UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: PSIQUIATRIA

TESE DE DOUTORADO

CITOCINAS INFLAMATÓRIAS COMO BIOMARCADORES NO TRANSTORNO BIPOLAR

Maurício Kunz

Orientador: Flávio Kapczinski

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Tese apresentada ao Programa de Pós-Graduação em Ciências Medicas: Psiquiatria da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do titulo de doutor em Psiquiatria

Porto Alegre, Outubro de 2010

"Chance favors the connected mind"

Steven Johnson

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SUMÁRIO

Serum levels of IL-6, IL-10 and TNF- α in patients with Bipolar Disorder and with Schizophrenia: Differences in pro and anti-inflammatory balance.

Brain-derived neurotrophic factor and inflammatory markers as predictors of outcome in bipolar disorder: prospective data from the Systematic Treatment Optimization Program for Early Mania (STOP-EM).

LISTA DE ABREVIATURAS

BDNF – Fator neurotrófico derivado do cérebro, do inglês *brain derived neurotrophic factor*

CRP – Proteina C Reativa

IFN-α – Interferon Alfa

IFN-γ – Interferon Gama

IL-1 – Interleucina 1

IL-1β – Inteleucina 1 Beta

IL1Rα – Receptor antagonista de interleucina 1

IL1B – Gene da Interleucina 1β

IL-2 – Interleucina 2

IL-3 – Interleucina 3

IL-4 – Interleucina 4

IL-6 – Interleucina 6

IL-8 – Interleucina 8

IL-10 – Interleucina 10

IL-12 – Interleucina 12

sIL-2R – Receptor solúvel da interleucina 2

TNF-α – Fator de Necrose Tumoral Alfa

TNFA – Gene do TNF-α

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RESUMO

Biomarcadores tem se tornado uma parte essencial da pesquisa clínica. Na psiquiatria, diversos biomarcadores séricos têm sido estudados. No Transtorno Bipolar, considerável atenção tem sido dada a marcadores inflamatórios e entre esses destacam-se as citocinas. No entanto, o entendimento dos diferentes padrões de envolvimento desses marcadores ainda não foi suficientemente estudado. Também, até o momento, poucos foram os estudos que avaliaram seu papel como marcadores de atividade ou preditores de curso de doença. A presente tese é composta por dois estudos: 1) uma comparação de citocinas inflamatórias entre pacientes eutímicos com um diagnóstico de Transtorno Bipolar em relação a pacientes com Esquizofrenia e controles saudáveis. 2) um estudo prospectivo de pacientes com Transtorno Bipolar avaliando medidas de citocinas no baseline em relação a variáveis clínicas de um ano de seguimento. Como um todo, os resultados apresentados corroboram o involvimento de citocinas inflamatórias no Transtorno Bipolar e na Esquizofrenia, no entanto evidenciando um padrão diferenciado de envolvimento nos dois transtornos. Também a IL-6 mostrou-se alterada em pacientes eutímicos que viriam a apresentar um ou mais episódios depressivos durante um ano de seguimento e seu aumento correlacionou-se com o numero de dias com sintomas depressivos. Esses achados fornecem apoio adicional para a investigação de citocinas como possíveis biomarcadores para a atividade da doença ou preditores de recorrência.

Palavras-chave: Transtorno Bipolar, Citocinas, Biomarcadores, Esquizofrenia

ABSTRACT

Biomarkers are becoming an essential part of clinical research. In Psychiatry, several serum biomarkers are currently being studied. In Bipolar Disorder, increasing attetion has been given to inflammatory markers and among these cytokines has received special attention. However, the understanding of the different patterns of inflammation has not yet been fully clarified. Few studies have focused on its properties as markers of disease activity and predictors of outcome. This thesis contains two different studies: 1) a comparison of cytokine levels in patients with Bipolar Disorder, Schizophrenia and healthy controls. 2) a prospective study of euthymic patients with Bipolar Disorder assessing baseline cytokines and theis association with clinical variables during follow-up. These studies reinforce the idea of an inflammatory response in both Bipolar Disorder and Schizophrenia; however, the pattern of reponse seems to be different between the two disorders. Also, increased levels of IL-6 were observed in euthymic patients that would later present a depressive episode during follow-up and this increase was correlated with the numbers of days depressed. These findings five further support for the investigation of cytokines as possible biomarkers of disease activity and predictors of recurrence.

Keywords: Bipolar Disorder, Cytokines, Biomarkers, Schizophrenia

1. