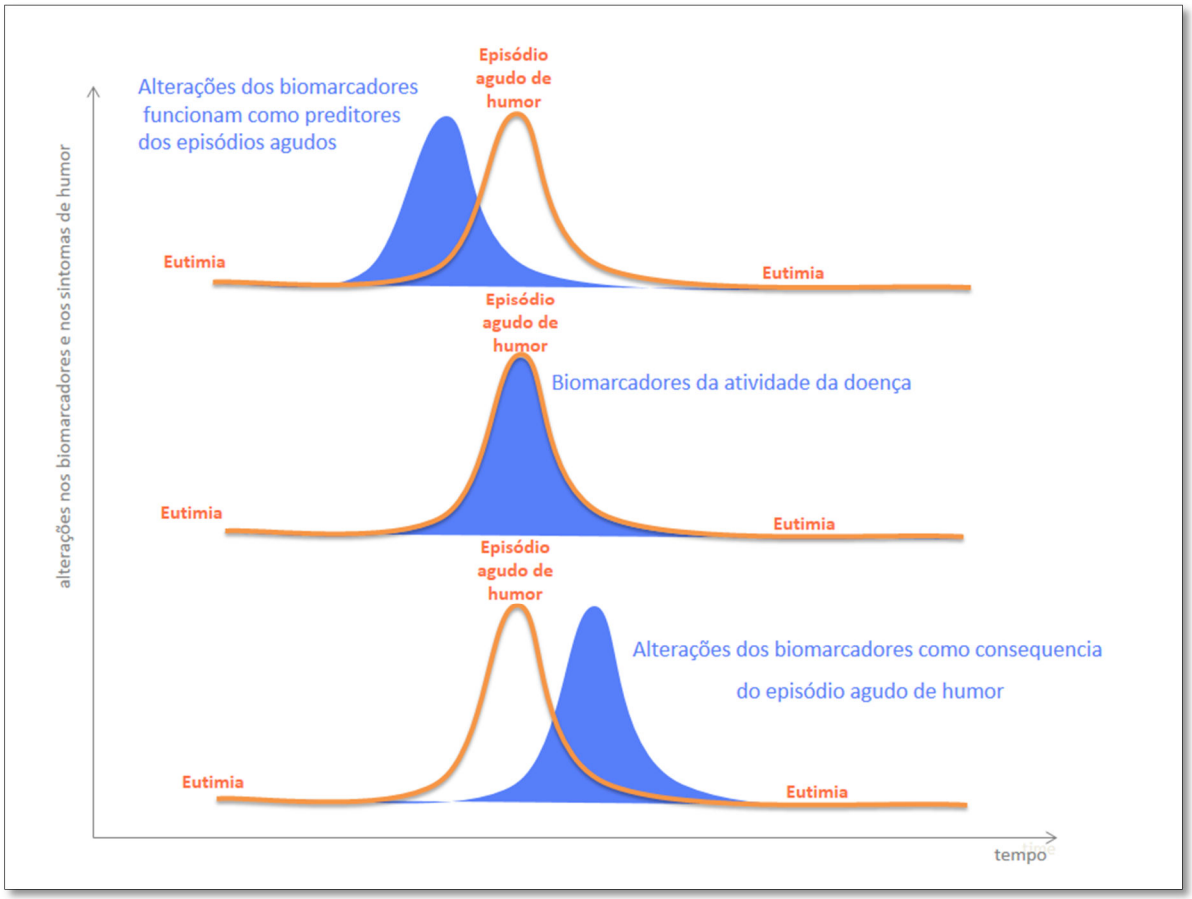

Figura 3. Alterações em biomarcadores periféricos em um episódio agudo de humor (mania ou depressão) poderiam seguir três padrões distintos. Primeiro, os biomarcadores poderiam sofrer alterações antes do começo do episódio agudo, demonstrando um potencial em prever esses eventos. Neste caso, os biomarcadores teriam um grande potencial terapêutico: eles poderiam auxiliar no planejamento/implantação de intervenções precoces e previnir/monitorar a resposta ao tratamento. Segundo, as alterações nos biomarcadores poderiam acontecer concomitantemente aos episódios agudos, refletindo a atividade da doença. Neste caso, eles seriam uma ferramenta útil no apoio de decisões clínicas que melhorassem o manejamento dos episódios agudos. Finalmente, os biomarcadores poderiam alterar após um episódio, ou seja, como uma consequência dele, o que poderia contribuir para melhorar nossa compreensão da patofisiologia do transtorno bipolar. Esta avaliação pode ser útil como um substituto da eficácia farmacológica, predizendo a resposta ao tratamento de um episódio agudo após o início da terapia. Os padrões das alterações de biomarcadores e sua relação temporal com os episódios de humor no transtorno bipolar permanecem desconhecidos.

Com relação a novas estratégias terapêuticas, evidências preliminares apoiam o papel de novas terapias adjuntivas na modulação de processos neurotróficos, inflamatórios, oxidativos e apoptóticos. Agentes neuroprotetores em potencial estão atualmente disponíveis, mas mais estudos

clínicos são necessários, assim como mais informações a respeito de quais subgrupos de pacientes teriam o maior benefício se usassem essas intervenções. Além disso, em virtude dos altos índices de mortalidade observados no TB, tem havido uma pressão para a busca de mecanismos subjacentes a toxicidade aguda e a atividada da doença. Esta perspectiva é a lógica por trás de uma abordagem em que a validação de novos indicadores biológicos irá melhorar as estratégias clínicas tradicionalmente empregadas.

Justificatica

Dessa forma, a presente tese tem como meta principal, ampliar os conhecimentos relacionados aos mecanismos que possam estar subjacentes aos episódios agudos de humor e à remissão clínica em pacientes com TB, focando especialmente em vias de epigenética, morte celular, inflamação e toxicidade sistêmica.

OBJETIVOS

Objetivo Geral

Avaliar, através de estudos pré-clínicos e clínicos, os mecanismos subjacentes ao episódio agudo de humor e à remissão clínica em pacientes com transtorno bipolar, focando em vias de epigenética, morte celular, inflamação e toxicidade sistêmica.

Objetivos Específicos

1. Avaliar se a atividade da enzima histona desacetilase, bem como os níveis de expressão gênica e proteica de BDNF, estão associados às esteriotipias comportamentais induzidas pela *d*-anfetamina em um modelo animal de mania.
2. Verificar em pacientes com TB, através de um estudo aberto longitudinal de 16 semanas, se um conjunto de marcadores de dano celular (DAMPs) está presente durante o episódio agudo de humor e como o mesmo se comporta após o tratamento farmacológico desses pacientes.
3. Avaliar como se comportam marcadores de morte celular, inflamação e antioxidantes em pacientes com TB que entrarem em remissão clínica após o episódio agudo de humor, comparando com pacientes com sepse e controles saudáveis.
4. Avaliar, baseado na literatura dos últimos 2 anos, se o TB pode ser considerado uma doença inflamatória e propor o mecanismo pelo qual ocorre a interação entre a progressão do TB, a inflamação periférica e a reorganização do substrato neuronal.

PARTE II

RESULTADOS

CAPITULO I

Histone deacetylase activity and brain-derived neurotrophic factor (BDNF) levels in a pharmacological model of mania

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Artigo publicado pela Revista Brasileira de Psiquiatria em 2013

Revista Brasileira de Psiquiatria. 2013;35:00–00

doi:10.1590/1516-4446-2013-1094

Histone deacetylase activity and brain-derived neurotrophic factor (BDNF) levels in a pharmacological model of mania

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Objective: In the present study, we aimed to examine the effects of repeated D-amphetamine (AMPH) exposure, a well-accepted animal model of acute mania in bipolar disorder (BD), and histone deacetylase (HDAC) inhibitors on locomotor behavior and HDAC activity in the prefrontal cortex (PFC) and peripheral blood mononuclear cells (PBMCs) of rats. Moreover, we aimed to assess brain-derived neurotrophic factor (BDNF) protein and mRNA levels in these samples.
Methods: We treated adult male Wistar rats with 2 mg/kg AMPH or saline intraperitoneally for 14 days. Between the 8th and 14th days, rats also received 47.5 mg/kg lithium (Li), 200 mg/kg sodium valproate (VPT), 2 mg/kg sodium butyrate (SB), or saline. We evaluated locomotor activity in the open-field task and assessed HDAC activity in the PFC and PBMCs, and BDNF levels in the PFC and plasma.
Results: AMPH significantly increased locomotor activity, which was reversed by all drugs. This hyperactivity was associated with increased HDAC activity in the PFC, which was partially reversed by Li, VPT, and SB. No differences were found in BDNF levels.
Conclusion: Repeated AMPH administration increases HDAC activity in the PFC without altering BDNF levels. The partial reversal of HDAC increase by Li, VPT, and SB may account for their ability to reverse AMPH-induced hyperactivity.

Keywords: Bipolar disorder; mood stabilizer; sodium butyrate; histone deacetylase; BDNF

Introduction

A growing body of evidence suggests an association between epigenetic mechanisms and the pathophysiology of bipolar disorder (BD), given the importance of long-lasting changes in neuroplasticity and gene expression to the mechanism of action of mood stabilizers.[1] The importance of gene-environment interaction in the development of different psychiatric disorders has been discussed elsewhere, in that external stimuli can cause modifications in gene expression without actually altering the genetic code.[2] These long-lasting changes based on epigenetic mechanisms would be desirable in a treatment approach as well. Among such mechanisms, a change in the methylation and acetylation state of genes and histones is the most well known.[3] Hyperacetylation of histones generally promotes chromatin decondensation and increased gene activity, whereas hypoacetylation leads to condensation and reduced activity.[4] Given that such modifications can alter and regulate the expression of specific genes, this mechanism could be potentially associated with acute mood symptoms in BD and with the mechanisms of action of psychotropic drugs, altering protein levels in parallel with behavioral changes. Sodium valproate (VPT) inhibits the enzyme histone deacetylase (HDAC), which catalyzes the removal of acetyl groups from histones.[5] Lithium (Li) seems to share some mechanism with HDAC inhibitors (HDACi), but whether it can inhibit this enzyme directly remains unclear.[6] So far, little is known about the association between HDAC activity and the behavioral effects of mood stabilizers in vivo.

One gene whose expression has been linked to mood-stabilizing effects is brain-derived neurotrophic factor (BDNF). Among its functions, BDNF acts to promote neuronal survival and synaptic plasticity[7] and has been extensively studied as a biomarker of illness activity in BD.[8] Serum BDNF levels are reduced in BD patients during manic and depressive episodes, whereas no differences are found in euthymic patients compared to healthy controls.[9] Moreover, BDNF levels seem to

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Submitted Jan 21 2013, accepted Mar 07 2013.

increase along with remission of symptoms[10] and after mood stabilizer treatment in vitro and in vivo.[11,12] Of note, BDNF transcription is at least partially modulated by epigenetic mechanisms.[13] Evidence suggests that BDNF increases after treatment with HDACi in vitro and in vivo, and this increase seems to occur in a promoter-specific way.[14]

Based on previous findings, the ability to inhibit HDAC and modulate behavior could be a way to screen for potential novel drugs with mood-stabilizing properties. Studies investigating other drugs capable of inhibiting HDAC as compared with known mood stabilizers in vitro suggest that the short-chain fatty acid sodium butyrate (SB) may be a promising candidate.[11,15] SB has been shown to exhibit antidepressant properties[16,17] and can increase BDNF levels in vitro.[11] Moreover, it has recently been shown that SB reverses and prevents D-amphetamine (AMPH)-induced hyperactivity in an animal model of mania,[18] but the extent to which it is associated with HDAC activity requires further research.

Considering that chromatin remodeling can lead to alterations in the expression of neuroprotective genes, the effects of AMPH and mood stabilizers on such parameters would be of great interest to the field of BD research and therapy. AMPH exposure has been consistently put forward as a model for mania due to its ability to increase dopamine levels upon administration.[19] AMPH-treated rats become hyperactive[12] and exhibit several memory deficits,[20] some of which are reversed by administration of mood stabilizers.[21] Moreover, the construct validity of this model has been increasing significantly over time, with several biomarkers found in human BD patients also altered in animals subjected to this model.[12] The present study aimed to evaluate if repeated exposure to AMPH would induce alterations in HDAC activity and if these alterations would be reversed by the administration of a classic HDACi (SB or VPT) or Li. Furthermore, we evaluated whether such HDAC alterations would be associated with a modulation of BDNF levels. To that end, we assessed the effects of the treatments on the locomotor behavior of rats and measured the activity of HDAC and BDNF protein and mRNA levels in the prefrontal cortex (PFC), a brain region known to be associated with BD pathophysiology.[22,23] We also evaluated HDAC modulation in peripheral blood mononuclear cells (PBMCs) and plasma BDNF levels to analyze whether the effects of these drugs would be mainly central or could also be systemic.

Methods

Animals

All experiments were conducted with male Wistar rats (age: 3-4 months; weight: 220-310 g) obtained from our breeding colony. Rats were housed five to a cage and kept on a 12-h light/dark cycle (lights on between 7 a.m. and 7 p.m.), with water and food available ad libitum. All study procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society of Neuroscience and Behavior (SBNeC) recommendations for animal care, and were approved by the Hospital de Clínicas de Porto Alegre Ethics Committee.

Treatment

The animals received daily intraperitoneal (i.p.) injections of 2 mg/kg AMPH (Sigma, St. Louis, MO, USA) or saline solution for 14 days. Between the 8th and the 14th day, AMPH or saline-treated rats were divided into four experimental groups: treatment with 47.5 mg/kg Li i.p. twice a day (n=12), 200 mg/kg VPT i.p. twice a day (n=12), 1.2 g/kg SB i.p. twice a day (n=12), or saline (n=21). All drugs were administered in the rats' home cages. On the 15th day, rats received either AMPH or saline, and their locomotor activity was measured 2 h later. The doses of the mood stabilizers tested are within the range of doses regularly used in other behavioral and functional studies with animal models of mood disorders. Of note, animals treated with Li had plasma Li levels in the range of 0.6-1.2 mEq/L, as recommended for the treatment of BD patients.[12]

Locomotor activity

Locomotor activity was assessed with the open-field task. The test was performed in a 40 x 60 cm open field surrounded by 50 cm-high walls made of brown plywood with a frontal glass wall. The open-field floor was divided into 12 equal rectangles by black lines. The animals were gently placed in the left rear quadrant and allowed to explore the arena freely for 5 min. Two observers who were blinded to the treatment status of the rats counted the number of times they crossed the black lines and performed rearing behavior. We considered the number of times that rats crossed the black lines to be a measurement of horizontal locomotor activity and the number of times they reared to be a measurement of vertical locomotor activity. The animals were euthanized by decapitation immediately after the open-field task.

Nuclear extraction and cytosolic fraction separation

The PFC (the anterior part of the frontal lobes of the rat brain) was fully dissected according to the stereotactic atlas of Paxinos and Watson.[24] The obtained samples were flash-frozen and stored at -80°C until nuclear proteins were extracted. Whole blood was collected by intracardiac puncture and PBMC separation was performed with Histopaque 1077 reagent (Sigma, St. Louis, MO, USA) according to manufacturer instructions. Tissue samples and PBMCs were subjected to a nuclear extraction protocol with a commercial Nuclear Extraction kit (Chemicon, USA). Briefly, samples were homogenized in cytoplasmic lysis buffer containing dithiothreitol (DTT) and protease inhibitors. The suspension was kept on ice for 15 min and was later centrifuged at 250 $\times$ g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in two volumes of cold cytoplasmic lysis buffer. The suspension was homogenized using a

small-gauge needle syringe and centrifuged at 8,000 × g for 20 min at 4°C. The resulting pellet contained the nuclear portion of the cell lysate. The supernatant containing the cytosolic fraction was transferred to another tube and stored at -80°C until the BDNF protein level assay was performed.

The pellet was resuspended in a nuclear extraction buffer containing DTT and protease inhibitors, and the suspension was homogenized with a small-gauge needle syringe. The resulting sample was kept in slow agitation for 30-60 min in an orbital shaker at 4°C. Later, the nuclear suspension was centrifuged at 16,000 × g for 5 min at 4°C, and the nuclear extract-containing supernatant was transferred to a new tube and stored at -80°C until further analysis.

HDAC activity

Nuclear extracts from the PFC and PBMCs were subjected to an HDAC activity assay with the HDAC Assay kit (Fluorometric Detection) according to manufacturer instructions (Upstate, USA). Briefly, 5 μL of nuclear extract were mixed with 5 μL of HDAC Assay Buffer and 5 μL of HDAC Assay Substrate in a 384-well plate and incubated at 30°C for 45 min. Concomitantly, a standard curve was performed with serial dilutions of deacetylated substrate and positive and negative controls were added to the plate. Afterwards, 10 μL of activator solution were added to the wells, and the plate was incubated at room temperature for 15 min. A fluorescence reading was obtained in a fluorescence plate reader, with 360 nm for excitation and 460 nm for emission. HDAC activity was calculated on the basis of the standard curve, and values are presented as μM/μg protein. Total protein was measured by a modified Lowry's method[25] using bovine serum albumin as the standard. HDAC activity was calculated as the micromolar concentration of deacetylated standard substrate per microgram of protein.

BDNF protein levels

Whole blood was collected by intracardiac puncture into a Vacutainer containing ethylenediamine tetraacetic acid (EDTA) to prevent clotting. Plasma samples were obtained by centrifugation (4000 rpm for 10 min) and frozen at -80°C until further analysis. BDNF levels in the cytosolic fraction from PFC and plasma samples were measured using an anti-BDNF sandwich enzyme-linked immunosorbent assay (ELISA) (CYT306), according to manufacturer instructions (Millipore, USA). Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples, diluted 1:2 in sample diluent, and the standard curve ranged from 7.8 to 500 pg/ml of BDNF. The plates were then washed four times with wash buffer, and a biotinylated monoclonal anti-BDNF rabbit antibody (ChemiKine, USA), diluted 1:1000 in sample diluent, was added to each well and incubated for 3 h at room temperature. After washing, peroxidase-conjugated streptavidin (ChemiKine, USA) (diluted 1: 1000) was added to each well and incubated at room temperature for 1 h. After the addition of substrate and stop solution, the amount of BDNF was determined by absorbance at 450 nm. The standard curve demonstrated a direct relationship between optical density (OD) and BDNF concentration. Total protein was measured by Lowry's method using bovine serum albumin (Sigma, St. Louis, USA) as standard.

RNA isolation

Tissues were subjected to an RNA isolation protocol with the use of TRIzol (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. Briefly, tissues (50 to 100 mg) were homogenized in 1 mL TRIzol and incubated at room temperature for 5 min. Afterwards, chloroform was added to the samples and the suspension was vigorously mixed for 15 s and incubated at room temperature for 5 min, followed by centrifugation at 12,000 × g for 15 min at 4°C. The aqueous phase was then placed in another tube to which isopropanol was added, followed by incubation at room temperature for 10 min and centrifugation at 12,000 × g for 10 min at 4°C. The supernatant was then discarded and the pellet was washed with 75% ethanol, followed by another centrifugation at 7,500 × g for 5 min at 4°C. After drying the pellet at room temperature, RNA was resuspended in 30 μL of DEPC-treated water. RNA samples were then quantitated with the Quant-iT RNA assay (Molecular Probe; Invitrogen, Carlsbad, CA, USA), treated with DNAse I, Amp Grade (Invitrogen, Carlsbad, CA, USA), and stored at -80°C until further analysis.

Primer design and quantitative real-time PCR

FAM-labeled TaqMan primers and a probe (Applied Biosystems, Foster City, CA, USA) specific for the rat BDNF gene were designed with the use of Primer Express software (Applied Biosystems, Foster City, CA, USA) based on the BDNF mRNA sequence obtained from NCBI GenBank (GenBank ID: 122427415, NM_012513). Primers and probe specificities were confirmed through comparison with other sequences available at the GenBank with the use of BLAST. The sequences used were as follows: forward primer, 5′-CTGACACTTTTGAGCACGTGATC-3′; reverse primer, 5′-CGTTGGGCCGAACCTTCT-3′.

Real-time RT-PCR reactions were performed in the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the use of the TaqMan One-Step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions. Reactions were performed in a final volume of 12 μL with 2 μL of total RNA (5 ng), 6 μL of Master Mix 2x, 0.3 μL of RT enzyme 40x, 0.6 μL of TaqMan Custom Gene expression assay, 0.6 μL of endogenous TaqMan gene expression assay, and 2.5 μL of DEPC-treated water. Expression values were normalized to beta-actin endogenous control expression using a TaqMan VIC/MGB endogenous control inventoried assay (Applied Biosystems, Foster City, CA, USA, 4352340E).

All reactions were performed in triplicate. Relative expression levels (RQ) were determined by the ΔΔCt method.[26]

Statistical analysis

Statistical analyses were performed using PASW Statistics 18.0 for Windows. Behavioral data (number of crossings and rearings), HDAC activity, and BDNF protein levels in the PFC fit a standard distribution curve and were therefore subjected to parametric analyses. For comparisons between groups, a one-way analysis of variance (ANOVA) test was performed, followed by the Tukey post-hoc test when the ANOVA was significant. P-values < 0.05 for a two-tailed distribution were considered statistically significant. BDNF mRNA and protein plasma levels did not fit a standard distribution curve and were thus subjected to nonparametric analyses. A Kruskal-Wallis test was performed to compare groups and p-values < 0.05 for two-tailed distributions were considered statistically significant. All biochemical data are expressed as % of control.

Results

Open-field test

In the open-field test (Figure 1), one-way ANOVA followed by Tukey's post-hoc test was performed to compare all groups. AMPH increased horizontal and vertical locomotor activities in saline-treated rats (ANOVA, crossings-$F_{7\text{-}82} = 6.673$, $p < 0.0001$, Figure 1A; rearings-$F_{7\text{-}82} = 7.980$, $p < 0.0001$, Figure 1B), whereas treatment with Li, VPT, and SB partially reversed this AMPH-induced hyperactivity. The administration of Li, VPT, and SB per se did not significantly alter the behavioral activity, indicating that their effects on AMPH-treated rats were not due to sedation.

HDAC activity

Figure 2 shows the amount of HDAC activity in the PFC and in PBMCs. In the PFC, AMPH treatment (AMPH + saline) significantly increased HDAC activity as compared with the control group (saline + saline) (ANOVA, $F_{7\text{-}79} =$

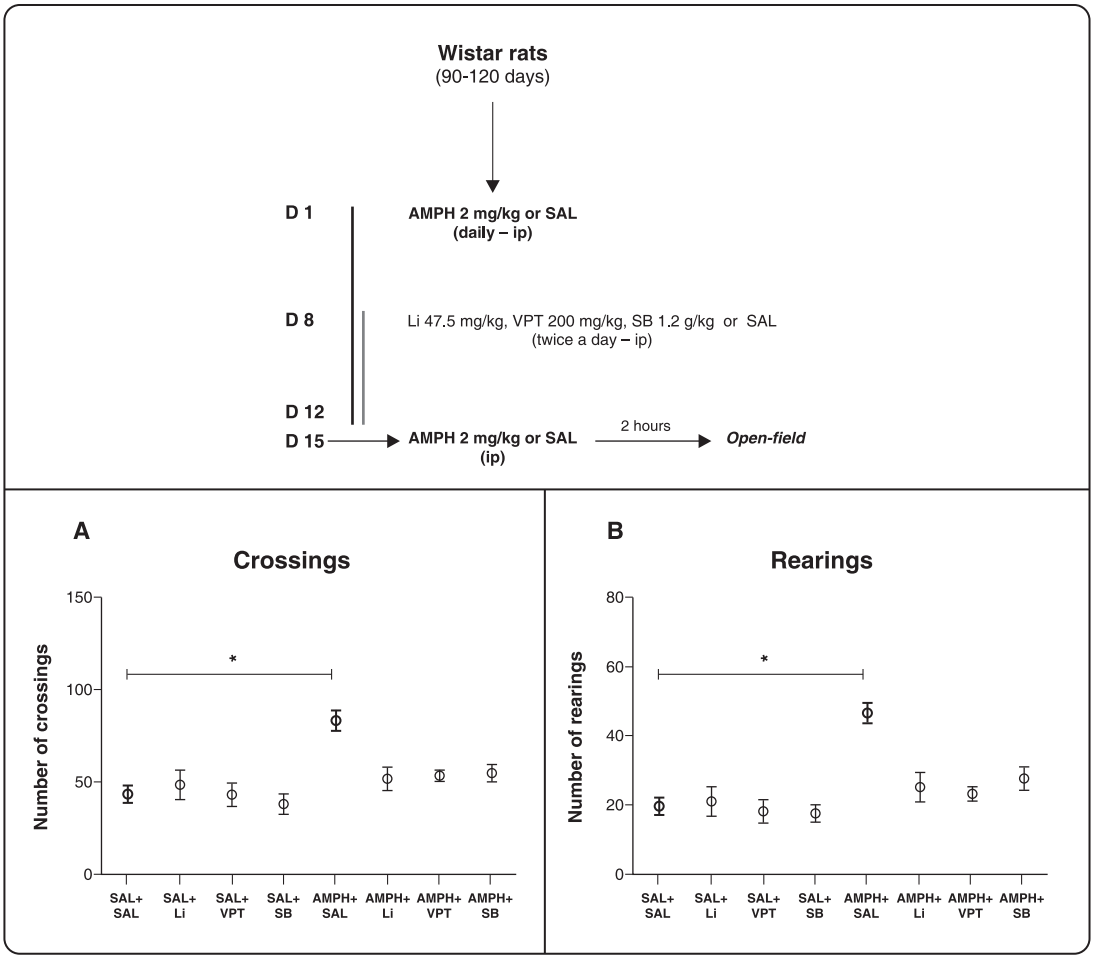

Figure 1 Schematic representation of treatment and open-field test. A) Number of crossings. B) Number of rearings. Rats were treated for 7 days with AMPH + 7 days of AMPH and Li, VPT, or SB. One-way ANOVA and Tukey's post-hoc test; * $p < 0.0001$ (different from the SAL + SAL group). The results are presented as mean ± standard error of the mean (n=11-21). AMPH = D-amphetamine; Li = lithium; SAL = saline; SB = sodium butyrate; VPT = sodium valproate.

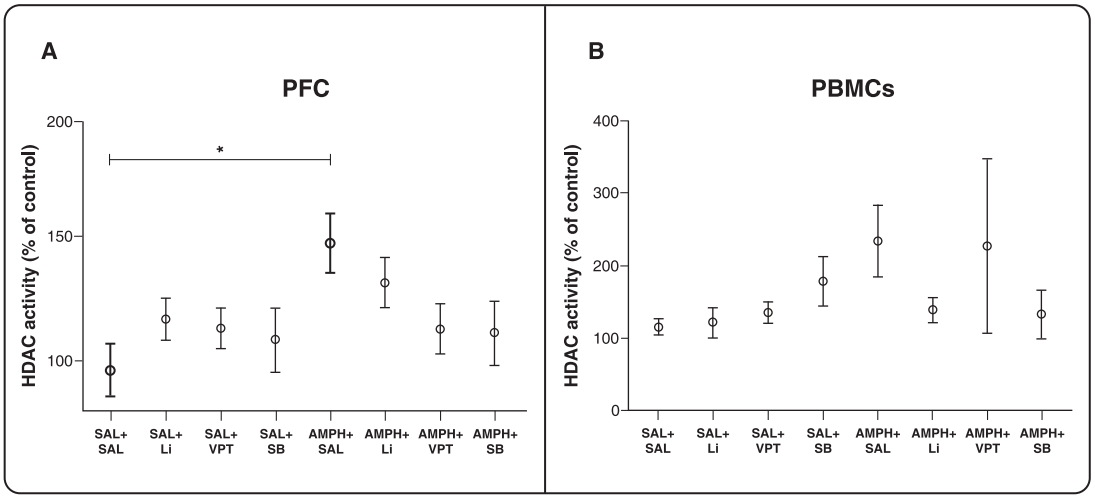

Figure 2 HDAC activity in the prefrontal cortex and PBMCs. A) Prefrontal cortex (n=8-18 per group). B) PBMCs (n=7-19 per group). One-way ANOVA and Tukey's post-hoc test; * p = 0.001. The results are presented as mean ± standard error of the mean. AMPH = D-amphetamine; HDAC = histone deacetylase; Li = lithium; PBMC = peripheral blood mononuclear cell; PFC = prefrontal cortex; SAL = saline; SB = sodium butyrate; VPT = sodium valproate.

2.173, p = 0.01, Figure 2A), and treatment with Li, VPT, and SB in AMPH-treated rats partially reversed this effect. In PBMCs, no differences in HDAC activity were found between groups ($p > 0.05$ for all comparisons, Figure 2B).

BDNF mRNA and protein levels in the PFC

Given that evidence suggests that BDNF expression may be linked to mood-stabilizing effects, it was hypothesized that BDNF levels would increase after treatment with HDACi. However, we did not observe any between-group differences in BDNF protein levels ($p > 0.05$ for all comparisons, Figure 3A) or mRNA levels ($p > 0.05$ for all comparisons, Figure 3B) the PFC. This indicates that HDAC modulation in the PFC might alter the expression of genes other than BDNF.

Plasma BDNF levels

To analyze whether the effects of the tested drugs would be mainly central or could also be systemic, we measured plasma BDNF levels (Figure 4). We did not find any between-group differences in these levels ($p > 0.05$ for all comparisons), suggesting that there are no systemic effects of AMPH-modulating HDAC in the BDNF gene.

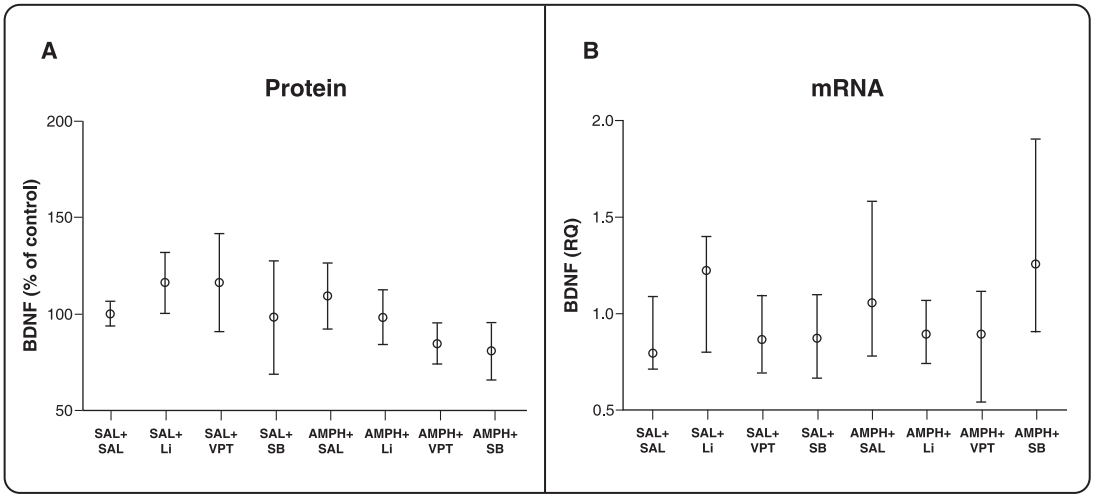

Figure 3 BDNF levels in prefrontal cortex. A) Protein levels. One-way ANOVA and Tukey's post-hoc test. The results are presented as mean ± standard error of the mean (n=7-12 per group). B) mRNA levels. A Kruskal-Wallis test was used to compare groups, $p > 0.05$. The results are presented as median and interquartile range (n=7-12 per group). AMPH = D-amphetamine; BDNF = brain-derived neurotrophic factor; Li = lithium; SAL = saline; SB = sodium butyrate; VPT = sodium valproate.

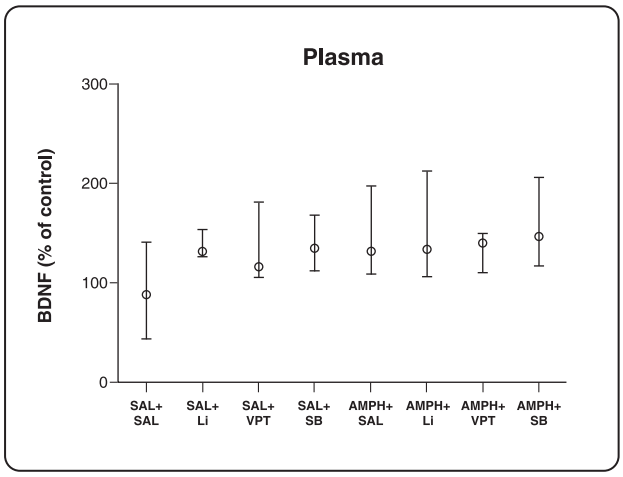

Figure 4 BDNF levels in plasma. A Kruskal-Wallis test was used to compare groups, $p > 0.05$. The results are presented as median and interquartile range (n=8-19 per group). AMPH = D-amphetamine; BDNF = brain-derived neurotrophic factor; Li = lithium; SAL = saline; SB = sodium butyrate; VPT = sodium valproate.

Discussion

Evidence suggests that HDACi may exert antimanic effects in different treatment regimens and animal models. Accordingly, our results show that treatment with SB, as well as with Li and VPT, was able to partially reverse AMPH-induced hyperactivity after repeated AMPH administration. Moreover, AMPH increased HDAC activity in the PFC, which was partially reversed by SB, VPT, and Li. No between-group differences were found in BDNF protein and mRNA levels. In addition, no differences were found in HDAC activity in PBMCs or in plasma BDNF levels, suggesting that the HDAC-modulating effects of AMPH are mainly central. The present study provides evidence that AMPH-induced behavioral effects may be associated with histone modifications in the PFC and that this can be partially reversed by HDAC inhibitors.

To the best of our knowledge, this is the first study showing that AMPH treatment increases HDAC activity in the PFC of rats. Although there is no evidence of a direct association between AMPH and HDAC, the ability of AMPH to increase dopamine levels in the synaptic cleft may have consequences at a nucleosomal level. Histone deacetylation has been associated with activation of D2 dopamine receptors, leading to the recruitment of a corepressor complex to the promoter of the prolactin gene.[27] Moreover, trichostatin A, which is an HDACi, has been shown to block cocaine-induced behavioral sensitization,[28] showing that other HDAC inhibitors may also have effects on dopamine-enhancing drugs. More recently, Arent et al. showed different effects of SB treatment in specific brain regions, suggesting that the antimanic effects of SB and of VPT are related to the amygdala, striatum, and, of special interest here, to the PFC, although not to the hippocampus.[29] More studies are warranted to clarify the effects of these HDAC inhibitors in different brain regions. Of note, the lack of between-group differences in HDAC activity in PBMCs in our sample suggests that the modulation of HDAC activity by the tested drugs is mainly central and cannot be observed in the periphery.

Based on our results, we cannot rule out the possibility that AMPH increases the expression of different HDACs, which would ultimately lead to an enhanced activity of this enzyme in our samples. Nevertheless, in this particular experiment, regardless of whether AMPH increased HDAC expression or only its activity, both scenarios would culminate in increased histone deacetylation and, thus, decreased gene expression. Furthermore, we did not assess whether this modulation of HDAC activity is associated with a change in acetylated H3 and H4 levels, which would contribute to the discussion of our results. In this sense, future studies are required to further explore these mechanisms.

Our results show that the reversal of AMPH-induced hyperactivity by Li, VPT, and SB was associated with the ability of these drugs to partially reverse the increase in HDAC activity. However, we found no effects of their administration on HDAC activity per se, suggesting more complex mechanisms regarding HDAC inhibition. Of note, there are two protein families with HDAC activity: the recently discovered SIR2 family of NAD^+- dependent HDACs, and the classical HDAC family. Members of the classical HDAC family fall into two different phylogenetic classes, namely class I and class II. Both of these protein families with HDAC activity share an ability to deacetylate histones, but they have different patterns of expression, different pharmacological profiles, different mechanisms of regulation, and different functions.[30] It is possible that different HDAC enzymes are differentially modulated by Li, VPT, and SB in the PFC. For instance, SB has relatively poor HDAC selectivity (disrupting the activity of multiple classes), which also limits our discussion on specific HDAC inhibition taking place in the brain. To address this, an analysis of second-generation compounds (such as hydroxamic acids and benzamides), which would potentially demonstrate the feasibility and importance of dissecting out the roles of particular HDACs in the brain, would be of great interest. In addition, it is also possible that the time elapsed between the last HDACi injection and euthanasia was too long, which would have prevented detection of any enzyme inhibition possibly induced by the drugs. Schroeder et al. showed that histone hyperacetylation in the hippocampus peaks 30 min after i.p. injection of SB in mice, returning to baseline within 1 h after administration.[17] Further studies are necessary to clarify these issues.

A growing body of evidence suggests a role for histone acetylation in the neurobiology and pharmacology of mood disorders.[3,31] Hobaro et al. found that the levels of HDAC2 and HDAC5 mRNA were increased in patients with major depressive disorder (MDD) while in the depressive state, and that HDAC4 mRNA levels were increased in depressive BD patients when compared with controls.[32] These data suggest that aberrant transcriptional regulation caused by altered HDAC expression

may be associated with the pathophysiology of mood disorders. HDAC class I and II inhibitors have shown efficacy in the treatment of neurodegenerative disorders,[33] neurodevelopmental disorders, cognitive deficits,[34] and psychiatric disorders such as depression and anxiety.[17,35] In this same vein, Ferrante et al. showed an attenuation of neuron loss and increased motor function after treatment with SB in an animal model of Huntington's disease.[36] Histone modifications are also associated with memory and learning processes, as shown by the increased formation of contextual fear memory in rats treated with SB.[37] In addition, treatment of mice with SB resulted in antidepressant-like effects when administered at a sufficiently high dose that induced global and transitory hyperacetylation of histones in the PFC and in the hippocampus.[17]

Amphetamine-induced hyperactivity in rats has been consistently put forward as a dopaminergic model of mania with predictive validity.[38] Therefore, the finding that AMPH-induced hyperlocomotion is partially blocked by SB treatment, as well as by Li and VPT treatment, suggests that SB may have mood-stabilizing properties.[18,29] Based on this pre-established relevance of the AMPH treatment as a valid animal model of mania, we are able to suggest that the results found in our experiments can be of relevance for human patients with BD. Nevertheless, clear extrapolations to the human condition regarding the doses of the tested drugs are less likely to be made, mostly due to differences in the metabolism of rats and humans and, therefore, in the kinetics of the drugs. Moreover, this finding suggests that other HDAC inhibitors should be further investigated as agents with possible therapeutic effects for manic episodes in patients with BD.

Finally, the use of HDAC inhibitors as mood stabilizers has been proposed on the basis of their ability to increase the expression of neuroprotective genes, some of which have already been investigated in patients with BD and other neuropsychiatric disorders.[39] The modulation of neurotrophin levels by HDAC inhibitors has already been shown in vitro,[11] and the differential pattern of HDAC inhibition in different brain regions is in line with previous findings of region-specific expression of neurotrophins.[40] We found no differences in BDNF protein or mRNA levels in the PFC of rats, even though AMPH increased HDAC activity in this brain region. Schroeder et al. did not find any change in BDNF levels after SB treatment in vivo either, and no other study has ever been conducted with this aim.[17] Although a significant body of evidence points to changes in serum BDNF levels in BD patients, there are still discrepancies between studies. Some did not find modulation of this factor in BD phases.[41] Several factors that may influence BDNF levels could explain these differences, such as medication, comorbidities, and staging of illness. Likewise, in studies with animals, some variables could have a direct impact on the results. In the present study, the absence of alterations in BDNF is likely to be related to the duration of HDACi treatment, which was brief. Fukumoto et al. demonstrated that only chronic Li treatment was able to increase BDNF protein levels in the rat cortex and hippocampus.[42] Furthermore, as we did not perform a dose-response experiment, one cannot rule out the possibility that higher doses would have stronger effects on BDNF. As far as we can assume, HDAC modulation in the PFC might alter the expression of genes other than BDNF. Further analyses are required to explore the SB-induced effects found in our model as well as in other behavioral tests, including evaluation of the dopaminergic system and of other structures, such as the hippocampus and the amygdala.

In conclusion, we demonstrated that repeated AMPH administration increased HDAC activity in the PFC without altering BDNF protein or mRNA levels. Moreover, Li, VPT, and SB administration partially reversed this increase in HDAC activity, which might account for the ability of these agents to reverse AMPH-induced hyperactivity. Further studies are required to explore the cellular consequences of HDAC inhibition in different brain areas, including assessment of the expression of other genes likely to be associated with these treatments. Nonetheless, the available results support the hypothesis that histone modifications are associated with the mechanisms of action of AMPH and mood stabilizers. In addition, the present data highlight the notion that HDAC inhibitors may hold important potential for the development of new drugs for the treatment of BD.

Acknowledgements

This work was supported by grants from National Science and Technology Institute for Translational Medicine (INCT-TM), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundo de Incentivo à Pesquisa – Hospital de Clínicas de Porto Alegre (FIPE-HCPA).

Disclosure

The authors report no conflicts of interest.

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CAPITULO II

Damage-associated molecular patterns in Bipolar Disorder

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Keywords: Bipolar disorder, cell death, circulating cell-free DNA, heat shock proteins, clinical remission, acute mood episode.

Category: Archival Reports

Word count (abstract): 247

Word count (body of the manuscript): 2549

References: 33

Figures: 1

Tables: 2

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Manuscrito a ser submetido à revista *Biological Psychiatry*

ABSTRACT

Background. Damage-associated molecular patterns (DAMPs) are a product of cell death (necrosis or apoptosis) and are highly immunogenic. Increased levels of early apoptosis have been recently demonstrated in bipolar disorder (BD), but no study so far has examined DAMPs levels in these patients. The aim of this study was to assess serum levels of DAMPs in drug-free BD patients and changes on these biomarkers after pharmacological treatment. **Methods.** Thirty-six drug-free BD patients in acute episode (mania/mixed or depression) and 55 matched healthy controls were recruited. Blood samples were collected at baseline and every week during a 16-week follow-up period among patients. **Results.** Higher levels of ccf DNA (nuclear (n)DNA ($p < 0.0001$) and mitochondrial (mt)DNA ($p = 0.032$)), as well as HSP70 ($p = 0.02$) were found in drug free bipolar patients compared to healthy controls . After pharmacological treatment, ccf nDNA ($p = 0.013$) and HSPs levels ($p = 0.025$) decreased in those patients that achieved clinical remission. **Conclusion.** DAMPs are altered in bipolar patients during acute episode. Those patients who achieve remission normalize the observed blood alterations. The present findings may be linked to the inflammatory activity previously described among bipolar patients, particularly during symptomatic periods**.**

INTRODUCTION

Bipolar disorder (BD) is a chronic mental illness of major clinical relevance with high rates of morbidity and mortality (1). Cognitive impairment and poor psychosocial functioning have been also found among bipolar patients (2, 3). In addition, there is increasing evidence showing that BD is associated with systemic alterations including increased inflammatory markers, oxidative stress, decreased BDNF, and DNA damage (4, 5). Such systemic alterations may be related to dysfunctions in cellular resilience mechanisms such as endoplasmic reticulum stress and mitochondrial damage (6, 7).

Furthermore, it has been showed that cellular death is associated with BD and probably involved in its pathophysiology (8, 9). For instance, a recent report showed increased early apoptosis in peripheral blood mononuclear cells from patients with BD (10). Another independent study found lower levels of anti-apoptotic factors such as HSP70 and BAX, in patients with BD (11). Furthermore, the percentage of apoptotic nuclei in the *ex vivo* cell culture tends to increase after exposure to the serum of patients with chronic mood disorders (8). In this regard, treatment with lithium seems to alter the balance of certain pro-and anti-apoptotic gene-expression, which is strongly associated to the heterogeneity in treatment response in BD (12).

Cell stress, damage or death release endogenous danger signals which may trigger several pattern-recognition receptors (PPRs), ultimately leading to the activation of an innate immune response (13). These pro-inflammatory factors are called damage-associated molecular pattern (DAMPs) (14) and the binding to PPRs upregulate genes such as *INF* and pro-inflammatory cytokines (15). In this sense, the presence of DAMPs could act as a trigger for the immune deregulation and systemic toxicity observed in BD (16, 17). Nonetheless, no study to date has examined DAMPs levels in patients with BD.

The aim of this study was to assess serum levels of DAMPs (circulating cell free (ccf) DNA and Heat Shock Protein (HSP)70, HSP90α, and HSP60) in drug-free BD patients during acute episodes matched for sex and age with healthy controls. We also investigated how the serum levels of DAMPs of drug free patients with BD changed over time when they received pharmacological treatment, and whether such differences were associated with the clinical remission.

METHODS AND MATERIALS

Subjects

Patients with BD (n=36) were recruited from subjects treated at the Bipolar Disorders Program of Clinical Hospital of Porto Alegre and University Hospital of Santa Maria, both in southern Brazil. All patients underwent a comprehensive clinical interview by a psychiatrist and diagnosis was based on the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Inclusion criteria included a diagnosis of BD and presence of a manic, depressive, or mixed episode at present according to DSM-IV-TR criteria. Patients also had to be medication-free for at least 2 weeks (6 weeks in the case of fluoxetine or depot antipsychotics), except for benzodiazepines. Exclusion criteria included DSM-IV diagnoses other than bipolar I or II, current abuse of illicit substances and history of a medical condition that would expose them to an undue risk of a significant adverse event, including hepatic, renal, respiratory, cardiovascular, endocrine, neurological, or hematological disease.

The control group (n=55) consisted of healthy subjects selected from the pool volunteers at the hospital. They had no current or previous history and no first-degree family history of major psychiatric disorders, including dementia or mental retardation, assessed by the non-patient version of the Structured Clinical Interview for DSM-IV (SCID).

The investigation was carried out in accordance with the latest version of the Declaration of Helsinki and the study design was reviewed by the Research Ethics Committee of Clinical Hospital of Porto Alegre. All participants provided written informed consent (which was approved by the local ethics committee) after the nature of the procedures had been fully explained.

Assessment

Sociodemographic and clinical data were collected in an extensive interview session conducted with the patients. A register of pharmacological treatment at study entry was also conducted. Manic and depressive symptoms were evaluated using the Young Mania Rating Scale (YMRS) and the Hamilton Depression Rating Scale, 21-item version (HDRS-21), respectively. Functioning was evaluated using the Global Assessment of Functioning (GAF) scale (18-20).

All patients were evaluated with this protocol at baseline and four follow-up periods: 2-weeks, 4-weeks, 8-weeks and 16-weeks. Remission criteria were defined by the HAM-D and YMRS scores of < 8 at follow-up period. Patients at week one were included in an open label treatment with quetiapine extended release or mood stabilizers.

Blood samples

Ten milliliters of blood were withdrawn from each subject by venipuncture prior to initial treatment (baseline) and every week during 16-weeks (follow-up). The blood without anticoagulant was immediately centrifuged at 2000×*g* for 10 min, and serum was kept frozen at −80◦C until assayed.

Serum circulating cell-free DNA quantification

Serum circulating cell-free DNA was extracted from 200 µL of serum using a commercial kit (QIAmp DNA Mini Kit; Qiagen) and eluted in a final volume of 100 µL. DNA was stored at -20 °C until use. For ccf nuclear (n)DNA analysis, the glyceraldehyde-3-phosphodehydrogenase (*GAPDH*) housekeeping gene was assessed with the following primers: 5'-CCCACTCCTGATTTCTGGAAAAGAG-3' (forward),

5’-GTCCCAGGGCTTTGATTTGC-3’ (reverse), and 5’-FAM-CAAGTTGCCTGTCCTTCC-MGB-3’ (probe). For ccf mitochondrial (mt)DNA analysis, a sequence of the mtDNA-encoded ATPase (*MTATP8*) gene starting at locus 8446 was amplified with forward primer 5’AATATTAAACACAAACTACCACCTACCTC-3’, reverse primer 5’-GTTCATTTTGGTTCTCAGGGTTTGTT-3’, and 5’-6-FAM-CCTCACCAAAGCCC-MGB-3’ as the probe. Singleplex real-time PCR was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, ABI). A total reaction volume of 12.5 μL was used, containing 2.5 μL DNA, 6.25 μL TaqMan® Universal PCR Master Mix, 4 primers, 2 probes (0.625 μL), and 3,125 μL distilled water. The mixture was incubated for 2 min at 50 °C, followed by an initial denaturation step at 95 °C for 10 min, and 40 cycles of 1 min at 60 °C and 15 s at 95 °C. Each sample was analyzed in duplicate. ccf DNA concentrations were estimated according to standard curves, using the known concentration of human genomic DNA with a dilution factor of 5 (starting with 5 ng/μL). To minimize errors, the two samples obtained from each patient (acute and remission phases) and their respective controls were always run in the same plate. The ccf nDNA and ccf mtDNA levels were expressed in genome-equivalents (GE) per mL of serum, based on the methodology described by Xia et al. (21).

Heat Shock Protein measurement

Heat-shock proteins (HSPs - Hsp70, Hsp90α, Hsp60) were measured in serum using commercially available ELISAs in accordance with manufacturer’s instructions (Enzo Biosciences). We optimize the dilution of the samples as followed: Hsp70 - 1:4, Hsp90α - 1:10, Hsp60 – no dilution.

Statistical Analysis

All biochemical results were Box-Cox transformed for parametric analysis (22). We used cluster analysis to aggregate serum measures of HSPs (Hsp70, Hsp90α, Hsp60) with the objective of maximizing within group and minimizing between-group similarities. Bipolar patients were divided into remitters and non-remitters based on *a priori* defined change from initial HAMD and YMRS scores. Remitters were defined as those having HAMD and YMRS <8 at the follow-up period (any week between 4 and 16). Patients who did not meet such criteria were classified as non-remitters. Baseline demographic, clinical and biochemical differences were assessed using t-tests. To evaluate the associations between the predictive variable (DAMPs) and clinical variables we used Generalized Estimating Equation (GEE) and Bonferroni post-hoc. All biochemical results are expressed as percentage of control.

RESULTS

Baseline assessment

The sample consisted of 36 drug free bipolar patients and 55 healthy controls. Sixty-seven percent of the patients were woman with an overall mean age of 41±12 years. There were no differences between patients and controls regarding sex ($p = 0.42$) or age ($p = 0.43$). Other clinical characteristics of the patients are presented in Table 1. Regarding pharmacological treatment, 50% of the patients received quetiapine extended release while 44% of them received mood stabilizers at study entry.

As expected, differences on DAMPs were found in patients compared to healthy controls at baseline (see Table 2). In particular, bipolar patients had a higher levels of ccf nDNA ($p < 0.0001$), ccf mtDNA ($p = 0.032$), and HSP70 ($p = 0.02$), indicating an increased peripheral apoptosis or necrosis process during acute episodes of BD. Remitters and non-remitters did not differ in terms of symptoms or baseline levels of DAMPs.

Remitters vs. Non-remitters: follow-up assessment

The proportion of patients who achieved symptomatic remission, as defined by HAM-D and YMRS < 8, was 63.8 % (n=23) during the follow-up period. Non-remitters group had longer duration of the illness, higher proportion of manic onset and bipolar II subtype compared to remitters group, but the differences were not statistically significant. Greater functioning was also found in remitters patients compared to non-remitters (70.52±13 vs. 61.42±15, p=0.074).

Figure 1 shows serum levels of DAMPs in bipolar patients in acute episode (baseline) and over the course following initiation of pharmacological treatment**.** Strikingly, ccf nDNA

and HSPs factor showed significantly lowers levels in patients who achieved clinical remission (Chi2 of Wald 11.94, df = 3, $P = 0.013$, Figure 1A, and Chi2 of Wald 8.33, df = 3, $P = 0.025$, Figure 1B, respectively). These results could indicate that such biomarkers may decrease in patients who achieve clinical remission after initiation of pharmacological treatment of BD. Noteworthy; we did not found differences in the levels of DAMPs when we compared the two pharmacological treatment (quetiapine *vs.* mood stabilizers, $p > 0.05$) at follow-up assessment. These findings suggest that DAMPs levels may decay in patients who achieve clinical remission after pharmacological treatment.

DISCUSSION

This is the first study to show increased DAMPs levels in serum from drug-free patients with BD. These changes were normalized in patients who achieved remission after treatment but not in patients that remained symptomatic. DAMPs are cellular constituents that can be identified by the innate immune system once released from dying cells (23). They include sugars, metabolites, lipids, and nucleic acids such as RNA and DNA (24), and can lead to the activation of signaling pathways resulting in a nonpathogenic-induced 'sterile' inflammation response (23). The increase of those molecules corroborates the evidences of increased cell death in bipolar patients (10), providing a mechanistic link between BD and increased inflammation and systemic toxicity (16). Interestingly, our results suggest that DAMPs may be used as markers of clinical response in the treatment of acute episodes.

In particular, we found increased serum levels of ccf nDNA in BD patients at baseline as compared to follow-up period. The ccf nDNA has a great pro-inflammatory potential, as it can bind to either TLR-9 receptor, activating the pathway dependent of myeloid differentiation factor 88 (MyD88), or to cytosolic sensors, such as Absent in melanoma 2 (AIM2), DNA-dependent activator of INF regulatory factors (DAI) and the IFN inducible protein 6 (IFII6) (15). Both pathways can culminate in the activation of NF-κB via the IKB kinase complex (IKK), thus increasing the transcription and secretion of cytokines such as TNF-α, IFN and IL-1, and are also able to increase the production of reactive oxygen species, which is another finding described among BD patients (25).

Herein, HSP70, HSP60, HSP90α levels were also greater in bipolar patients at baseline than follow-up, indicating dysfunctions on "heat shock factors", and consequently higher vulnerability of these individuals under stressful conditions. It is important to notice that the presence of DAMPs in environments that do not relate to the cells of which they were released can induce a response that lose its importance because it is out of context (26). For instance,

HSPs can be up-regulated as a response to cellular stress, inducing the maturation of dendritic cells and increasing the immunogenic properties of the agonizing cells after binding to TLR4 (27). On the other hand, HSP70 and HSP90 can also engage inhibitory receptors like CD24 and block their pro-inflammatory effects (28). The same signal, depending on the context, can activate and limit an inflammatory response (26).

Cells under early apoptosis, such as those found in patients with BD (10), have been shown to present a limited membrane permeabilization in blebs and apoptotic bodies that allows the release of proteins and other DAMPs (23). Once the inflammation is established, the risk for clinical comorbidities significantly increases, which is in accordance with the high prevalence of comorbidities found in patients (29). In this sense, the recurrence of episodes has been correlated with higher rates of comorbidity (30), again suggesting the role of the episode-associated toxicity on cell death and its consequences. Excessive responses to DAMPs can facilitate the development of autoimmunity, as well as sepsis. A recent work showed that individuals with BD have a greater Th1 pro-inflammatory profile as well as a reduced number of Treg cells (31), which may indicate a difficulty in restoring homeostasis after an inefficient clearance of apoptotic cells remnants. Altogether, we can hypothesize that enhancing cell survival and resilience might lead to reduced release of DAMPs, ultimately reducing inflammation and associated comorbidities.

The fact that only remitters presented reduced serum levels of DAMPs after treatment suggests that cellular resilience is a key mechanism in the remission of symptoms. It is likely that all subjects activate resilience and cell survival pathways in response to the systemic toxicity that takes place during an acute mood episode; however, based on individual differences on resilience mechanisms, only some of the patients might manage to sufficiently counteract these damaging stimuli and thus significantly attenuate cell death. Of note, individual differences might lead to different responses to any given medication in patients. In

this context, the expression of pro-apoptotic (BAK1 and BAD) and anti-apoptotic genes (Bcl2 and insulin receptor substrate-2) has been shown to differ between lithium responders and non-responders (12). As suggested by pharmacogenetic studies, these differences may rely on individual genetic fingerprints that can modulate one's response to treatment, including polymorphisms and epigenetic markers (32). For instance, a recent study has shown that only patients with major depressive disorder who present increased baseline DNA methylation at the *BDNF* gene are likely to benefit from antidepressant pharmacotherapy (33). In this particular study, only those that presented increased methylation were able to increase serum BDNF levels during the treatment, which was associated the symptom's remission. Therefore, we could hypothesize that individual biochemical and epigenetic markers might be altering one's ability to protect cells from the toxicity seen in the acute episode, and by that is not able to appropriately achieve remission. Consequently, the systemic toxicity is extended, ultimately amplifying inflammation and thus completing a feed-forward mechanism of cell death-induced toxicity in BD.

Limitations of the present study include a relatively small sample-size, admixture of patients with BDI and BDII and the lack of assessment of systemic toxicity in patients, which does not allow us to rule out the possibility of increased basal toxicity in non-remitter patients (which could then significantly interfere with their clinical response to the medications).

In summary, our findings showed altered DAMPs levels in bipolar patients, suggesting a potential link between inflammation, systemic toxicity, cell death and the pathophysiology of BD. Additionally, we showed that DAMPs levels may decrease over time after initiation of pharmacological treatment, indicating that individual differences in the treatment response may occur at the level of biochemical parameters. Finally, our finds suggest that a better understanding of the mechanisms underlying the heterogeneity of treatment response may lead to improved methods for individualizing treatment for BD in the future.

Acknowledgments

Laura Stertz and Gabriel R. Fries were supported by scholarships from Coordination for the Improvement of Higher Education Personnel (CAPES), Brazil. Dra. Adriane R Rosa was supported by scholarships from the *National* Counsel of Technological and Scientific Development (CNPq), Programa Ciência sem Fronteiras, modalidade Atração de Jovens Talentos and L'Oréal Brasil, Academia Brasileira de Ciências, and Comissão Nacional da UNESCO, "For Women in Science".

Financial Disclosure

Professor Kapczinski has received grants/research support from AstraZeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD, and the Stanley Medical Research Institute; has been a member of speakers' boards for AstraZeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier. Dr Kauer-Sant'Anna is on speaker/advisory boards for, or has received research grants from NARSAD, Stanley Medical Research Institute, CNPq-Universal, CNPq/INCT-TM, CNPq productivity award II, FIPE-HCPA and Eli-Lilly.

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Table 1. Demographic and clinical characteristics of patients

Variable	*Baseline (n=36)*	*Follow- up*	
		Remitters (n=23)	*Non-remitters (n=13)*
Age at enrolment (years)*	41±12	39±13	43±9
Gender (Woman)	66.7%	56.5%	84%
Bipolar Disorder			
Tipe I	75%	78.3%	69.2%
Tipe II or NOS	25%	21.7%	30.8%
Any psychiatric comorbidity	38.9%	39.1%	38.5%
Duration of disorder (years)*	14±12	13±12	17±10
Episode at study entry			
Depressive type	42%	34.8%	23%
YMRS*	1±2	1±1	3±5
HAMD*	23±8	6±6	11±5
GAF*	57±17	69±12	66±14
Manic type	33%	39.1%	54%
YMRS*	28±11	2±3	25±19
HAMD*	6±8	2±3	5±5
GAF*	35±20	74±14	53±11
Mixed type	25%	26.1%	23%
YMRS*	20±11	2±2	3±5
HAMD*	20±9	6±5	14±10
GAF*	39±18	68±13	57±23
Treatment followed			
Mood stabilizers	44.4%	47.8%	38.5%
Quetiapine extended release	50%	43.5%	61.5%
Antipsychotics	5.6%	8.7%	0.0%
Total duration of treatment			
Four weeks	N/A	43%	69%
Eight weeks	N/A	30%	15%
Sixteen weeks	N/A	26%	15%

*Values are indicated as mean ± SD. # Values are indicated as median (25-75 interquartile range).
BD, Bipolar disorder; NOS, not otherwise specified; YMRS, Young Mania Rating Scale; HDRS, Hamilton Depression Rating Scale; GAF, Global Assessment of Functioning scale.

Table 2. DAMPs levels in patients with bipolar disorder (BD) compared to healthy controls at baseline.

Variable	*Control (n = 55)*	*BD Baseline (n = 36)*	*p-value*
Ccf nDNA (% of control)[a]	100.14±8	114.43±18	t = 4.27, p<0.0001*
Ccf mtDNA (% of control)[a]	99.96±10	107.29±18	t = 2.24, p = 0.032*
HSP90α (% of control)[b]	100(80-248)	130 (82-177)	Z = -0.25, p = 0.79
HSP70 (% of control)[b]	101.2(86-133)	110(100-201)	Z = -2.33. p = 0.02*
HSP60 (% of control)[b]	91.55(52-156)	127 (40-221)	Z = -0.42, p = 0.66

[a] Values are indicated as mean ± SD. [b] Values are indicated as median (25-75 interquartile range). * Statistically significant. ccf nDNA, circulanting cell free nuclear DNA; mtDNA, mitochondrial DNA; HSP, Heat Schock Proteins.

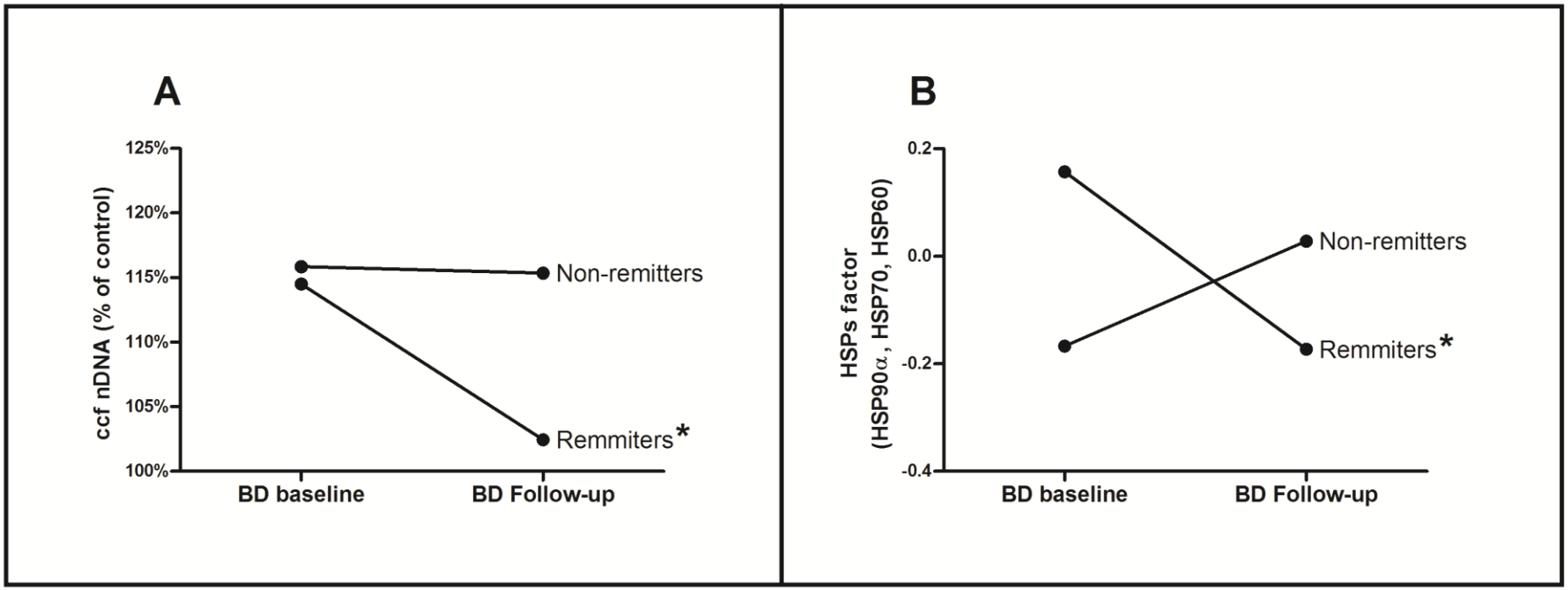

Figure 1. Serum levels of DAMPs differed over time between BD patients who achieve clinical remission. A) ccf nDNA levels are significativally lower in patients who achieved clinical remission (Chi2 of Wald 11.94, df = 3, P = 0.013). Results are shown as mean of GE/ml % of control. **B)** The mean values of the factor HSPs compose by HSP70, HSP60 and HSP90α are shown. Only patients who achieved clinical remission shown differences from baseline (Chi2 of Wald 8.33, df = 3, P = 0.025). *Differences were considered statistically significant at p≤ 0.05. ccf *nDNA: circulating cell-free nuclear DNA; HSPs: heat shock protein.*

CAPÍTULO III

Uric acid and TNF-α as biological predictors of clinical remission in bipolar disorder

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Word count (abstract): 215

Word count (body of the manuscript): 2097

References:

Figures: 1

Tables: 2

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Manuscrito a ser submetido à revista *Journal of Affective Didorders*

ABSTRACT

The biological underpinnings of clinical remission after treatment of bipolar disorder (BD) are still poorly understood. Therefore, the aim of this 16-week follow-up study was to assess serum levels of several biomarkers in drug-free BD patients (baseline, N = 20) and changes occurring after treatment during the clinical remission (follow-up). Among biomarkers, we included markers of cellular death (Cytochrome C), antioxidants (uric acid), and pro- and anti-inflammatories cytokines (Interleukin (IL)-1α ,IL-6, IL10, Tumor Necrosis Factor (TNF)-α, IL-12p70, IL-17A, Interferon (INF)-γ, IL-13, Granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand 11 (CCL-11) and Prostaglandin (PG)E2). We also included two control groups at baseline: one comprising healthy subjects, which was taken as a negative control (N = 20), and another group comprising patients with sepsis, taken as a positive control for toxicity (N=20). We found that PGE2 was significantly increased in BD when compared with negative controls (p=0.034) at baseline. Moreover, we found a decrease in the levels of TNF-α (p=0.005) and an increase in the levels of uric acid (p=0.006) in patients who achieved clinical remission after treatment. These results may add to the notion that certain biomarkers may function as indicator of illness activity in bipolar episode, and may help in the development of more targeted and personalized treatments for patients under acute episodes of BD.

Key words: biomarkers, cytokines, acute mood episode, bipolar disorder, clinical remission

1. OBJECTIVES

The understanding that acute mood episodes of Bipolar Disorder (BD) can contribute with the worse outcomes in this pathology is not a recent trend. Since 1999, studies indicate that the number of episodes is correlated with a reduction in the response to treatment (Franchini et al., 1999; Swann et al., 1999). In this sense, a growing body of evidence suggests that cognitive decline is associated with the number of illness episodes (El-Badri et al., 2001; Robinson and Ferrier, 2006), particularly that greater neuropsychological dysfunction is associated with a worse prior course of illness, especially the number of manic episodes. Of note, patients with higher number of previous episodes tend to present a treatment-resistant illness and a higher tendency to relapse when compared to those that have had fewer episodes (Berk et al., 2011). Also, an analysis using data from STEP-BD shows that patients with more than ten episodes tend to have worse longitudinal functioning and quality of life (Magalhães et al., 2012). Furthermore, MRI studies have suggested that the recurrence of mood episodes leads to abnormal neural development (Schneider et al., 2012). In summary, these data indicate that the acute episodes could be damaging several critical factors and may impair the recovery of these patients.

Notoriously, not only clinical alterations have been observed after a mood episode. As we have previously proposed, systemic toxicity, which consists of an increase in the levels of several peripheral markers, seems to be implicated in the potential toxicity related to acute mood episodes (Kapczinski et al., 2011). Moreover, several lines of evidence indicate increased levels of cellular death in BD (Gigante et al., 2011; Herberth et al., 2011; Politi et al., 2008). However, the biological underpinnings of the clinical response and the decreased systemic toxicity in patients with BD after clinical remission remain unknown.

Therefore, the aim of this 16-week follow-up study was to assess serum levels of several biomarkers, including markers of cellular death (Cytochrome C), antioxidants (uric acid), as well as pro- and anti-inflammatories cytokines [Interleukin (IL)-1α ,IL-6, IL10, Tumor Necrosis Factor (TNF)-α, IL-12p70, IL-17A, Interferon (INF)-γ, IL-13, Granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand 11 (CCL-11) and Prostaglandin (PG)E2] in drug-free BD patients (at baseline) and their changes occurring after treatment during the clinical remission (follow-up). We also included two control groups at baseline: one comprising healthy subjects, taken as a negative control; and another group comprising patients with sepsis, used as a positive control for toxicity.

2. **MATERIAL AND METHODS**

Subjects

Patients with BD (n=20) were recruited from subjects treated at the Bipolar Disorders Program of Hospital de Clínicas de Porto Alegre and Hospital Universitário de Santa Maria, both in southern Brazil. All patients underwent a comprehensive clinical interview by a psychiatrist. Inclusion criteria included a diagnosis of BD and presence of a manic, depressive, or mixed episode at present according to DSM-IV-TR criteria. Except for benzodiazepines, patients also had to be medication-free for at least 2 weeks (6 weeks in the case of fluoxetine or depot antipsychotics). Exclusion criteria included any DSM-IV diagnoses other than bipolar I or II, current abuse of illicit substances, history of a medical condition including hepatic, renal, respiratory, cardiovascular, endocrine, neurological, or hematological disease.

The use of both a negative and a positive control groups in this case-control longitudinal study was based on a previous work (Kapczinski et al., 2010). Negative controls (n=20) were individuals with no history of psychiatric illness, mostly family members of people using other health care facilities at the same hospital. Positive controls were septic patients (n=20) selected

at an intensive care unit and diagnosed according to American College of Chest Physicians/Society of Critical Care Medicine criteria (Bone et al., 1992). All participants provided written informed consent, and procedures were approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre.

Assessment

Sociodemographic and clinical data were collected in an extensive interview session conducted with the patients. All patients were evaluated using the same protocol at baseline and over a 16-week follow-up period. Longitudinal assessments were performed at weeks 2, 4, 8, and 16 and patients were medicated at the discretion of the therapist beginning at week one.

Manic and depressive symptoms were evaluated using the Young Mania Rating Scale (YMRS) and the Hamilton Depression Rating Scale, 21-item version (HDRS-21), respectively (Williams, 1988; Young et al., 1978). Functioning was evaluated using the Global Assessment of Functioning (GAF) scale (Jones et al., 1995). We defined *a priori* that only remitters would be included in this study (remitters were defined as those BD patients having a score of ≤8 on HDRS-21 and YMRS at any visit between 4 and 16 weeks after beginning of treatment).

Blood collection and processing

Ten milliliters of blood were withdrawn from each subject by venipuncture into an anticoagulant-free vacuum tube for serum analysis at baseline and at 16-week follow-up. Blood without anticoagulant was immediately centrifuged at 2000×g for 10 min, and serum was kept frozen at -80 °C until assayed.

Assays

All assays were performed in serum samples according to the manufacturer's instructions. Uric acid was measured by an enzymatic colorimetric assay from Analisa (Gold Analisa Diagnóstica Ltda, Minas Gerais, Brazil) and values are expressed as mg/dL.

Cytochrome C was measured with an ELISA test from eBiosciences (San Diego, CA) and values are expressed as ng/mL. PGE2 was determined by a competitive immunoassay from Enzo Life Sciences (New York, USA) and values are expressed as pg/mdL.

Cytokines levels were determined by the CBA Human Enhanced Sensitivity Flex Set System (BD Biosciences, San Diego, CA). Briefly, serum samples and a standard curve ranged from 0 (negative control) to 200.000 fg/mL (IL-6, IL10, TNF-α, IL-12p70, IL-17A and IFN-γ) were coated with the Capture Beads of interest for 2 hours. Afterwards, we added the mixed Human Detection Reagent (Part A) to each assay well and coated for 2 hours at room temperature. After washing, the Enhanced Sensitivity Detection Reagent (Part B) was added and the samples were incubated for 1 hour. A final wash was performed and the samples were analyzed using FACSCalibur flow cytometer. The final concentration of cytokines was calculated using Excel program for Macintosh. Values are expressed as fg/mL. For the measurement of IL-13, GM-CSF, IL-1α and CCL-11 we used a similar method. Briefly, serum samples were incubated with the cytokine capture beads for 1.5 h, then washed and incubated for more 1.5 h with PE-conjugated detection antibodies, both incubations at room temperature and protected from light. Afterwards, samples were washed and data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA). Values are expressed as pg/mL.

Statistical Analysis

All biochemical results were Box-Cox transformed (Osborne, 2010). To analyze differences between BD patients in acute episode at baseline and controls (positive and

negative) we use Analysis of covariance (ANCOVA) controlling for age. To evaluate the associations between the predictive variables and clinical variables we used Generalized Estimating Equation (GEE). Differences were considered statistically significant at $p \leq 0.05$. Statistical dependence between two variables was measured by Fisher's rank correlation coefficient.

3. RESULTS

Demographic and clinical characteristics of the subjects assessed are shown in **Table 1**. All participants were matched by age and sex, except for septic patients, who were selected by convenience. No differences were found between sexes in any of the biochemical measures. Since there were significant differences in age in the positive control group, we used this variable as a covariable in all analyses. Twenty patients with BD were included in the study, of which 30% were in a quetiapine monotherapy protocol and the other 70% were in a naturalistic treatment protocol. Of them, 50% were having manic episodes, 30% depressive episodes, and 20% mixed episodes.

There were significant differences at baseline in the levels of GM-CSF ($p = 0.006$), IL12p70 ($p < 0.0001$), IL-6 ($p < 0.0001$), IL-10 ($p = 0.023$) and PGE2 ($p = 0.008$) between groups (**Table 2**). However, when compared to negative controls, we only found differences in PGE2 levels, whose levels were significantly higher in BD patients ($p = 0.039$).

After treatment, we analyzed if there were any differences between the assessment at baseline and after clinical remission (*follow-up*) in BD. We found that patients at follow-up had an increase in uric acid levels (Chi square of Wald 7.66, df = 1, $p = 0.006$, **Figure 1A**) and a concomitant decrease in the levels of TNF-α (Chi square of Wald 7.25, df = 1, $p = 0.007$, **Figure 1B**). Moreover, we found that uric acid had a moderate negative correlation with YMRS ($r = -0.366$, $p = 0.04$), as well as a positive correlation with TNF-α levels ($r = 0.338$, $p = 0.05$).

4. DISCUSSION

Our results indicate that uric acid and TNF-α can be taken as biological markers of clinical remission in BD. Surprisingly, the levels of these two markers were similar to the control group at baseline.

In particular, we found increased serum levels of uric acid in BD patients at baseline when compared to follow-up. In the purine metabolism, uric acid is the final oxidation product and is normally excreted in the urine. Usually, uric acid is soluble inside the cells, but once in the extracellular space it can form microcrystals in a reaction to the higher sodium levels (Bianchi *et al.,* 2007). These microcrystals present major inflammatory properties and can engage the inflammasome, ultimately producing IL-1β and IL-18 (Martinon *et al.,* 2006). When the uric acid can maintain itself soluble in plasma and serum, it can act as a strong reducing agent and a potent antioxidant, constituting almost half of the antioxidant capacity of these fluids (Maxwell et al., 1997). In our study, we demonstrated that increased levels of soluble uric acid seem to support the remission of symptoms in e patients, given that greater levels of uric acid were associated with lower scores of YMRS. Previous works had already demonstrated increased oxidative damage in proteins and lipids in the serum of bipolar patients (Banerjee et al., 2012; Kapczinski et al., 2011). Contrary to our findings, a significant association between improvement in manic symptoms and a decrease in plasma uric acid levels has been demonstrated (Machado-Vieira et al., 2008), This may be explained by the fact that our patients did not demonstrated differences at baseline from healthy controls. Moreover, the levels at *follow-up*, despite being increased, were not beyond the normal levels (reference levels for men and woman are between 1.5 and 7 mg/dL; the highest level we found in a bipolar patient was 4.9 mg/dL).

On the other hand, we found that TNF-α levels in serum decreased after treatment. This cytokine acts in the process of cell survival, cellular resilience and apoptosis (Stertz et al., 2013). In addition, its effects may be influenced by other cytokines (pro-and anti-inflammatory), which orchestrate a series of reactions that lead (or not) to a state of inflammation. *In vitro*, mood stabilizers are able to reduced TNF-α levels in therapeutics concentrations (Himmerich et al., 2013). Surprisingly, the levels of TNF-α, as for the uric acid levels, were similar to the control group in BD baseline. Despite that, both were correlated with the YMRS scores, showing that increased levels of uric acid and lower levels of TNF-α seem to support the remission symptoms in patients.

Finally, the treatment and the clinical remission did not seem to be able of revert the increase in the levels PGE2 seen at baseline. PGE2 has been reported to have ambiguous effects, acting as a pro-inflammatory factor with imunossupressor activity (Kalinski, 2012). Beyond the fact that it mediates the early stages of inflammation, it is also capable of supressing the production of several pro-inflammatory cytokines. In BD, studies in the late 80s showed several alterations in arachidonic acid metabolism and increase in the serum levels of PGE2 (Lieb et al., 1983; Nishino et al., 1989). However, animal studies showed that mood stabilizers are able to downregulate the PGE2 levels in rats (Rao and Rapoport, 2009). In our study, we were not able to observe differences in PGE2 levels induced by the treatment, indicating that PGE2 might be a trait marker rather than a state marker. Further studies evaluating the role of PGE2 in the progression of BD should help to clarify these results.

In conclusion, we suggest that uric acid, TNFα and PGE2 are important and promising biological markers for clinical remission in BD. Moreover, our results indicate that important biological mechanisms may be involved in improving symptoms of patients after treatment. Further studies are necessary to access how treatment is hindering cell death and preventing associated symptoms.

Acknowledgments

Laura Stertz and Gabriel R. Fries were supported by scholarships from Coordination for the Improvement of Higher Education Personnel (CAPES), Brazil. Dra. Adriane R Rosa was supported by scholarships from the *National* Counsel of Technological and Scientific Development (CNPq), Programa Ciência sem Fronteiras, modalidade Atração de Jovens Talentos and L'Oréal Brasil, Academia Brasileira de Ciências, and Comissão Nacional da UNESCO, "For Women in Science".

Financial Disclosure

Professor Kapczinski has received grants/research support from AstraZeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD, and the Stanley Medical Research Institute; has been a member of speakers' boards for AstraZeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier.

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Table 1. Demographic and clinical profile of the participants.

Variable	*Negative control (n=20)*	*Positive control (n=20)*	*BD Baseline (n=20)*
Age at enrolment (years)*	35±13	59±13*	35±13
Female sex #	11 (58%)	4 (20%)	12 (60%)
Self-reported clinical illness#	-	-	6 (40%)
Protocol followed (week one)			
Naturalistic treatment#	-	-	14 (70%)
Treatment with quet #	-	-	6 (30%)
Treatment (week one)			
Mood stabilizers#	-	-	13 (65%)
Antipsychotics#	-	2 (10%)	18 (80%)
Antidepressants#	-	-	0 (0%)
Benzodiazepines#	-	-	9 (45%)
Quetiapine #	-	-	6 (30%)
Current episode			
Depressive type (296.5x)#	-	-	6 (30%)
Manic type (296.46)#	-	-	10 (50%)
Mixed type (296.6x)#	-	-	4 (20%)
Disorder duration*	-	-	14±13
YMRS*	-	-	19±14
HAMD*	-	-	13±11
GAF*	-	-	41±19

*Data shown as mean ± standard deviation. # Data shown as N (percentage).
BD, Bipolar disorder; YMRS, Young Mania Rating Scale; HDRS, Hamilton Depression Rating Scale; GAF, Global Assessment of Functioning scale.

Table 2. Levels of peripheral biomarkers at baseline.

Variable	*Negative control (n=20)*	*Positive control (n=20)*	*BD Baseline (n=20)*	*P-value*
Uric acid	1±0.34	0.99±0.54	1.15±0.25	F(2, 53)=0.54, p=0.58
Cytochrome C	0.27±0.19	0.31±0.18	0.26±0.17	F(2, 53)=0.008, p=0.99
IL-1α	1.32±0.31	1.18±0.52	1.36±0.3	F(2, 50)=1.42, p=0.24
IL-13	3.83±1.27	3.09±0.94	3.77±1.06	F(2, 50)=0.35, p=0.7
GM-CSF	2.26±0.23	1.96±0.41	2.20±0.3	F(2, 50)=5.68, p=0.006*
IL-17A	6.42±2.99	7.75±2.22	6.07±2.45	F(2, 52)=1.43, p=0.24
IL-12p70	18.15±10.78	36.10±3.47	16.15±10.05	F(2, 52)=14.78, p<0.0001*
CCL-11	16.23±7.23	15.44±6.44	17.46±5.16	F (2, 52)= 1.207, p=0.307
TNF-α	36.47±15.35	26.81±12.14	37.22±6.31	F(2, 48)=1.64, P=0.25
IL-6	6.67±2.08	10.72±1.4	7.08±1.43	F(2, 50) = 12.87, P<0.0001*
IL-10	8.91±3.84	13.57±3.74	8.25±2.31	F(2, 50)=4.07, P=0.023*
INF-γ	38.18±10.66	41.75±9.94	37.58±7.24	F(2, 51)=0.69, p=0.5
PGE2	7.38±1.86	5.98±2.27	9.46±3.34	F (2, 53)=5.23, P=0.008*#

Data shown as mean ± standard deviation. Analysis of covariance (ANCOVA) controlling for age.
* Statistically significant. # BD *vs*. negative control (p = 0.039). IL – Interleukin; TNF-α - Tumor Necrosis Factor alpha; IFN – Interferon; GM-CSF - Granulocyte-macrophage colony-stimulating factor; CCL11 - chemokine (C-C motif) ligand 11 e PGE2 - Prostaglandin E2

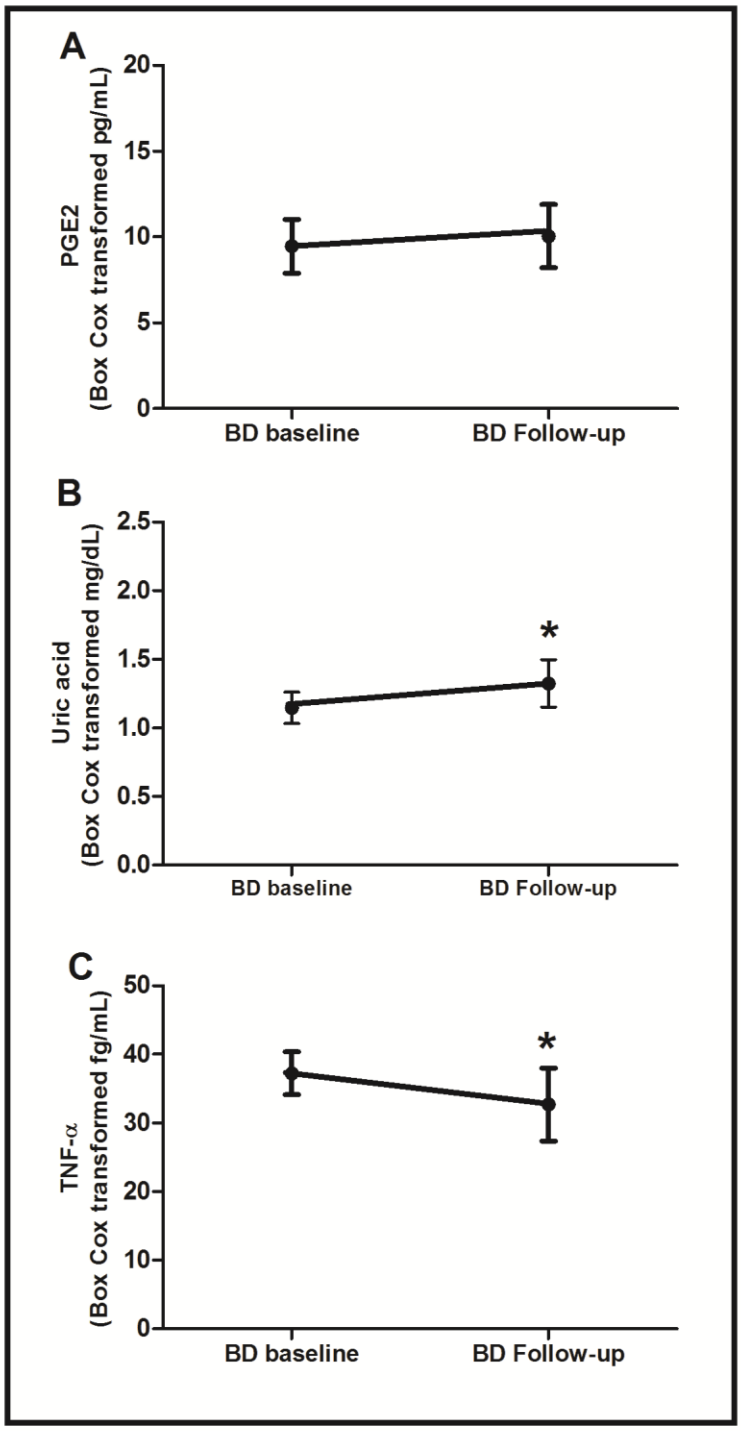

Figure 1. Serum levels of uric acid and TNF-α but not PGE2 differed over time in BD. A) Serum levels of PGE2 did not differ over time (Chi square of Wald 0.21, df = 1, $p = 0.886$). B) Levels of uric acid are significativally higher over time in patients asymptomatic (Chi square of Wald 7.66, df = 1, $p = 0.006$). C) Patients presented a decrease in the levels of TNF-α after clinical remission (Chi square of Wald 7.25, df = 1, $p = 0.007$). Data are represented as 95% of confidence level. * Differences were considered statistically significant at $p \leq 0.05$. BD – bipolar disorder.

CAPITULO IV

Is bipolar disorder an inflammatory condition? The relevance of microglial activation

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Artigo publicado pela revista *Current Opinion in Psychiatry* em 2013

Is bipolar disorder an inflammatory condition? The relevance of microglial activation

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Purpose of review
Literature published over the past few years indicates that bipolar disorder has an inflammatory component but does not explicitly define bipolar disorder as an inflammatory or a noninflammatory condition.

Recent findings
Recent studies have shown that bipolar disorder involves microglial activation and alterations in peripheral cytokines and have pointed to the efficacy of adjunctive anti-inflammatory therapies in bipolar depression.

Summary
The presence of active microglia and increased proinflammatory cytokines in bipolar disorder suggests an important role of inflammatory components in the pathophysiology of the disease, as well as a possible link between neuroinflammation and peripheral toxicity.

Keywords
bipolar disorder, inflammation, microglial activation, neuroinflammation, systemic toxicity

INTRODUCTION

Whether or not bipolar disorder should be considered an inflammatory condition will depend on an unambiguous definition of inflammatory condition and immune response. Inflammation is a part of the nonspecific immune response that takes place after any type of bodily injury or microbial invasion. Many of these reactions involve cytokines, especially interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and IL-6, produced by dendritic cells, macrophages, and other types of cells. Inflammatory responses are also accompanied by increased levels of acute-phase reactants [such as high sensitivity C-reactive protein (hsCRP)] and complement factors [1▪].

The immune system is often involved with inflammatory disorders, such as allergic reactions and skin disorders, many of which result in abnormal inflammation. Wounds and infections would never heal without inflammation, but chronic inflammation, if not controlled, can also lead to a number of pathological conditions, such as inflammatory bowel disease and rheumatoid arthritis. This is one of the reasons why the inflammation is so closely regulated by the body.

Over the past decade, bipolar disorder has been consistently associated with clinical comorbidities [2]. Recent data from the Systematic Treatment Enhancement Program for Bipolar Disorder show that over 50% of patients with chronic bipolar disorder have at least one associated comorbidity [3▪]. Prominent in this group are cardiovascular disease, diabetes, obesity, dyslipidemia, and insulin resistance – all metabolic syndrome components [2,4▪]. This overlap is one of the reasons why great emphasis has been placed on systemic mechanisms related to bipolar disorder-related impairment [4▪]. Up to the present moment, two clinical studies, one conducted at a specialized clinic [5▪] and another assessing the general population [6▪], have shown that subtle proinflammatory states are characteristic of the peripheral pathophysiology of bipolar disorder [7▪].

Progressive impairment of different cognitive functions has been consistently described in bipolar

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Curr Opin Psychiatry 2013, 26:19–26
DOI:10.1097/YCO.0b013e32835aa4b4

KEY POINTS

- Microglial activation may be involved in synaptic pruning in bipolar disorder.
- Systemic toxicity and microglia may work together as part of a positive feedback system.
- The inflammatory changes observed in bipolar disorder appear to be associated with disease progression rather than to integrate a causal model.

disorder, corroborating a potential role of neuroinflammation in this illness [8,9]. Immune signaling in the brain is of special interest because it provides a relevant explanatory link between progressive dysfunction, cognitive impairment, medical comorbidity, and premature mortality [10]. Neurocognitive alterations include impairment of attention, executive function, and verbal memory [11▪]. These changes can be influenced by inflammatory mediators through the shaping of synaptic transmissions. Inflammation can influence the role of microglia in synaptogenesis (synaptic formation) and pruning, that is, reduction of the overall number of neurons and synapses, leaving only more efficient synaptic configurations [12▪▪,13▪▪]. Also, TNF-α influences dendritic arborization, modulates long-term potentiation (a mechanism of memory consolidation), and affects neurotransmitter pathways [14▪▪].

Many of these impairments and comorbidities have been described in clinical populations after the occurrence of mood episodes. Because the pathophysiology of bipolar disorder tends to differ in early versus late stages, the term neuroprogression has been used to describe these changes [7▪]. Structural and functional modifications change as the illness progresses and as patient age increases. MRI studies have suggested abnormal neural development already in the early stages of the disorder, with progressive changes as mood episodes occur [15▪]. Neuroprogression in bipolar disorder underlies changes in inflammatory cytokines and neurotrophins, mitochondrial dysfunction, oxidative stress, and epigenetic effects [7▪]. These parameters can be sensitive to the progression of illness and have, therefore, been used as first biochemical indicators in the staging of bipolar disorder [16].

In this review, we will focus on lines of investigation that establish a link between neuroinflammation and peripheral toxicity in bipolar disorder, in an attempt to define bipolar disorder as an inflammatory or a noninflammatory condition, using data published mainly in the past 18 months.

MICROGLIAL ACTIVATION AND NEUROINFLAMMATION

In the framework of the nervous system, inflammation can be viewed as a collection of immune responses aimed at dealing with a threat to the neuronal environment. Inflammation is accompanied by synaptic degeneration and neuronal loss (for a detailed revision on the role of inflammation in the developing brain, see Harry and Kraft [17▪▪]), and it can be induced by disease, physical trauma, ischemia/hypoxia, or cellular damage due to multiple initiating stimuli, including exposure to neurotoxicants [18▪▪].

The brain is rich in resident macrophages, called microglia, which become activated in response to tissue damage or brain infections and can be the first to detect critical changes in neuronal activity and health [17▪▪]. The microglial activation can be divided into two types: classical M1 (first line of defense) and alternative M2 (anti-inflammatory). In the M2 case, microglia increase the production and release of anti-inflammatory cytokines and neurotrophic factors, and the production of cytoactive factors involved in repairing and restructuring damaged extracellular matrix in the brain [19▪]. In the M1 case, microglial activation leads to the synthesis of an array of proinflammatory mediators, which can clear infections and repair tissues. However, if not controlled, this response may perpetrate bystander neural insult [20▪▪].

Activated microglia secrete innate proinflammatory cytokines TNF-α and IL-1β, which can directly injure neurons at supraphysiological levels [20▪▪]. TNF-α, for instance, interacts with two receptors: p55 (TNF-RI) and p75 (TNF-RII). Binding of TNF-α to either receptor can activate an apoptotic signaling cascade when ligand binding occurs. The TNF-R then associates with the TNF receptor-associated death domain. This results in recruitment and internalization of Fas, activation of caspase-8, and cell death [21].

The threshold for microglial activation, however, may be higher than that of macrophage activation in other tissues. Healthy neurons maintain microglia in an inactive state via secreted and membrane-bound signals, including CD200, CX3CL1 (fractalkine), neurotransmitters, and neurotrophins [17▪▪]. If this control fails (e.g., as a result of neuronal injury or loss of regulatory signals), activated microglia may participate in a form of chronic neuroinflammation, which has been implicated in the pathoetiology of a number of neurodegenerative diseases [20▪▪].

There is recent, still limited, evidence indicating the involvement of neuroinflammation in bipolar disorder. A 2010 study reported that markers of

neuroinflammation were significantly upregulated in post-mortem frontal cortex from patients with bipolar disorder. In particular, those authors observed the activation of the IL-1 receptor (IL-1R) cascade involved in microglial activation. The same work found increased astroglial and microglial markers (glial fibrillary acidic protein, inducible nitric oxide synthase, c-fos, and CD11b), another evidence of microglial activation [22]. Recently, patients experiencing one or more manic/hypomanic episodes during the previous year were shown to have significantly higher levels of IL-1β in cerebrospinal fluid levels when compared with patients without a recent manic/hypomanic episode. This indicates a relationship between the presence of acute episodes and activation of the IL-1R cascade [23▪].

The mechanisms described above suggest microglial activation in bipolar disorder. Some forms of cognitive decline, including the one observed in bipolar disorder [24▪], involve remodeling or destruction of specific regions of neuronal dendrites in response to changes in synaptic activity, neurite dysfunction, or excess extracellular neurotransmitters. Microglia monitor synaptic activity and may contribute to the remodeling of impaired synapses [18▪▪]. In this vein, the activation induced by the IL-1R cascade could indicate not only an inflammation process but also a 'synaptic adaptation attempt' to cope with the insult caused by the acute episode (Fig. 1a).

SYSTEMIC TOXICITY

The understanding of severe psychiatric disorders as systemic conditions is not a recent trend. Since the publication in 2002 of an article that already has classic status [25], emphasis has been placed on early mortality due to natural causes and the burden related to medical comorbidities in patients with bipolar disorder [11▪,26▪] – parts of a spectrum that we have been calling systemic toxicity [27]. As we have earlier proposed, systemic toxicity consists of an increase in the levels of several peripheral markers implicated in bipolar disorder as mediators of allostasis (the adaptation by which living organisms maintain homeostasis) [7▪]. Inflammatory markers account for some of the primary components of this cumulative load. Table 1 [1▪,5▪,23▪,28▪,29▪▪,30▪,31▪] describes the primordial functions of cytokines and recent alterations found in bipolar disorder.

Most evidence supporting the implication of inflammation in the pathophysiology of psychiatric disorders comes from circulating inflammatory markers, especially TNF-α. Serum TNF-α levels seem to be elevated not only during acute episodes [28▪] but also in response to treatment with lithium. A significant increase in TNF-α levels has been observed in patients with a poor response to lithium when compared with those with a good response [29▪▪].

Additionally, studies have demonstrated that bipolar disorder is associated with both cytokine alterations and acute-phase reactants, such as hsCRP, produced by the liver in response to IL-1 and IL-6 [1▪,30▪]. Serum hsCRP levels are significantly higher in bipolar patients (in both acute mania and partial remission) when compared with controls [30▪]. Moreover, hsCRP levels are positively associated with hypomanic/manic symptoms [31▪]. Recently, investigators from the Psychiatric Center Copenhagen published an extensive systematic review and meta-analysis on cytokine alterations in bipolar disorder. The authors found that altered levels of TNF-α, soluble TNF receptor type 1 and soluble IL-2 receptor were most strongly associated with bipolar disorder [28▪].

ANTI-INFLAMMATORY POTENTIAL OF ESTABLISHED TREATMENTS AND CURRENT EVIDENCE ON NEW ADJUNCTIVE TREATMENTS

There is considerable preliminary evidence suggesting that traditional mood stabilizers modulate neuroinflammation. Very recently, lithium was shown to have neuroprotective activity in two preclinical studies [32▪▪,33]. In rat glial cells, pretreatment with lithium showed a significant anti-inflammatory potential, decreasing lipopolysaccharides-induced secretion of TNF-α, IL1-β, prostaglandin E (2), and nitric oxide. Similarly, in an intracerebral hemorrhage model, lithium reduced cell death, cyclooxygenase (COX) 2 expression, and reactive microglia in perihematomal regions in rats. Interestingly, valproate has also shown anti-inflammatory properties in preclinical models, modulating both systemic and central nervous system (CNS) responses [34▪]. Nevertheless, not enough clinical evidence exists to support that these would exert neuroprotective effects in general, and specifically through immune and inflammatory pathways in particular [35].

The adjunctive use of drugs with anti-inflammatory properties, such as omega-3 fatty acids (fish oil), COX inhibitors, minocycline, and statins, is another arena that has recently started to be explored [36▪▪]. Omega-3 are nutritionally important fatty acids that include α-linolenic acid (C18 : 3), docosahexaenoic acid (DHA, C22 : 6), and eicosapentaenoic acid (EPA, C20 : 5). A recent meta-analysis showed that EPA is a more effective component in the treatment of major

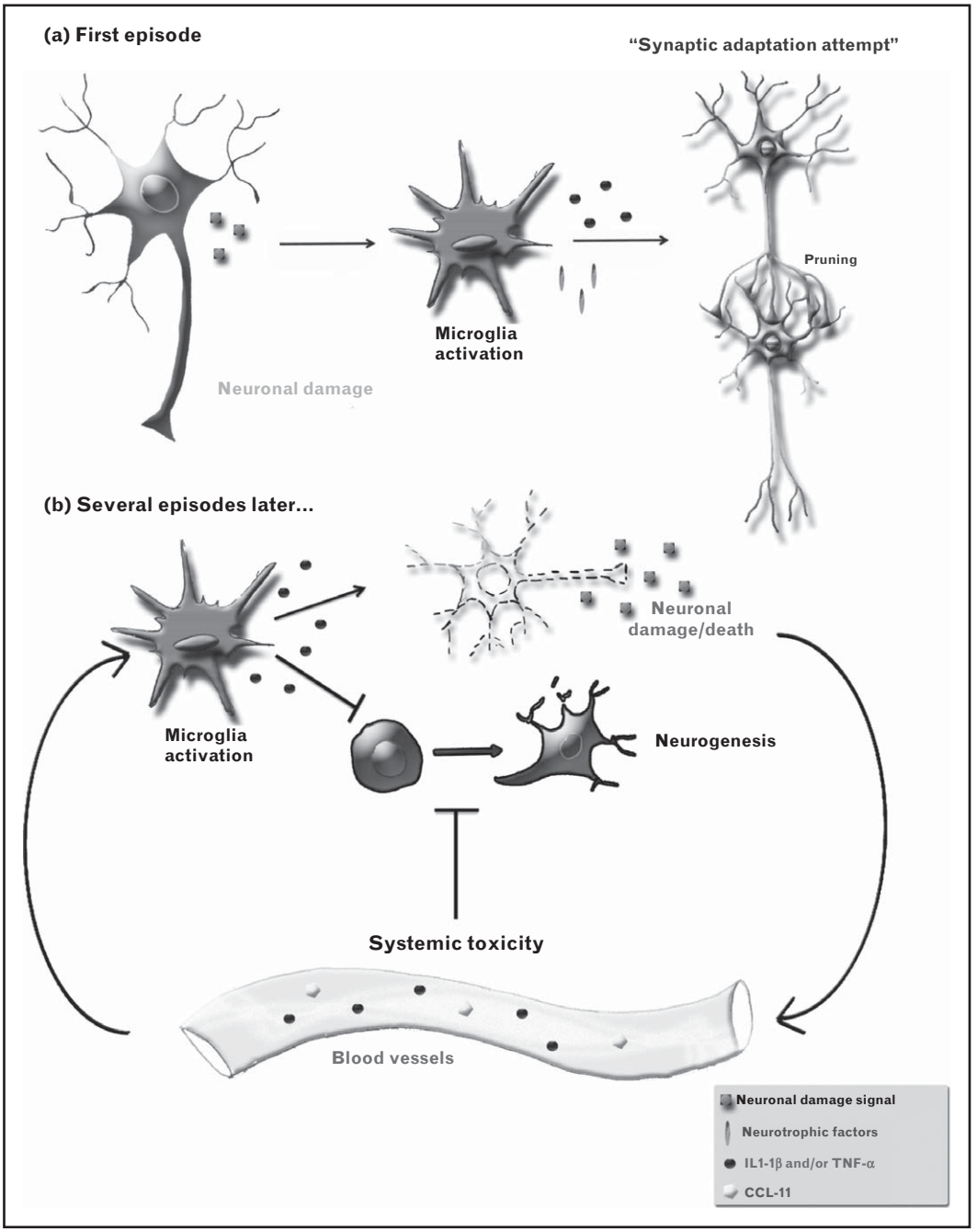

FIGURE 1. The hypothetical role of inflammation in the pathophysiology of bipolar disorder. (a) After the first acute episode, neuronal injury causes the release of damage-associated molecules that in turn activate the microglia. Activated microglia release both proinflammatory cytokines and neurotrophic factors. These molecules induce modifications of the synaptic environment by synaptic pruning, in a 'synaptic adaptation attempt' to cope with the insult caused by the acute episode. (b) After several episodes, the excessive production of proinflammatory cytokines, which exceeds the downregulatory capacity of the system in response to an acute induction, maintains the microglia in a constantly activated state. The constant presence of tumor necrosis factor (TNF) α and interleukin (IL) 1β in the extracellular medium inhibits neurogenesis in damaged neurons. Molecules associated with neuronal damage/death or failure of the negative feedback control system potentially perpetuate systemic toxicity. At the same time, CCL11 inhibits neurogenesis and peripheral cytokines may continue to activate microglial cells.

depressive episodes than DHA [37▪]. These molecules are supposed to compete for the biotransformation of inflammatory eicosanoids (such as prostaglandins and leukotrienes). In fact, competition for the biosynthesis of inflammatory mediators could be partially responsible for their anti-inflammatory effects

Table 1. Primordial cytokine functions and recent findings in bipolar disorder

Cytokine	Acronym	Major cellular source	Biological function	Inflammatory stimulus	Recent findings in bipolar disorder (compared with healthy controls)
Interleukin-6	IL-6	Macrophages, endothelial cells, T cells	Synthesis of acute-phase proteins, proliferation of B cells	Proinflammatory	Increased [28▪]
					Decreased [23▪]*
Tumor necrosis factor-alpha	TNF-α	Macrophages, T cells	Cellular apoptosis, synthesis of acute-phase proteins, neutrophil activation and inflammation	Proinflammatory	Increased [5▪]
Interleukin-1 beta	IL-1β	Macrophages, endothelial cells	Synthesis of acute-phase proteins, inflammation	Proinflammatory	Increased [23▪]*
CCL2	CCL2	Macrophages, endothelial cells	Mixed leukocyte recruitment		Increased [29▪▪]
CCL11	CCL11	Macrophages, endothelial cells	Eosinophil, basophil and TH2 recruitment	Proinflammatory	Increased [30▪]
CCL24	CCL24	Macrophages, endothelial cells	Eosinophil, basophil and TH2 recruitment	Proinflammatory	Increased [30▪]
CXCL10	CXCL10	Macrophages, endothelial cells	Effector T-cell recruitment	Proinflammatory	Increased [30▪]
CXCL8	CXCL8	Macrophages, endothelial cells	Neutrophil recruitment	Proinflammatory	Decreased [30▪]
Interleukin-10	IL-10	Macrophages, T cells	Inhibition of IL-12 (proinflammatory cytokine)	Antiinflammatory	Increased [5▪]
Interleukin- 1 receptor antagonist	IL-1Ra	Macrophages	Competitive antagonist of IL-1	Antiinflammatory	Increased [31▪]
Other					
Soluble tumor necrosis factor receptor 1	sTNF-R1	Macrophages, T cells	Low concentrations stabilize the activity of TNF-α. High concentrations may antagonize the biological effects of TNF-α	Antiinflammatory and proinflammatory	Increased [28▪]
Long pentraxin 3	PTX3	Macrophages, endothelial cells	Facilitates pathogen recognition by macrophages	Proinflammatory	Increased [29▪▪]
C-reactive protein	CRP	Hepatocytes	Activates the complement system	Proinflammatory	Increased [31▪]

CCL, chemokine (C-C motif) ligand; CXCL, chemokine (CXC motif) ligand; TH2, Type 2 helper T cells.
Data about cytokines collected from [1▪].
*Results from cerebrospinal fluid.

[38▪]. Although current evidence does not support the adjunctive use of omega-3 in the treatment of bipolar mania, some studies have demonstrated its efficacy in bipolar depression [39▪,40▪].

The antibiotic and anti-inflammatory effects of minocycline inhibit apoptosis by attenuating microglial release of proinflammatory cytokines IL-1β, TNF-α, and IL-6, while at the same time promoting release of anti-inflammatory cytokine IL-10. However, the efficacy of minocycline has not been formally tested in mood disorders [41▪▪]. Recently, a clinical trial with minocycline and aspirin was proposed and is currently underway [42▪▪].

Acetylsalicylic acid (ASA) irreversibly inhibits COX-1 and modifies the enzymatic activity of COX-2. COX-1 and COX-2 differentially modulate leukocyte recruitment during neuroinflammation. The clinical use of low-dose ASA has been primarily driven by its role as an antithrombotic and thrombolytic agent. Given the high rates of death from cardiovascular events in bipolar disorder, this action might be potentially advantageous in the management of bipolar disorder. Nevertheless, recent literature also supports the use of low-dose ASA in the management of the mood disorder itself, more specifically to ameliorate depressive symptoms [42▪▪]. The COX-2 inhibitor celecoxib was tested in the treatment of depressive or mixed episodes in bipolar disorder in a short-term randomized controlled trial [43]. That study showed some benefits of celecoxib in the treatment of depressive symptoms, but it remains unclear whether those benefits outweigh the risks at this point. Another trial is currently underway [36▪▪].

PROBLEMS WITH THE INFLAMMATORY SYSTEM OR WITH NEGATIVE FEEDBACK?

The immune system is a good example of how connections between the brain and the body can have multiple relevant facets. Communication with the peripheral immune system occurs via vagal afferents, circumventricular organs, and directly at the blood–brain barrier [44▪▪]. For instance, systemic administration of CCL11, a proinflammatory chemokine, may decrease adult neurogenesis and impair learning and memory in young mice [45▪▪]. Also, vagal afferent stimulation by systemic inflammation elicits 'sickness behavior' in healthy humans, for example, sleep and appetite disturbances, psychomotor slowing, and memory impairment [46▪]. At the same time, efferent processes from the CNS affect and regulate inflammatory response by inducing the secretion of glucocorticoids, epinephrine, norepinephrine, and α-melanocyte-stimulating hormone, all of which inhibit the production of cytokines. Another descending mechanism occurs via the vagal efferent arm, regulating cytokine production, controlling the immune response system, and preventing excessive inflammation [47].

Altogether, these lines of evidence allow us to consider the role of inflammation in the pathophysiology of bipolar disorder (Fig. 1). At first, different insults, perhaps caused by the acute episode itself, may trigger inflammatory signaling and microglial activation. These events can induce a proinflammatory environment that may change or damage surrounding neurons and synapses. Microglial activation may affect synaptic transmission through proteolytic modifications of the synaptic environment or by synaptic pruning (Fig. 1a). After several acute episodes, the negative feedback control system may fail, and systemic toxicity occurs. These alterations may be related to microglial senescence and their inability to perform normal activities, or to an excessive production of proinflammatory cytokines, exceeding the downregulatory capacity of the system in response to an acute induction [18▪▪] (Fig. 1b). This state of toxicity may contribute to a better understanding of bipolar disorder in which the management of the disorder does not depend only on the correct use of medications, but also on a number of other palliative measures, such as the control of comorbidities and of a persistent proinflammatory state.

CONCLUSION

The original question contained in our title still remains: is bipolar disorder an inflammatory condition? We believe it is not, or at least not a primarily inflammatory condition. On the basis of the data currently available, the inflammatory changes observed in bipolar disorder appear to be associated with disease progression rather than to integrate a causal model. Microglial activation and its role in the disorder are not yet completely understood and deserve further investigation. However, systemic inflammation does not seem to be the only key aspect of bipolar disorder. The inefficacy of anti-inflammatory drugs in the treatment of acute manic episodes does not necessarily mean that these patients will not benefit from this approach. Rather, inflammation may be one of the reasons why patients in more advanced stages of the disorder do not properly respond to treatment (i.e., as a result of disease progression). At present, clinicians should be aware from the outset that the early use of mood-stabilizing medication may help prevent comorbid conditions, ultimately resulting in better outcomes.

For patients in late bipolar disorder stages, there is a possibility that adjunctive treatments could ameliorate symptoms [48▪]. The adjunctive use of anti-inflammatory drugs, however, still needs to be formally tested.

Acknowledgements

L.S. is the recipient of a scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. P.V.S.M. is the recipient of a postdoctoral scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. Professor F.K. has received grant/research support from Astra-Zeneca, Eli Lilly, the Janssen-Cilag, Servier, CNPq, CAPES, NARSAD, and the Stanley Medical Research Institute; he has also been a member of the speakers' boards for Astra-Zeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier.

We thank MSc Dinler Amaral Antunes for his support in creating Fig. 1. We also thank MSc Gabriel Rodrigo Fries for reviewing and suggesting improvements to the article.

Conflicts of interest

There are no conflicts of interest.

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PARTE III

DISCUSSÃO E CONCLUSÕES

DISCUSSÃO

Dados clínicos, cognitivos e de resposta ao tratamento sugerem que o TB apresenta um curso progressivo, principalmente no caso dos pacientes inadequadamente medicados (Post, R. M., 2010). Dentre os diversos mecanismos que podem estar relacionados com a neuroprogressão, mecanismos epigenéticos são fortes candidatos. Estresse, abuso de substâncias e comportamentos do tipo depressivo podem induzir alterações epigenéticas relativamente estáveis (Tsankova *et al.,* 2007), podendo representar uma "memória" da vulnerabilidade ao episódio de humor, culminando em anormalidades comportamentais e neurobiológicas. Além disso, regulações transcricionais e alterações epigenéticas induzidas por mecanismos de sensibilização podem estar direcionando algumas alterações neurológicas vistas clinicamente, incluindo baixos níveis de BDNF e aumentos persistentes de CRH (do inglês *Corticotropic releasing hormone*) e de glicocorticoides (Post, R. M., 2010a, Post, R.M. *et al.,* 2010b).

No primeiro capítulo dessa tese, nós demonstramos que a anfetamina (AMPH), um potente psicoestimulante, é capaz de aumentar a atividade da enzima histona desacetilase (HDAC) no CPF de ratos. Esse aumento mostrou-se associado a indução de estereotipias comportamentais (hiperatividade) comumente vistas em modelos animais de mania. Demonstramos ainda, que a reversão desse comportamento está associada com a capacidade de fármacos como lítio (LI), VPT (valproato de sódio) e butirato de sódio (SB, do inglês *sodium butyrate*) em atenuar o aumento de atividade na HDAC. Outro achado interessante desse trabalho foi o fato de que não foram encontradas diferenças na atividade da HDAC nas células mononucleares periféricas, sugerindo que os efeitos de modulação da HDAC pela AMPH são principalmente centrais.

Diversos estudo apontam para o envolvimento da acetilação de histonas na neurobiologia dos transtornos de humor. Como já discutido no capítulo I, modificações de histonas estão associadas com processos de memória e aprendizagem, como por exemplo o aumento da formação de memória contextual em ratos tratados com SB (Levenson *et al.,* 2004). Outro trabalho

mostrou que a expressão da HDCA4 estava elevada em pacientes com TB em estado agudo de depressão, quando comparado ao grupo controle (Hobara *et al.,* 2010). Estudos *post mortem* também revelaram alterações na expressão gênica e proteica das HDAC 1, 2 3 5 entre pacientes com depressão maior, esquizofrenia e TB (Covington *et al.,* 2011, Hobara *et al.,* 2010). Recentemente, mostrou-se que o composto 60 (Cpd-60), um inibidor dos membros da família HDAC de classe 1 (que inclui a HDAC1 e 2), foi capaz de atenuar a atividade locomotora de camundongos após desafio agudo com AMPH. Essas alterações foram associadas com significativas modificações transcricionais em regiões comumente envolvidas na regulação do humor (CPF, núcleo *accumbens* e hipocampo), além de ser mediadas por modificações epigenéticas (aumento na acetilação de histonas nas regiões promotoras dos transcritos regulados positivamente) (Schroeder *et al.,* 2013).

A investigação da acetilação de histonas no contexto dos transtornos de humor pode fornecer novas pistas para o uso de tratamentos baseados em mecanismos epigenéticos. Nós observamos, pela primeira vez, que não somente os inibidores diretos de HDAC (VPT e SB), mas também o popular estabilizador de humor Li, foram capazes de reverter o aumento da atividade da HDAC induzido pela AMPH. Estudos anteriores mostraram que o Li por si só não é capaz de alterar a atividade da HDAC, visto pela inabilidade do mesmo em aumentar a acetilação de histonas. Entretanto, se usado em combinação com diferentes inibidores (i) de HDAC, promove potencialização da acetilação de histonas *in vitro* (Leng *et al.,* 2008), indicando uma via de sinalização comum entre os fármacos.

Estudos anteriores demonstraram a associação da inibição da HDAC com o aumento da expressão de fatores neuroprotetores, entre eles o BDNF (Kim, H. J. *et al.,* 2007a, Leng *et al.,* 2008). Surpreendentemente, no nosso modelo animal não houve alterações nem gênica nem proteica de BDNF no CPF, embora a AMPH tenha sido capaz de aumentar a atividade HDAC. Baseado nestas evidências, nós podemos supor que a modulação da HDAC no CPF está alterando a expressão de outros genes e não do BDNF. Apesar de evidências apontarem para mudanças nos níveis séricos de BDNF em pacientes com TB, ainda existem discrepâncias entre os estudos. É preciso considerar que

tanto em estudos animais como em estudos clínicos, algumas variáveis (por exemplo, tempo de tratamento, intervalo entre a administrações e doses) podem refletir diretamente nos resultados. Foi demonstrado que apenas o tratamento crônico com lítio é capaz de aumentar níveis proteicos de BDNF no córtex e no hipocampo de ratos (Fukumoto *et al.,* 2001). Outro estudo mostra que ocorre um pico na hiperacetilação de histonas no hipocampo de camundongos, 30 minutos após a injeção de SB, retornando a níveis basais 1 hora após a administração (Schroeder *et al.,* 2007). No nosso trabalho, os animais foram eutanasiados 2 horas após a administração dos fármacos, o que pode justificar porque os iHDAC sozinhos não inibiram a atividade da enzima. E por fim, apesar das doses usadas terem sido baseadas na faixa terapêutica, não podemos descartar que o uso dos fármacos em maior concentração poderiam ter induzido efeitos mais pronunciados nos níveis de BDNF dos que encontramos no nosso estudo.

Os resultados do nosso estudo pré-clínico sugerem que efeitos epigenéticos podem ser mediadores da neuroprogressão, enfatizando que fatores ambientais podem direcionar algumas das alterações neurobiológicas vistas no TB. Além disso, apesar de alguns efeitos epigenéticos poderem permanecer por toda a vida, o fato de que pelo menos parte deles pode ser amenizada por tratamentos farmacológicos nos dá esperanças para a criação de uma nova geração de farmacoterapias baseadas em epigenética.

Além de mecanismos epigenéticos, alterações em marcadores periféricos dos mecanismos de resiliência celular e inflamação também tem sido implicadas na neuroprogressão no TB. Uma maior vulnerabilidade celular pode ser vista pelos diversos estudos que mostram o aumento de processos associados a morte celular no TB (Andreazza *et al.,* 2007b, Benes *et al.,* 2006, Fries *et al.,* 2014). Quando uma célula morre, é estressada ou sofre algum tipo de dano ela acaba liberando ou expondo moléculas intracelulares chamadas padrões moleculares associados ao dano (DAMPs, do inglês *damaged- assossiate molecular patterns*) ou moléculas associadas à morte (Seong *et al.,* 2004). Essas moléculas tem a capacidade de modular o sistema imune, alterando a maturação e o *status* de ativação de células apresentadoras de antígenos (APCs) (Garg *et al.,* 2009). Pode-se classificar DAMPs

em vários subgrupos, baseados na localização celular ou no mecanismo pelo qual são liberados (Ver tabela 1).

O papel da inflamação na morte celular é limpar debris, estimular a substituição das células perdidas, detectar células mortas por agentes infecciosos, alertar o hospedeiro para a necessidade de defesa e, possivelmente, reforçar a barreira exógena contra a oncogênese (Zitvogel *et al.,* 2010). Normalmente os processos necróticos são acompanhados por processos inflamatórios, mas já se sabe que processos apoptóticos ou tentativas frustradas de lidar com o estresse celular também podem ativar a resposta imune (Krysko *et al.*). Respostas excessivas aos DAMPs podem facilitar o desenvolvimento de autoimunidade, bem como sepse. Um trabalho recente demonstrou que os indivíduos com TB tem um perfil pro-inflamatório Th1 maior, bem como uma diminuição do número de células T regulatórias (Treg) (do Prado *et al.,* 2013), o que pode indicar uma dificuldade em restaurar a homeostase após uma limpeza ineficiente de células apoptóticas remanescentes.

Considerando os diversos indícios de morte celular no TB, é apenas lógico deduzir que também poderia existir um aumento nos níveis de DAMPs nos pacientes com TB. No capítulo II, num estudo longitudinal de 16 semanas, nós mostramos que pacientes com TB não-medicados e durante o episódio agudo de humor (mania/depressão/misto) apresentam maiores níveis séricos de DAMPs. Mais ainda, esses valores são normalizados em pacientes que alcançam a remissão clínica, mas não naqueles pacientes que continuam sintomáticos. Com base nesses resultados, podemos sugerir que, num primeiro momento, todos os pacientes ativam vias de resiliência e sobrevivência celular como resposta à toxicidade sistêmica que ocorre durante os episódios agudos de humor. Entretanto, devido a fatores genéticos e epigenéticos, somente uma parte dos indivíduos seria capaz de gerar uma resposta suficiente para atenuar a morte celular e, consequentemente, a liberação de DAMPs.

Tabela 1. Diferentes DAMPs com suas características, distribuição celular e receptor associado.

	Característica	*Distribuição Celular*	*Receptor associado*
DAMPs associados às células			
HMGB1	Liga a cromatina	Todas as células	RAGE, TLR-2, TLR-4
Ácido Úrico (monocristais)	Catabolismo de purinas	Todas as células	TLR-2, TLR-4, CD14
DNA/ Cromatina	Nuclear	Todas as células	TLR-9, DAI, AI2, IIF-6
HSP70	Proteína de estresse celular	Todas as células	CD14, CD91, LOX-1, CD40
grp96	Proteína de superfície celular que se liga a adenosina	Todas as células	TLR-2, TLR-4, CD91, SREC-I, receptores *scavenger* da classe A
Galectinas	Lecitina citosólica que recruta monócitos	Leucócitos e endotélio	CD2 e outros com β-galactose
Tioredoxina	Enzima com atividade antioxidante	Todas as células	?
Proteínas S100	Proteínas Ligadoras de Cálcio	Vários Tipos celulares	RAGE para S100A12 e S100B
Catelicidinas	Efeito sinérgico com defensinas	Abundantes em neutrófilos	FPRL-1
Defensinas	Peptídeos citotóxicos microbicidas	Leucócitos	CCR6 e TLR-4
Adenosina e ATP	Energia Celular	Todas as células	Receptores P_1, P_2X e P_2Y
DAMPs associados às mitocôndriais			
Peptídeos mitocondrias que carregam o grupo N-formil	Origem procariótica	Mitocôndrias	FPR e FPRL1
HSP60	Chaperonina	Citoplasma e mitocôndrias	TLR-2, TLR-4, CD14
mtDNA	DNA Mitocondrial	Mitocôndrias	TLR-9
TFAM	Fator de Transcrição mitocondrial	Mitocôndrias	FPRL1
Citocromo C	Catalisa a transferência de elétrons entre os complexos III e IV da cadeia respiratória	Mitocôndrias	LPG
Cardiolipina	Fosfolipídio aniônico da membrana mitocondrial	Mitocôndrias	?
CPS-1	Proteína mitocondrial	Mitocôndrias	?

Abreviaturas: HMGB1, do inglês *high mobility box-1;* RAGE, do inglês *receptor for advanced glycation end products*; TLR, do inglês *toll-like repector*; HSP, do inglês *heat shock protein*; LOX-1, do inglês *lectin-like oxidized LDL receptor-1*; SREC-1, do inglês *scavenger receptor expressed by endhotelial cell-1*; CCR6, do inglês *chemokine (C-C motif) receptor 6*; FPR, do inglês *formyl peptide receptor*; FPRL1, do inglês *formyl peptide receptor 1*; ATP, Adenosina Trifosfato; TFAM, do inglês *mitochondrial transcription fator A*; CSP-1, do inglês *carbamoyl phosphate synthetase-1*; LPG, do inglês *leucine-rich alpha-2-glycoprotein-1*. Tabela baseada nos trabalhos de Rock e cols., 2008 e Krysko e cols., 2011.

É importante perceber que de um grande conjunto de DAMPs (Tabela 1), apenas cinco foram analisados nesse trabalho. Em particular, nós verificamos o aumento dos níveis séricos de DNA nuclear livre circulante (ccf nDNA, do inglês *circulanting cell-free nuclear DNA*) em pacientes com TB durante o episódio agudo de humor. Ácidos nucleicos circulantes (principalmente DNA dupla fita) tem um grande potencial pró-inflamatório, já que podem ligar-se tanto ao receptor TLR9 (do inglês *toll-like receptor 9*), ativando a via dependente de fator de diferenciação mielóide 88 (MyD88), como a sensores citosólicos, como o ausente em melanoma 2 (AIM2, do inglês *absent in melanoma 2*), DAI (do inglês *DNA-dependent activator of INF regulatory factors*) e a proteína IFN induzível 6 (IFII6, do inglês *IFN inducible protein 6*) (Sirisinha*,* 2011). Ambas estas vias podem culminar com a ativação do NF-κB através do complexo da cinase IκB (IKK), aumentando assim, a transcrição e a secreção de citocinas (como TNF-α, IFN-γ e IL–1), bem como a produção de espécies reativas de oxigênio (Ermakov *et al.,* 2013).

Além de ccf nDNA, nós também demonstramos no capítulo II, que os níveis de HSP70 HSP60 e HSP90α estão elevados em pacientes com TB durante o episódio agudo de humor, indicando possíveis disfunções sobre os “fatores de choque térmico" e, consequentemente, maior vulnerabilidade celular à condições estressantes. As HSP podem ser positivamente reguladas como uma resposta ao estresse celular, podem induzir a maturação de células dendríticas e podem aumentar as propriedades imunogênicas das células após ligação ao TLR4 (Spisek *et al.,* 2007). Por outro lado, a HSP70 e HSP90 podem também se ligar a receptores inibitórios, como o CD24, e bloquear os efeitos pró-inflamatórios (Chen, G. Y. *et al.,* 2009). Sendo assim, o mesmo sinal, dependendo do contexto, pode ativar e limitar a resposta inflamatória (Zitvogel *et al.,* 2010).

O próximo passo do trabalho foi avaliar se, nesse grupo de pacientes que respondeu ao tratamento, haveria possíveis biomarcadores periféricos (principalmente inflamatórios) que pudessem clarificar os mecanismos envolvidos na remissão clínica (capitulo III). Para isso, avaliamos três grupos de biomarcadores séricos: DAMPs específico de mitocôndrias (representado por Citocromo C); antioxidantes não enzimáticos (representado pelo ácido úrico); e, principalmente,

marcadores inflamatórios (representados por IL-1, IL-6, IL-10, TNF-α, IL-12p70, IL-17A, INF-γ, IL-13, GM-CSF, CCL-11 e prostaglandina (PG)E2). Como controle positivo de toxicidade nós usamos um grupo de pacientes com sepse, visto que já foi demonstrado altos níveis de DAMPs nesses pacientes (Rhodes *et al.,* 2012). Como esperado, pacientes com sepse apresentaram níveis elevados de diversos marcadores. Porém, apenas PGE2 se mostrou aumentada durante o episódio agudo em pacientes com TB, quando comparados aos controles saudáveis. As prostaglandinas são pequenas moléculas derivadas do AA produzidas por cicloxigenases (COX). A PGE2 é a prostaglandina mais abundante no encéfalo dos mamíferos e é conhecida por ter um efeito ambíguo: atua como um fator pró-inflamatório com atividade imunossupressora (Kalinski*,* 2012). Nos transtornos de humor, estudos do final da década de 80 mostraram várias alterações no metabolismo do AA e nos níveis séricos de PGE2 (Lieb *et al.,* 1983, Nishino *et al.,* 1989). Adicionalmente, estudos em animais mostraram que os estabilizadores do humor são capazes de regular negativamente os níveis de PGE2 em ratos (Rao *et al.,* 2009). Em nosso estudo, mesmo após a estabilização dos sintomas, não houve diminuição nos níveis de PGE2. É importante salientar que, esta prostaglandina, é capaz de induzir uma mudança no padrão da resposta imune de um perfil de células Th1 (pró-inflamatório) para um padrão menos agressivo mediado por células Th2 e Th17 (Kalinski*,* 2012). Como já mencionado anteriormente, pacientes com TB apresentam uma forte tendência ao perfil Th1 (do Prado *et al.,* 2013). Sendo assim, nós propomos que esses altos níveis de PGE2, mesmo após a remissão clínica, são uma tentativa de suprimir essa resposta Th1 e ativar uma resposta mais moderada e menos deteriorativa.

No nosso estudo observamos diminuição nos níveis séricos de TNF-α após o tratamento farmacológico. Apesar de não haver alteração durante o episódio agudo de humor, o TNF-α apresentou uma correlação negativa com os valores de YMRS, indicando que a diminuição nos níveis dessa citocina pode auxiliar na estabilização dos sintomas em pacientes com TB. Sendo um dos principais mediadores pró-inflamatórios, o TNF-α age em vias de neuroplasticidade, resiliência e sobrevivência celular, podendo induzir a morte celular apoptótica. Seus efeitos são influenciados por outras citocinas (pró- e anti-inflamatórias), que orquestram uma série de reações que podem levar ou

não a um estado agudo de inflamação. Junto com a IL-1β, eles representam os chamados mediadores inflamatórios primários que, ativando o fator de transcrição NF-κB, ativam a produção de outras citocinas, incluindo a IL-6, a IL-8 e o IFN-γ (Brietzke *et al.,* 2008). *In vitro*, os estabilizadores de humor tem sido capazes de reduzir os níveis de TNF-α em concentrações terapêuticas (Himmerich *et al.,* 2013).

Outro fator importante demonstrado no capítulo III foi o envolvimento do ácido úrico na remissão dos sintomas dos pacientes. Trabalhos anteriores já haviam demonstrado um maior dano oxidativo a proteínas e lipídios no soro de pacientes com TB (Banerjee *et al.,* 2012, Kapczinski *et al.,* 2011). Em nosso estudo demonstramos que o aumento dos níveis de ácido úrico solúvel parece acompanhar a remissão dos sintomas em pacientes, uma vez que maiores níveis níveis de ácido úrico foram associados com menor pontuação na YMRS. O ácido úrico é o produto final de oxidação no metabolismo de purinas, sendo excretado na urina. Esse composto é normalmente solúvel no interior das células, mas, uma vez no espaço extracelular, entra em contato com os altos níveis de sódio e pode formar microcristais (Bianchi*,* 2007). Estes microcristais apresentam grandes propriedades inflamatórias e podem ativar a produção de IL - 1β e IL - 18 (Martinon *et al.,* 2006). Se o ácido úrico for capaz de se manter solúvel no plasma e no soro, ele pode atuar como um forte agente de redução e um potente antioxidante, representando quase a metade da capacidade antioxidante destes fluidos (Maxwell *et al.,* 1997). Ao contrário dos nossos achados, uma associação significativa entre a melhora dos sintomas maníacos e a diminuição dos níveis de ácido úrico plasmáticos já foi demonstrada (Machado-Vieira *et al.,* 2008). Essa discrepância pode ser explicada pelo fato de que nossos pacientes não demonstraram aumento durante o episódio agudo quando comparados a controles saudáveis, indicando um mecanismo de sinalização e regulação mais específico. Além disso, os níveis de ácido úrico no período de remissão, apesar de estarem aumentados quando comparado à linha de base*,* não se encontravam acima dos níveis normais (níveis de referência para homens e mulheres são entre 1,5 e 7mg/dL; o nível mais alto que encontramos em um paciente com TB foi de 4,9 mg/dL).

Os resultados encontrados nos capítulos II e III levantam uma importante questão: o TB é uma doença inflamatória? No capítulo IV nós propomos possíveis respostas para essa questão. O reconhecimento de que a inflamação pode representar um mecanismo comum entre doenças tem sido estendido para incluir transtornos psiquiátricos, inclusive o TB. As citocinas podem acessar o SNC e interagir com quase todos os domínios fisiopatológicos relevantes aos transtornos de humor, incluindo o metabolismo de neurotransmissores, funções neuroendócrinas e plasticidade neuronal (Dantzer *et al.,* 2008). Além disso, vários estudos indicam que o estresse pode ativar uma resposta inflamatória periférica ou central. Por exemplo, estresse crônico, como discórdias matrimoniais, está associado com aumentos nos níveis de PCR e IL-6 (McDade *et al.,* 2006). Além disso, a ativação da microglia ocupa o papel principal na resposta do SNC à citocinas induzidas por estresse (Frank *et al.,* 2007).

Ao todo, essas linhas de evidência nos permitem considerar o papel da inflamação na fisiopatologia do TB. Nó propomos um modelo de integração entre sistemas onde, em primeiro lugar, diferentes danos (provocadas pelo episódio agudo em sí) induzem a liberação de DAMPs e a ativação microglial. Depois de ser ativada pela primeira vez por eventos estressores, a microglia é sensibilizada para o estresse e se mantem em um constante estado de pré-ativação (Frank *et al.,* 2007). A ativação microglial pode alterar as vias de transmissão sináptica através de modificações proteolíticas na matriz extracelular dos botões sinápticos ou através do *pruning* (poda) das sinapses. Nós acreditamos que essas modificações são uma tentativa de lidar com o insulto causado pelo episódio de humor (cortisol, citocinas inflamatórias, morte celular, DAMPs). Com a recorrência dos episódios de humor, o SNC perde o controle dos demais sistemas (endócrino e imune), que deveriam estar recebendo uma sinalização inibitória. Essa perda de controle pode estar relacionada à sensibilização da microglia ao estresse e à excessiva produção de citocinas pró-inflamatórias. A constante presença de TNF-α e IL-1β no meio extracelular acaba inibindo a neurogênese e danificando ainda mais os neurônios. Falhas no sistema de retroalimentação negativa periférica ou DAMPs potencialmente perpetuam a

toxicidade sistêmica, que ativa ainda mais a microglia. Assim, o ciclo vicioso se completa e já não necessita mais de um estímulo externo (como o episódio de humor) para se manter.

Nós propomos no capítulo IV que, as alterações inflamatórias vistas no TB, podem estar mais associadas com a progressão do transtorno ao invés de integrar um modelo causal. A ativação da microglia e seu papel na doença ainda não são completamente entendidos. Na tentativa de identificar padrões microgliais de ativação, nós realizamos um estudo piloto com um modelo animal de mania. Dados imunohistoquímicos preliminares indicaram a presença de uma leve microgliose no hipocampo de ratos *wistar* duas horas após a última administração de anfetamina (dados não mostrados). Entretanto, esses dados necessitam de uma análise mais profunda antes de maiores conclusões.

Por fim, nossos resultados apontam uma forte relação entre epigenética, morte celular, inflamação, toxicidade sistêmica e a patofisiologia do TB, ressaltando que diferenças individuais na resposta ao tratamento de pacientes com TB podem ocorrer ao nível de parâmetros bioquímicos e epigenéticos.

CONCLUSÃO

A partir dos resultados obtidos no presente trabalho, concluímos que:

- A AMPH é capaz de aumentar a atividade da enzima HDAC no CPF de ratos *Wistar*, sendo que esse aumento pode estar associado a indução de estereotipias comportamentais (hiperatividade) comumente vistas em modelos animais de mania. Ainda, devido a capacidade do LI, VPT e SB em atenuar a atividade dessa enzima após a administração de AMPH, propomos que a capacidade terapêutica desses fármacos está associada a inibição da HDAC no CPF. Em virtude de não encontrarmos diferenças na atividade da HDAC nas células mononucleares periféricas, sugerimos que os efeitos de modulação da HDAC pela AMPH são tecido-específicos.
- Pacientes com TB não-medicados e durante o episódio agudo de humor (mania/depressão/misto) apresentam maiores níveis séricos de DAMPs, em concordância com achados que mostram aumento de células apoptóticas no TB. Esses níveis são normalizados em pacientes que alcançam a remissão clínica, mas não naqueles pacientes que continuam sintomáticos.
- O ácido úrico e o TNF-α são possíveis marcadores de estado e a PGE2 pode ser uma biomarcadora de traço no TB, sugerindo importantes mecanismos de inflamação e de estresse oxidativo envolvidos na melhora dos pacientes após o tratamento clínico e farmacológico. Entretanto, mais estudos são necessários para validar a sensibilidade e a especificidade desses biomarcadores.
- Por fim, nós propomos que as alterações inflamatórias vistas no TB estão mais associadas com a progressão do transtorno ao invés de integrar um modelo causal.

Em conclusão, o presente trabalho ressalta que, no TB, os pacientes podem apresentar diferenças individuais nas respostas ao tratamento em virtude de particularidades a níveis

epigenéticos (inibição da HDAC) e bioquímicos (morte celular, DAMPs e inflamação), aumentando assim, a necessidade de farmacoterapias cada vez mais individualizadas.

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ANEXO I

EXPERT REVIEWS

Neurotrophins, inflammation and oxidative stress as illness activity biomarkers in bipolar disorder

Expert Rev. Neurother. 13(7), 827–842 (2013)

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Recent studies highlight the presence of systemic toxicity as an integral dimension of bipolar disorder pathophysiology, possibly linking this mood disorder with other medical conditions and comorbidities. This review summarizes recent findings on possible peripheral biomarkers of illness activity, with a focus on neurotrophins, inflammation and oxidative stress. The possible mechanisms underlying the systemic toxicity associated with acute episodes in bipolar disorder are also discussed. Finally, the authors outline novel therapies that emerge from this new research and the assessment of multiple biomarkers as a potential approach to improving management strategies in bipolar disorder.

Keywords: bipolar disorder • endoplasmic reticulum stress • glial dysfunction • illness activity • inflammation • mitochondrial dysfunction • neurotrophins • novel therapies • oxidative stress • peripheral biomarkers • systemic toxicity

Bipolar disorder (BD) is a severe chronic illness in which recurrent episodes of mania and depression alternate with periods of clinical remission (euthymia). BD has been commonly associated with significant disability, morbidity, and premature mortality [1,2]. The recurrence of acute episodes and illness progression often translate into worse long-term outcomes, for example, higher rates of clinical comorbidities, functional and cognitive impairments and lower responsiveness to treatment [3–6]. Moreover, patients with BD are at higher risk for developing a wide range of medical conditions, including cardiovascular and cerebrovascular disease, neurological disorders and metabolic syndrome [7].

One of the hypotheses that has been proposed to explain the mechanisms underlying the heavy medical burden and cumulative damage related to BD is the allostatic load theory [8–10]. According to this theory, the chronic activation of mechanisms to restore homeostasis after stressful conditions leads to wear-and-tear in the body and brain that has been called allostatic load [8,11]. These events are vital adaptive functions, but they may also promote maladaptive effects on brain plasticity, as well as on metabolic, immune, and cardiovascular pathophysiology, whenever mediators are excessive in number or remain active [12]. Recently, the allostatic load paradigm has been incorporated into a new concept of neuroprogression in BD, described as a pathological brain rewiring process-taking place when clinical and cognitive deterioration is observed as a result of disease progression [13]. In this sense, there is a growing interest in understanding the systemic pathophysiological mechanisms that contribute to dysfunction resulting from multiple mood episodes in BD, and especially in identifying the pathways associated with allostatic mediators involved in neuroprotection, oxidative stress and inflammation.

Within this scope, several studies have been performed to detect peripheral biomarkers that could work as indicators of cellular impairment and toxicity in patients with BD [14,15]. Different biomarkers could be associated with illness activity (indicating whether the illness is active or in remission), illness neuroprogression, or both. Of note, systemic markers have already been implicated in BD as mediators of allostasis [8,13,16]. These studies may be important in improving our understanding of illness activity

 10.1586/14737175.2013.811981 ISSN 1473-7175

and progression, and also in providing insights for new approaches to treatment and biomarkers.

The aim of the present communication is to review current evidence available on possible biomarkers of illness activity in BD. Special attention will be given to neurotrophins, inflammation and oxidative stress, as well as to the possible mechanisms whereby these metabolic routes are activated in acute mood episodes. Finally, the authors will outline emerging therapeutic opportunities in the field of BD. For recent reviews on the role of systemic pathophysiology and neuroprogression in BD, please see the reports of Grande *et al.* [9] and Fries *et al.* [17].

Peripheral biomarkers in bipolar disorder

There is considerable interest in incorporating biomarkers into psychiatry [18], using them as biological indicators to more accurately assess psychiatric conditions. A biological marker or biomarker is a feature that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (National Institutes of Health Definition Working Group, 2001) [19]. Biomarkers may be genes, proteins or other molecules, as well as morphological characteristics identified on the basis of physiological or biological mechanisms.

In addition to improving diagnosis, biomarkers could assist in predicting illness prognosis and the potential risk of developing a disorder, with valuable applications in monitoring illness status and responses to a therapeutic intervention or management strategy [20]. Moreover, biomarkers could help discover new therapeutic targets and are likely to contribute to uncover illness mechanisms in complex psychiatric disorders [21]. Particularly in BD, biomarkers may become useful tools in detecting illness activity associated with different mood states (a state marker) or in identifying specific features of the long-term course of illness (a trait marker) [22].

Only validated biomarkers can be used in the clinical setting. In other words, in order to be used clinically, a biomarker has to prove it is accurate, has high sensitivity and specificity for the expected outcome, is highly reproducible in standardized, cost effective, fast assays, is minimally invasive and acceptable to the patient, and also that it provides a clinically relevant result, with easily interpretable information [20]. Among the strategies adopted for biomarker discovery and application in BD, a great interest in peripheral biomarkers is observed: certain proteins found in peripheral blood may be transported through the blood–brain barrier and enter the CNS [23]. For instance, proteins of the neuregulin (NRG) family, that could enhance myelination of neurites, and brain-derived neurotrophic factor (BDNF) could enter the spinal cord and brain by a saturable receptor-mediated mechanism [24,25]. A recent study has found a correlation between cerebrospinal fluid and plasma BDNF levels in drug-naive, first-episode psychotic subjects [26].

This approach, using peripheral biomarkers, has several advantages, including easy collection, low cost, wide availability and feasibility for large-scale studies. Several peripheral markers have been studied as mediators of allostasis in BD [8,13]. Studies have focused primarily on biological pathways related to neuroplasticity in BD, including the role of neurotrophins, inflammation, oxidative stress and underlying processes.

Neurotrophins

Neurotrophic factors are small-secreted proteins that act in a range of biological functions related to interaction with different receptors, their local distribution and transport in the CNS [27]. Nerve growth factor was the first neurotrophin to be identified, by Levi-Montalcini in 1966. After that, several studies have discovered other neurotrophins, such as BDNF, glial cell-line derived neurotrophic factor (GDNF), neurotrophin 3 and neurotrophin 4/5 (NT-4/5), all playing major roles in synaptic plasticity, dendritic arborization, and neuronal connectivity. In addition, all have been shown to be altered in BD, as will be discussed below [8,28].

BDNF is the most abundant and widely distributed neurotrophin in the CNS and also the most studied one; current studies show that an altered expression of BDNF contributes to several disorders, including BD. A correlation between serum BDNF levels and other markers of CNS injury has also been suggested [29,30]. Moreover, a growing body of evidence points towards a relationship between peripheral BDNF levels and illness activity in BD.

Serum BDNF has been found to be reduced in BD during manic and depressive episodes when compared with euthymic patients and healthy controls, even in drug-free patients [31–34]. In unmedicated manic children and adolescents, a decrease in both mRNA levels of lymphocyte-derived BDNF and protein levels in platelets has been found in relation to healthy controls [35]. Despite some discrepancies between studies [15,36–39], meta-analyses have been performed to measure the effect size of differences in BDNF levels between patients in different mood states and controls [40,41]. The latest of these meta-analyses demonstrated that peripheral BDNF levels decrease during manic and depressive states and those patients who have experienced more episodes present lower BDNF levels.

In this context, the discrepancies found in the peripheral levels of BDNF could be associated with the difference on the methodology used in these studies. Further, this could be related to a study that verified that patients at different stages of the illness differ in the BDNF levels, showing changes in the late stages but not in early stages of illness compared with controls [42]. In addition, another study observed that BDNF levels were inversely related with age and length of illness [43]. Taken together, these findings suggest that the toxicity and cognitive impairment observed in patients with BD would be related to the number of episodes; each new episode would lead to further damage and therefore lower levels of BDNF [44].

Regarding treatment, mood stabilizers have been shown to increase BDNF levels [40]. Patient recovery from a manic episode after treatment with lithium has been associated with an increase in serum BDNF levels [45]. In the same vein, Rybakowski and Suwalska found that excellent lithium responders showed higher plasma BDNF levels compared with non-responders, and similar

levels compared with controls [46]. Other data have shown a significant increase in BDNF levels after lithium monotherapy for the management of manic episodes, suggesting a direct role of the regulatory effects of lithium on BDNF levels in mania [47]. A very recent open-label longitudinal trial in previously medication-free patients measured serum BDNF sequentially for 16 weeks. Relevantly, BDNF levels tended to increase with treatment, but only in patients acutely depressed at baseline. Those in manic or mixed episodes, in turn, showed a decrease in BDNF levels in the first weeks of treatment. These results suggest that manic and mixed episodes may be particularly toxic compared with depression, perhaps requiring a longer treatment time for BDNF to return to its baseline levels [9]. All these studies describing an important role of BDNF in the pathophysiology of BD have led to further research into novel targets of this neurotrophin, in addition to the already known therapeutic action of mood stabilizers.

Changes in other neurotrophic factors have also been reported in patients with BD, such as increased serum neurotrophin 3 levels during manic and depressive episodes compared with euthymic patients and healthy controls [48,49] and increased serum NT-4/5 levels in patients versus healthy controls, regardless of symptomatic state [50]. A recent work found increased GDNF plasma levels in euthymic patients compared with manic patients and healthy controls [51], although another study observed increased levels of GDNF in manic and depressive patients but not in euthymic patients when compared with control group [52]. Moreover, a previous study had found decreased serum levels of GDNF in patients during mania and depression, and increased levels after remission [53]. In line with this, a study has shown decreased levels of GDNF in remitted patients [54]. Taken together, these findings reinforce the implication of GDNF in the BD pathophysiology, but with a still unclear role. Additional evidence is needed to assess whether peripheral levels of GDNF are correlated with CNS levels of the neurotrophin [55].

Inflammation

Over the past few years, the number of publications focusing on immunological abnormalities involved in the pathophysiology of BD has grown substantially. Immune disturbances have been related to the severity and recurrence of mood episodes [42], illness progression [56,57], high rates of comorbidities [56] and drug effects [58,59].

Overall, mood episodes have been characterized as pro-inflammatory states [22], based on findings that show increased peripheral levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, during depressive episodes, and of IL-2, IL-4, IL-6 and TNF-α in mania when compared with euthymic patients and healthy subjects [60–62]. A recent review and meta-analysis has found that, during mania, patients show increased levels of TNF-α, soluble TNF receptor type 1 (sTNF-R1) and soluble IL-2 receptor (sIL-2R) when compared with healthy subjects, and high levels of sTNF-R1 and TNF-α when compared with euthymic patients [63]. Another recent meta-analysis has found that manic patients show increased TNF-α and sIL-2R levels and a trend toward higher sTNF-R1 concentrations when compared with euthymic patients, in addition to increased IL-1 receptor antagonist (IL-1RA) and a trend toward higher IL-6 levels when compared with healthy controls. Increased IL-10 levels were also found in patients in depression versus controls, however not reaching significance between acute phases. Finally, some cytokines, such as IL-1RA, have shown altered levels during euthymia compared with controls [64].

An aberrant inflammatory gene expression signature has also been demonstrated in monocytes from patients with BD; some of them were related to the cytokines most commonly correlated with BD, namely, TNF and IL-6 [65]. In this study, mRNA expression of chemokine ligand 2 and MAPK-6 was found to be significantly greater in monocytes during manic and depressive episodes. In addition, IL-6, PTX3, and cell survival/apoptosis signaling genes *EMP1* and *BCL2A1* were overexpressed during the depressive phase compared with euthymic patients, suggesting a differentiated activation of the inflammatory response system. The study mentioned above showed that the inflammatory state in monocytes from patients is familial, which means that similar results were found in the offspring of these patients, but the study did not evaluate the possible interaction between the existence of an aberrant proinflammatory gene expression signature and environmental factors. In a follow-up study, Padmos *et al.* have shown that the pro-inflammatory activation of monocytes in monozygotic and dizygotic twins is most likely due to shared environmental factors [66]. In the same direction, this group showed that schizophrenic patients also present an inflammatory activation of monocytes. This signature is such like the one found in patients with BD, an upregulation of ATF3, DUSP2, EGR3 and MXD1, and differs of BD signature in PTPN7 and NAB2 [67].

However, this increased inflammatory signature at the transcriptomic level has not been demonstrated at the protein level in patients with BD. In addition, Herberth *et al.* identified altered expression of seven pro-inflammatory and five pro-/anti-inflammatory protein analytes in the serum of euthymic patients [68]. This serum was used to treat peripheral blood mononuclear cells and was observed to decrease cell viability, pointing to an increased inflammatory response and likely cell death in the immune system of patients with BD.

In accordance with the aforementioned abnormalities, adolescents with BD have also been shown to present some type of immune disturbance. Preliminary findings obtained in a sample of adolescents with BD have indicated an association between severity of manic symptoms and high-sensitivity C-reactive protein (hsCRP), as well as a negative association between serum IL-6 and BDNF protein levels [32]. In the same vein, Padmos *et al.* demonstrated that the offspring of patients with BD also had an altered expression of inflammation-related genes [65].

Changes mentioned above serve as a source of information on the biological bases of BD, however these changes have not been found every time when tested and the same cytokines are not always implicated. Therefore, the use of inflammatory markers as biomarkers for predicting prognosis is still limited, but these findings could point to targets for treatment and monitoring of these patients in order to improve their quality of life.

The origin of immunological imbalance in BD is still unknown. However, some studies have pointed to factors such as sleep and circadian rhythm alterations, stress, immune activation by retrovirus infection or autoimmune dysfunction [69], unhealthy lifestyle, long-term exposure to drugs and some specific mechanisms that should be the focus of further studies [70,71].

Patients with BD are known to be at a higher risk of developing medical comorbidities, including cardiovascular disease, metabolic syndrome and diabetes [32,72,73]. The main connection between these disorders seems to be the presence of chronic systemic inflammation, or high levels of the inflammatory markers mentioned above. In fact, it is precisely because of the growing evidence suggesting chronic mild inflammation in the periphery and brain of patients with BD [60,74,75] that BD has been referred to as a multisystemic inflammatory disease by some authors [56,76].

A major confounding factor present in almost all studies designed to investigate inflammation in BD is the exposure of patients to drugs. Some studies have proposed that lithium can restore the inflammatory imbalance observed in BD [77]. Guloksuz *et al.* found a correlation between lithium response and TNF-α levels, where patients with a poor response to lithium showed increased serum TNF-α levels [78]. Further studies are needed to elucidate the relationship between inflammatory markers, treatment and the development of medical comorbidities in BD.

Oxidative stress

A growing body of evidence has demonstrated that oxidative stress plays an important role in the pathophysiology of BD [79–81]. Oxidative stress is defined as an imbalance between oxidant and antioxidant agents, potentially leading to cellular damage. Decreased levels of antioxidants or an increased production of pro-oxidants will result in an oxidative stress state, ultimately causing damage to macromolecules such as lipids, proteins (receptors and enzymes), carbohydrates and DNA [82].

The CNS is particularly vulnerable to oxidative injury, due to high oxygen consumption and hence the generation of free radicals, and also because of the relatively low antioxidant capacity of this structure [83]. Increased neuronal oxidative levels may have deleterious effects on signal transduction, plasticity and cellular resilience [84]. The antioxidant system is the major line of defense against oxidative stress, and can be divided into the enzymatic system, comprising the key enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx), and the nonenzymatic system [85]. The most important nonenzymatic cellular antioxidant and redox-regulator is glutathione (GSH), the brain's dominant antioxidant [86].

Under physiological conditions, mitochondria are a major source of free radicals (oxidants), produced in electron transport chain complexes [87]. In BD, the prevalent hypothesis is that a greater burden of oxidative stress is generated as a result of a disturbed mitochondrial function [13]; this hypothesis has been supported by postmortem studies reporting alteration in mitochondrial complex I activity [88] and decreased levels of GSH [89] in the prefrontal cortex of patients. Mitochondrial dysfunction in BD will be reviewed in another section.

Clinical studies have demonstrated systemic alterations in diverse oxidative stress parameters and antioxidant enzymes in patients with BD. Some of these changes have been related to mood episodes. For instance, Andreazza *et al.* reported that SOD activity is increased during manic and depressive phases, but not in euthymia [90]. This finding was confirmed by Machado-Vieira *et al.* [33] who showed increased SOD activity in unmedicated manic patients, as well as by Kunz *et al.* [91] who also reported increased SOD activity in acute phases of BD, but not during euthymia. However, others studies have shown decreased SOD activity in acute phases of BD and in the euthymia [92–94]. Furthermore, Raffa *et al.* did not found differences in the SOD levels in patients when compared with healthy controls [95]. Catalase activity was also decreased in euthymic patients [90,94,95], but increased in medication-free patients during mania [33]. These results suggest that alteration in antioxidant enzymes can change due the treatment and the phases of the illness.

An increased frequency of DNA damage possibly caused by oxidative stress has been shown in patients with BD and was correlated with severity of depression and manic symptoms [96]. Conversely, a meta-analysis investigating markers of oxidative stress in BD showed that thiobarbituric acid reactive substance, a marker of lipid peroxidation and nitric oxide, a reactive nitrogen species, were significantly elevated in all phases of BD [79], suggesting a relevant role of these parameters as possible biomarkers of illness traits.

Parenthetically, evidence from preclinical, clinical and epidemiological studies suggests a benefit for adjunctive antioxidant compounds in BD [97]. *N*-acetylcysteine, for instance, proved safe in two randomized trials as an adjuvant to mood stabilizers [98–100]. Preliminary data also suggest clinical effects of antioxidant compounds in mania and depression, and a particularly strong effect in patients with comorbid medical conditions [100,101].

Mechanisms underlying cellular alterations & toxicity in bipolar disorder

Mechanisms leading to reduced resilience to stressful conditions associated with acute episodes in BD probably involve cell signaling pathways and organelles that are typically responsible for maintaining cellular homeostasis, for example, the mitochondrion and endoplasmic reticulum (ER), and could affect cells from both the periphery and the CNS, for example, neurons and glial cells. Basic research experiments have significantly contributed to the understanding of these mechanisms, partially explaining the toxicity related to cumulative mood episodes in BD. In this section, the authors attempt to summarize some of the mechanisms underlying toxicity in BD.

Mitochondrial dysfunction & the role of chronic stress

A growing body of evidence has suggested a key role of mitochondrial dysfunction in BD [102]. Impaired energy metabolism, alterations in respiratory chain complex enzymes, altered levels of

cytoplasmic calcium, and downregulation of mitochondria-related genes are some of the abnormalities reported [102]. In addition, several postmortem, imaging, and genetic studies have pointed to an association between mitochondrial dysfunction and BD [103]. Mean cerebrospinal fluid lactate concentrations are significantly higher in patients when compared with controls, which indicates increased extra-mitochondrial and anaerobic glucose metabolism and is consistent with impaired mitochondrial metabolism in BD [41]. More recently, a decrease in attachment of hexokinase 1 to the outer mitochondrial membrane in postmortem brain parietal cortex tissue of individuals with BD has been reported, associated with increased activity of an alternative anaerobic pathway of glucose metabolism [104]. In the same vein, alterations in mitochondrial shape and distribution could be one of the underlying causes of energy dysfunction in BD, as shown in the prefrontal cortex of postmortem brains and in peripheral cells from patients with BD [103]. The role of mitochondrial dysfunction in BD is further supported by studies reporting that known mood stabilizers and antidepressants can enhance mitochondrial function [102,105,106]. For instance, lithium has been shown to stimulate the activity of mitochondrial respiratory chain enzymes at clinically relevant concentrations [107].

To a greater extent, abnormalities may be associated with the consequences of chronic exposure to stress, which seems to play a role in the pathophysiology of BD [108]. The stress hormone axis, more commonly known as the hypothalamic–pituitary–adrenal axis, is clearly altered in mood disorders, as suggested by the high number of patients with BD that inefficiently suppress cortisol release on the dexamethasone suppression test [109]. This deficiency of the hypothalamic–pituitary–adrenal axis results in a feed-forward production of cortisol in response to stress and in a decreased ability to return to resting levels once stress exposure is ceased [110]. As a consequence, patients with BD in the three phases of the disorder present similarly increased levels of cortisol, higher than those observed in controls [111]. These increased cortisol levels may have important long-term consequences in patients. For instance, *in vitro* and animal model studies have shown that chronic stress and chronic exposure to glucocorticoids can induce mitochondrial dysfunction, causing reductions in oxygen consumption, mitochondrial membrane potential, and calcium holding capacity and ultimately leading to apoptosis [112,113]. Glucocorticoids may also aggravate inflammation and induce toxicity in the CNS, making neurons less capable of removing glutamate from the synapse and quenching free radicals [114]. In addition, neuronal toxicity and damage could be generated by an increase in synergists of inflammation, oxidative stress and mitochondrial dysfunction [115].

Altogether, the authors hypothesize that some of the impairments in mitochondrial functions in patients with BD are induced and further stimulated by chronic stress. As a consequence, dysfunctional mitochondria are likely to impair cellular resilience to environmental stimulus, ultimately inducing activation of caspases and apoptosis. Once dead, these cells may end up releasing immunostimulatory molecules and therefore induce alterations in inflammatory markers. These alterations may be then responsible for detrimental effects on peripheral cells, possibly inducing apoptosis and completing a vicious cycle of peripheral toxicity and reduced cellular resilience.

ER stress

The ER plays a central role in Ca^{2+} storage and signaling, and also in the synthesis, folding and quality control of secretory and membrane proteins [116]. Alterations in the ER luminal environment, such as changes in the redox state and in calcium homeostasis, nutrient deprivation, or defects in protein post-translational modifications, may affect the function of this organelle and subsequently result in accumulation of unfolded proteins. This condition is known as ER stress, and the cellular response to this condition is called unfolded protein response (UPR), an adaptive physiological process in which cells activate protective mechanisms to restore homeostasis in the ER. Prolonged ER stress (e.g., when UPR is not sufficient to restore the balance) leads to cell death [117,118].

Some studies have suggested an involvement of UPR dysfunction in the pathophysiology of BD. For instance, a decreased response of XBP1 (a transcription factor that induces the expression of ER chaperones) and CHOP (a transcription factor that induces ER stress-induced apoptosis) was found in lymphoblastoid cells from patients exposed to two ER stress inducers [119]. Other findings have confirmed these results, reporting a reduction in stress-induced splicing of XBP1 and in the expression of GRP94 (another ER chaperone) in patients with BD [120]. Moreover, pharmacological evidence suggests that mood stabilizer valproate modulates ER stress response [121–123]. In a recent study, lymphocytes from patients with BD, in contrast to healthy controls, failed to induce UPR-related proteins and presented higher cell death levels in response to *in vitro*-induced ER stress, suggesting that this dysfunctional response to ER stress may reflect an increased cellular susceptibility [124].

Taken together, these findings suggest that patients with BD show a dysfunctional ER stress response, inappropriate and insufficient to maintain homeostasis. This impaired response to ER stress may be related to several neural function impairments reported for these patients, given that UPR components are also involved in neural development and plasticity, maturation and transport of several receptors and calcium signaling [125–127].

The ER is closely linked with mitochondria, both morphologically and functionally; Ca^{2+} exchange is possibly the main way of communication between both organelles [128]. ER-derived Ca^{2+} signals modulate mitochondrial bioenergetics. As a result, alterations in ER–mitochondria interactions, such as changes in cellular Ca^{2+} levels, influence the regulation of cellular metabolism and could cause mitochondrial dysfunction, metabolic imbalance and ultimately lead to cell death [129]. Of note, changes in intracellular calcium levels are a consistent finding in BD [130].

Harmful crosstalk between both organelles has also been shown to be involved in oxidative damage [131]. Taking into consideration the prolonged ER stress and mitochondrial dysfunction observed in BD, the disruption of ER–mitochondria interactions may

potentially be responsible for metabolic alterations and peripheral toxicity associated with the disorder. ER stress may also be related to neurotrophic pathways [132,133] that may contribute to maintaining oxidative damage and systemic inflammation in BD, as these processes are intimately interrelated [134,135].

Glial alterations

In 1858, Rudolf Virchow described glial cells as a connective tissue that binds nervous elements together [136]. As we know today, the role of these cells goes far beyond: glial cells are functional components of the nervous system. Sometimes called neuroglia, some of their functions include maintaining homeostasis (astrocytes), forming myelin (oligodendrocytes), and providing support and protection for neurons in the brain (microglia). Glial cells are capable of responding to changes in the cellular and extracellular environment, and, possibly through a glial network, have communication skills that complement those of the neurons [136]. Given the fact that these cells play an important role in the CNS, it is natural to think that they will also play an important role in the establishment and development of neurological disorders. Indeed, several studies have demonstrated alterations in glial cells in psychiatric disorders, including a decreased glial density in the amygdala of patients with major depression [137] and upregulation of extracellular matrix proteins in astrocytes of the amygdala and entorhinal cortex of schizophrenic patients [138]. More directly in BD, the results in the last 5 years are scarce.

Histological observations as well as imaging studies support findings of myelin abnormalities and glial alterations in BD [125]. Oligodendrocytes express transferrin, an iron mobilization protein that acts as a trophic and survival factor for neurons and astrocytes, pointing to another important function of oligodendrocytes in addition to myelination [139]. A postmortem study has shown that transferrin is underexpressed in the internal capsule of patients with BD; in contrast, two astrocyte-associated genes (*GFAP* and *ALDH1L1*) showed higher mean levels in all brain regions [140]. These results could indicate an impaired functioning of oligodendrocytes and some degree of astrocytosis (increase in astrocyte markers). Another study has reported that astroglial and microglial markers (glial fibrillary acidic protein, inducible nitric oxide synthase, c-fos and CD11b) were significantly upregulated in the postmortem frontal cortex of patients with BD, in particular the IL-1 receptor (IL-1R) cascade involved in microglial activation [141]. Microglia are the brain resident macrophages, which become activated in response to tissue damage or brain infections [142]. Moreover, the fact that neuregulin (*NRG*), a gene involved in oligodendrocyte development and myelination of the CNS, is located at one of the genetic loci for BD [143] is another indicator that glial alterations deserve further attention. In fact, it is possible that glial dysfunction in BD could result in abnormal neuronal–glial interactions, as already reported for mania [144].

We speculate that the abnormalities described above could be interrelated, affecting cellular resilience and function both in the periphery and in the brain of patients with BD. In line with previous hypotheses [8], there is likely a set of complex, interacting processes occurring in BD that could lead to cell endangerment and be related to the toxicity found in patients during acute episodes [15]. In order to better understand the mechanisms underlying toxicity in BD, further studies addressing the association between these processes and mood states are required.

Novel therapies for bipolar disorder

In light of the pathways known to be implicated in illness activity, novel therapies can be designed and proposed for a better management of BD. Thinking of the more immediate future, interesting alternatives may involve adjuvant therapies that act on the pathways mentioned in this review [14,15]. Some of these agents, with antioxidant, anti-inflammatory and neuroprotective effects, will be described in more detail below.

N-acetylcysteine (NAC), a precursor of GSH, has been shown, in both basic and clinical studies, to attenuate oxidative stress, modulate inflammation and act on neurogenesis and glutamatergic and dopaminergic pathways [59,99]. Supplementation of conventional treatment for BD with substances that act on oxidative stress has been investigated in clinical trials. NAC treatment adjunctive to usual medication for BD in the maintenance phase significantly improved depressive symptoms, quality of life and functioning in a double-blind, randomized, placebo-controlled trial with large effect sizes [99]. A secondary exploratory analysis revealed that adjunctive NAC showed promising effectiveness for participants with a syndromal diagnosis of bipolar depression [100]. More recently, a double-blind, randomized, placebo-controlled trial investigating the maintenance effects of NAC failed to find significant differences in recurrence or symptomatic outcomes during the maintenance phase [98]. Further randomized trials assessing adjunctive NAC for BD are required to more reliably determine the effect size of this treatment approach.

In addition to influencing the redox state, the neuroprotective properties of NAC may be associated with its ability to induce neurogenesis, which is likely related to mitochondria-protective mechanisms [145]; also, the modulating effects of NAC on inflammation [146] may be fundamental for its efficacy as a mood-stabilizing agent, considering the already described relevance of systemic inflammation in BD pathophysiology [15]. Therefore, even though very few studies have investigated the use of anti-inflammatory agents as an adjunct therapy for BD, inflammatory pathways seem to be another group of potential new therapeutic targets for the development of more effective treatments for BD. Conventional mood stabilizers have been described to have effects on both pro- and anti-inflammatory cytokines [78,147]. Among anti-inflammatory drugs, cyclooxygenase-2 (COX-2) inhibitor celecoxib was studied in a double-blind, randomized, placebo-controlled study as an adjunct in the treatment of patients with BD during depressive or mixed episodes. Treatment with celecoxib was associated with a more rapid improvement of depressive symptoms after 1 week compared with placebo, but the difference was statistically significant only for subjects who completed the full 6-week trial. This

finding suggests a potential antidepressant effect of COX inhibitors [58]. In this context, studies have demonstrated that mood stabilizers approved for the treatment of BD decrease expression of markers of the rodent brain arachidonic metabolic cascade, and reduce excitotoxicity and neuroinflammation-induced upregulation of these markers [148]. Recent papers demonstrating neuroinflammation, excitotoxicity [141] and upregulated arachidonic acid metabolism [149] in the postmortem brain of patients with BD support the hypothesis of altered arachidonic acid cascade in BD.

Another compound currently under investigation is minocycline, a tetracycline antibiotic that crosses the blood–brain barrier and has shown antioxidant, anti-inflammatory and neuroprotective effects [150]. Given that these pathways overlap with the pathophysiological mechanisms observed in BD, the use of minocycline has been pointed out as a potential adjunctive treatment. More specifically, minocycline inhibits microglia-mediated release of proinflammatory cytokines IL-1b, TNF-α, IL-6 and p38, and promotes the release of anti-inflammatory cytokine IL-10 [151]. It is also an effective scavenger of reactive oxygen species and protects against glutamate-induced excitotoxicity [152]. Case reports of individuals with psychiatric disorders have shown benefits of minocycline treatment for the severity of symptoms. Currently, a clinical trial is testing the efficacy of minocycline and/or aspirin in the treatment of bipolar depression and evaluating the anti-inflammatory effects of these compounds [153].

Supplementation with ω-3 polyunsaturated fatty acids (ω-3 PUFAs) has also been considered a potential new treatment for BD, as these fats have shown neuroprotective and antioxidant capacity in animal models [154]. A recent review of clinical trials using nutraceuticals in combination with standard treatment for BD has shown that ω-3 PUFAs improved bipolar depression symptoms [155]. The BDNF signaling pathway is one of the possible mechanisms of action by which ω-3 PUFAs mediate mood regulation in patients with BD [156]. Further double-blind, placebo-controlled, randomized clinical trials with long follow-up periods and adequate power-effect sizes are needed before we can gain a better understanding of this relationship and of the therapeutic role of ω-3 PUFAs in BD.

Neurotrophic factors are emerging as promising therapeutic targets in BD. Lithium, the classical mood stabilizer, has been shown to be effective in restoring peripheral BDNF levels in patients with BD [40,47]. In this sense, studies that attempt to prevent, treat and reverse molecular impairments are interesting therapeutic avenues for novel and improved therapies in BD [157]. In particular, delivery of neurotrophic factors from biomaterial scaffolds seems to be a promising area of research for the treatment of any disorder affecting the CNS. This drug delivery system allows to control the site and time of release of therapeutic agents, ensuring that biologically active agents, for example, neurotrophic factors, will be transported to the desired location to help treat a disorder [158]. In a recent review, the advantages and challenges associated with different drug delivery systems were evaluated, and the possibility to combine drug delivery systems with gene therapies was raised, suggesting that the drug delivery device could be adjusted to provide a controlled release of neurotrophic factors [159].

Regarding the mechanisms of action of the mood stabilizers traditionally used for the treatment of BD (lithium and valproic acid), hypothesis involving pathways discussed in this review are often highlighted. A recent work has reviewed preclinical findings showing that these drugs, in addition to other roles, regulate the transcription and expression of factors involved in neuroprotective, neurotrophic and anti-inflammatory effects. Moreover, oxidative stress pathways and cell survival signaling cascades may further underlie beneficial actions of these already established treatments [160].

In summary, the identification of specific therapeutic targets commonly modulated by these drugs may reveal new avenues for the effective use of add-on therapies, with the primary aim of treating acute mood episodes and preventing their recurrence.

Peripheral biomarkers & illness activity in bipolar disorder

As discussed above, an increasing body of evidence points to changes in neuroplasticity, oxidative stress and inflammation pathways in BD, mainly during mood episodes. However, these peripheral biomarkers have usually been investigated individually, contrary to the proposal that single biomarkers are unlikely sufficient to identify complex disorders. Rather, research should be geared towards sets of biomarkers, reflecting different processes implicated in a given condition [18,20].

To evaluate these biomarkers simultaneously, Kapczinski *et al.* conducted an *en bloc* assessment of a set of targets related to oxidative stress, neurotrophins and inflammation, all previously described as individual biomarkers of mood episodes in BD. The results demonstrated significant correlations among most biomarkers, which were then used to extract a systemic toxicity index. Patients in manic and depressive episodes showed higher systemic toxicity than euthymic patients and healthy controls, but lower systemic toxicity was seen when compared with patients with sepsis ('positive' control group for extreme peripheral illness; Figures 1 & 2) [14,15].

The findings above associating acute episodes with significant systemic toxicity in BD corroborate the idea that BD can be seen as a multisystemic illness of which peripheral pathophysiology is a major component [1]. However, these data alone are unable to explain how peripheral changes correlate with brain changes. The brain coordinates all physiological processes and is therefore sensitive to systemic damage [12]. Of note, central and peripheral pathophysiology could be connected in pro-oxidant states [161], possibly via changes in blood–brain barrier permeability. Peripheral toxicity has been shown to significantly alter brain oxidative stress [162]. Indeed, as mentioned above, there may be a link between inflammation, oxidative stress and neuroplasticity pathways in BD. For instance, inflammation has been demonstrated to cause oxidative stress through activation of calcium-dependent proteins and direct inhibition of the mitochondrial electron transport chain [163]. Changes in oxidative status, in

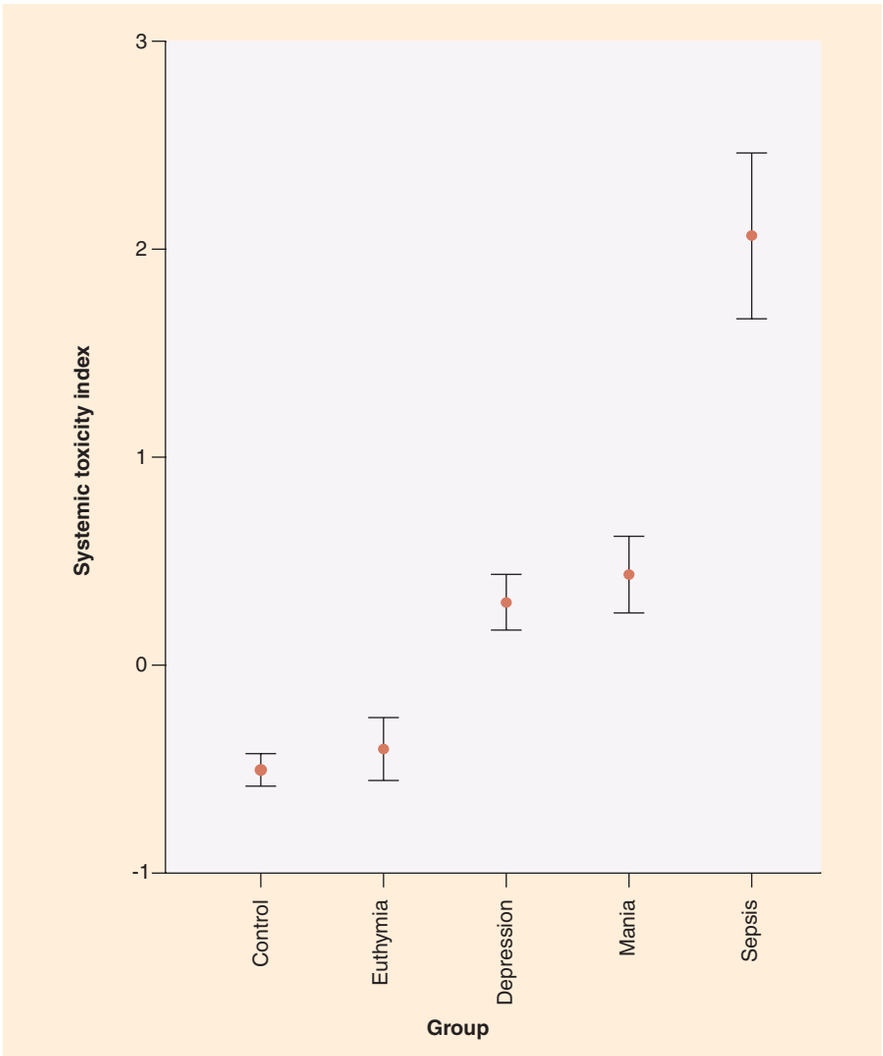

Figure 1. Systemic toxicity index to assess peripheral changes in mood episodes. To evaluate the peripheral toxicity associated with illness activity in patients with bipolar disorder, Kapczinski et al. conducted an en bloc assessment of a set of targets related to oxidative stress, neurotrophins and inflammation, all previously described as individual biomarkers of mood episodes. These peripheral biomarkers were measured in different acute mood states and in healthy subjects. Moreover, they also were evaluated in patients with sepsis ('positive' control group for extreme peripheral illness) with the aim of highlighting the relevance of potential changes between groups. More specifically, the biomarkers assessed were neurotrophins (brain-derived neurotrophic factor, neurotrophin-3), oxidative stress markers (protein carbonyl content, thiobarbituric acid reactive substances, and total reactive antioxidant potentials) and inflammatory markers (IL-6, IL-10 and TNF-α). The results demonstrated significant correlations among most biomarkers, which were then used to extract a systemic toxicity index. Patients in manic and depressive episodes showed higher systemic toxicity than euthymic patients and healthy controls; however, it was lower when compared with patients with sepsis. Figure reproduced with permission from [15].

turn, may be mechanistically associated with the reduced levels of BDNF observed in patients during acute mood episodes [164].

In summary, peripheral biomarkers have been consistently demonstrated to differentiate between patients with BD in manic or depression episodes and euthymic subjects. Whereas changes in one single biomarker usually have small effect sizes, the assessment of multiple biomarkers, especially primary mediators, could be a practical approach to improving diagnostic strategies and promoting earlier interventions [16,18].

Expert commentary

This review highlights the systemic toxicity related to acute mood episodes in BD and discusses possible mechanisms underlying these processes. Clinical and preclinical research gives overall support to the view of illness episodes as toxic to multiple elements in the body. Regarding the use of peripheral biomarkers as means for assessing illness activity, promising candidates at the moment can be subsumed in three main general areas: oxidative stress, inflammation and neurotrophins, in particular BDNF. These three groups do not yet meet the empirical characteristics of a traditional biomarker; however, they are relevant inasmuch as they provide information on the pathophysiology of BD and on illness activity. The assessment of systemic toxicity through a set of peripheral biomarkers may facilitate understanding of the body and brain damage associated with recurrent mood episodes and of the way it affects illness management.

Perhaps the most relevant upshot of having validated illness activity biomarkers would be the identification of biological features that indicate either the onset of an episode before specific symptoms occur or the lingering of illness activity despite an apparent response. Another potential application could be the detection of early response, before symptom resolution. Peripheral biomarker alterations in an acute mood episode could follow three different patterns, all of which would be powerful tools in guiding therapy (Figure 3).

Regarding new therapeutic strategies, preliminary evidence supports a role for novel adjunctive therapies in modulating neurotrophic, inflammatory, oxidative and apoptotic processes. Potential neuroprotective agents are currently available, but further clinical trial data are needed, as is information regarding which subgroups would benefit most from such interventions. Furthermore, in view of the high comorbidity rates observed in BD, there has been a push towards understanding the mechanisms underlying acute toxicity and illness activity. This perspective is the rationale behind an approach where the validation of

novel biological indicators will enhance the clinical strategies traditionally employed.

Five-year view

Well-documented studies evaluating potential peripheral biomarkers in BD have reported disturbances in inflammatory, neurotrophic, and oxidative stress markers in patients versus healthy individuals. However, pertinent questions remain about the translational applications of biomarkers in BD within the next years. It is expected that, in the future, translational approaches will be applied to the diagnosis and treatment of BD, using peripheral biomarkers to predict outcomes and identify high-risk individuals. This could guide the planning of more personalized clinical strategies and help monitor treatment interventions. In addition, a better understanding of illness activity mechanisms could advance the development of novel and more effective treatments. If changes in biomarkers can be reversed with treatment, we could ultimately consider that some pathological mechanisms are alterable, and thus allow interventions and secondary prevention (especially of the deleterious effects associated with multiple episodes).

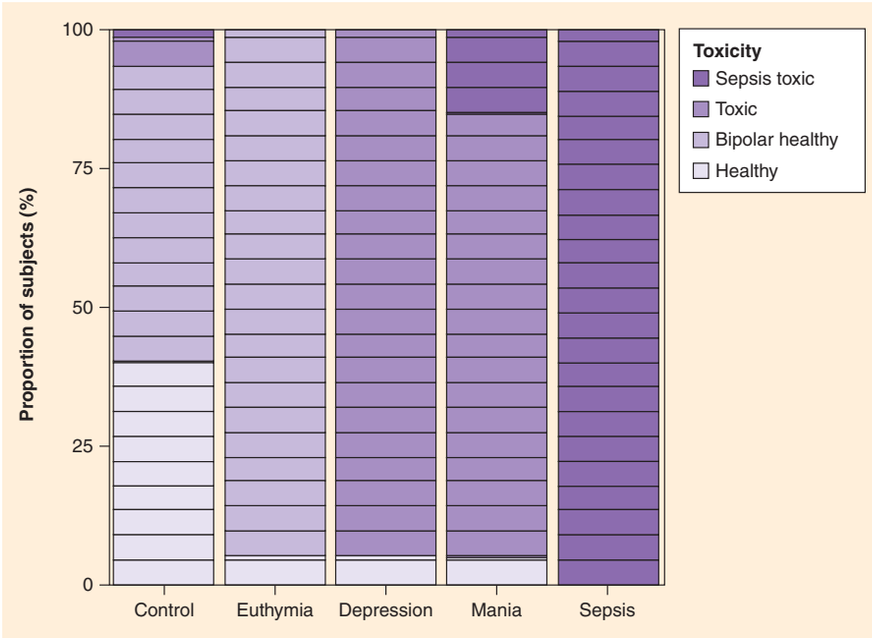

Figure 2. Peripheral biomarkers and illness activity in bipolar disorder. The graph shows the proportion of study subjects classified into four different categories: the category deemed most toxic, including all septic patients, was named 'sepsis toxic'. The less toxic category, which contained mostly healthy controls, was termed 'healthy'. The category that had serum biomarker levels in the toxic direction and included the patients in mood episodes was called 'toxic'. The last category was intermediary in terms of serum biomarkers and mostly contained euthymic patients, and was termed 'bipolar healthy'. Figure reproduced with permission from [14].

Future studies assessing biomarkers in large-scale, prospective cohorts (for an increased statistical power) and testing candidate biomarkers for sensitivity and specificity (to address overlaps with related disorders) will be quite valuable in determining the applicability of these biological markers in different rigorous scientific approaches [22]. In the past few years, much research has been undertaken to better understand individual biological markers related to the pathophysiology of BD; in the 5 years to come, an important direction will be measuring and comparing several biomarkers together, that is, not only the levels of each biomarker but also the correlations between them. Of note, several of these systemic toxicity mechanisms do not seem to be unique to BD, but may also be present in other psychiatric disorders presenting alterations related to illness activity (in cases of acute episodes followed by euthymia, such as in major depressive disorder or schizophrenia). However, the combination of peripheral alterations may differ between pathologies. For instance, neurotrophic alterations seem to go in an opposite direction in schizophrenia compared with BD [165]. In addition, inflammatory markers are different among different diagnosis and populations, which suggests peculiar means of activation of inflammation associated with specific disorders [166–168].

The common limitations of clinical studies will become an even more pressing issue in the investigation of more rigorous biomarkers for BD. For instance, until now, most research has been conducted with chronic patients treated for BD at tertiary care centers. Therefore, further studies, with other groups of patients with BD and other medical and psychiatric conditions, are required to increase the representativeness of the findings and to evaluate the effects of long-term medication use. In addition, studies conducted in community samples will be interesting to study individuals that are not usually seeking treatment at these healthcare facilities, thus avoiding a selection bias. Finally, among the clinical samples to be investigated, children, adolescents and young adults with BD are a group of great interest: evaluation of peripheral biomarkers in these individuals could contribute to a better understanding of primary illness changes [74] and some neurodevelopmental aspects, focusing on early interventions and, especially, on prevention attitudes.

Longitudinal studies will be able to confirm mood state-related findings and the hypothesis that these indices of peripheral abnormalities are related to course of illness, cognitive/functional impairment, and medical burden. Prospective studies assessing a set of measures, in turn, will be relevant to determine whether these peripheral biomarkers of illness activity may predict course of illness or medication response. In either way, the utility of these biomarkers will have to be validated via assessment of peripheral biological changes following specific therapies, for example, with anti-inflammatory or antioxidant agents [70,169], and treatment efficacy will have be evaluated based on mental health outcomes. If the biological changes suspected to occur during mood episodes are confirmed, novel

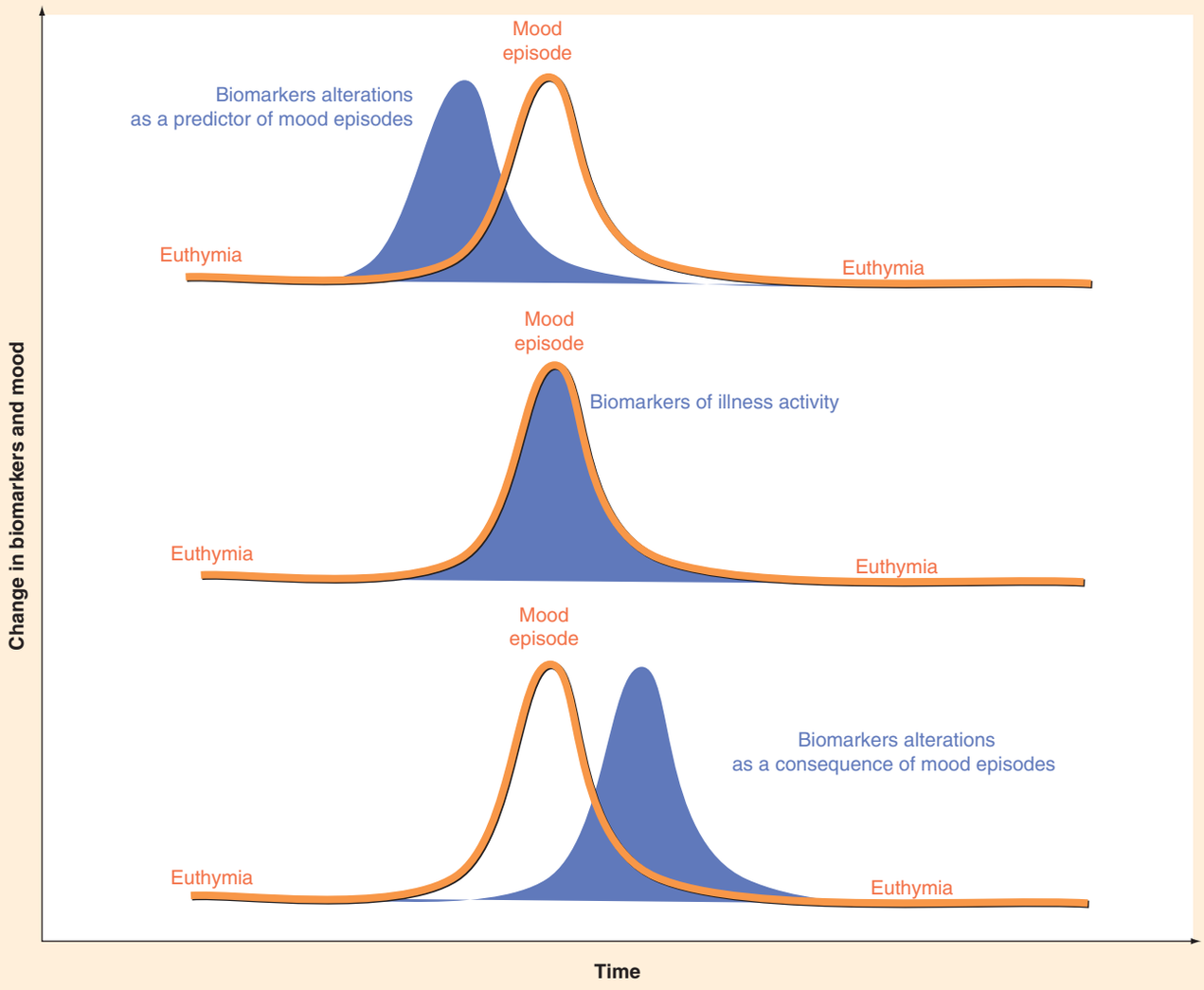

Figure 3. Peripheral biomarker alterations in an acute mood episode (mania or depression) could follow three different patterns. First, biomarkers could change before the beginning of a mood episode, showing a potential to predict these events. In this case, biomarkers would have a major therapeutic potential: they could help plan/implement early interventions and prevent/monitor treatment response. Second, biomarker changes could occur concomitantly with mood episodes, reflecting illness activity. In this case, they would be a useful tool in supporting clinical decisions for a better management of acute episodes. Finally, biomarkers could change after a mood episode, that is, because of it, which could contribute to improve our understanding of the pathophysiology of bipolar disorder. This assessment could be useful as a surrogate of pharmacological efficacy, predicting response to treatment of an acute episode after therapy initiation. The alteration patterns of biomarkers and their temporal relationship with mood episodes in bipolar disorder remain unknown.

treatment strategies should involve agents that act on pathways related with illness activity in BD. These findings could be useful not only to develop a more efficient, personalized approach to treat mood symptoms, but also to understand and perhaps revert biological changes associated with the illness, potentially bringing psychiatry into a new era of preventive psychopharmacology.

Financial & competing interests disclosure

F Kapczinski has received grant/research support from AstraZeneca, Eli Lilly, Janssen-Cilag, Servier, CNPq, CAPES, NARSAD and the Stanley Medical Research Institute; has been a member of the speakers' boards for AstraZeneca, Eli Lilly, Janssen and Servier; and has served as a consultant for Servier. B Pfaffenseller, GR Fries, B Wollenhaupt-Aguiar and L Stertz are supported by scholarships from Coordenacao de Aperfeicoamento de Pessoal de Nıvel Superior (CAPES), Brazil. PVS Magalhães is supported by a postdoctoral scholarship from Fapergs/CAPES, Brazil. GD Colpo and B Panizzutti are supported by scholarships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The recurrence of acute mood episodes in bipolar disorder (BD) often translates into several worse outcomes; for example, higher rates of clinical comorbidities, functional and cognitive impairments and decreased responsiveness to treatment.
- There is a growing interest in understanding the pathophysiological mechanisms that contribute to dysfunction resulting from multiple mood episodes in BD, especially those pathways involved in neuroprotection, oxidative stress and inflammation.
- Among neurothophins, consistent evidence suggests a possible role of brain-derived neurotrophic factor in the pathophysiology of BD: brain-derived neurotrophic factor levels are reduced during manic and depression episodes, and treatment with mood stabilizers is able to increase its levels.
- Mood episodes in BD have been characterized as pro-inflammatory states based on findings reporting alterations in the levels of cytokines and their receptors and an aberrant inflammatory gene expression.
- Several studies have demonstrated systemic alterations in diverse oxidative stress parameters in patients during mania or depression; for example, increased lipid peroxidation and nitric oxide levels and alterations in antioxidant enzymes superoxide dismutase and catalase.
- Mechanisms leading to reduced resilience associated with acute episodes probably involve organelles typically responsible for maintaining cellular homeostasis, for example, the mitochondrion and endoplasmic reticulum (ER), and could affect cells from both the periphery and the CNS, such as neurons and glia.
- A growing body of evidence suggests a key role of mitochondrial dysfunction in BD, including impaired energy metabolism, alterations in respiratory chain complex enzymes, altered levels of cytoplasmic calcium and downregulation of mitochondria-related genes. Patients with BD also seem to show a dysfunctional ER stress response, failing to stimulate an appropriate or sufficient response to maintain homeostasis under stress situations.
- Glial dysfunction and activation of a proinflammatory process by the release of damage-associated molecular patterns, as well as disruption of ER–mitochondria interactions, may be responsible for metabolic alterations and peripheral toxicity in BD.
- In light of the pathways known to be implicated in illness activity, novel therapies can be proposed for a better management of acute mood episodes and to prevent their recurrence. These could include adjuvant therapies with antioxidant, anti-inflammatory and neuroprotective agents.
- The assessment of systemic toxicity through a set of peripheral biomarkers may facilitate understanding of the body and brain damage associated with recurrent mood episodes and of the way how it impacts illness management.

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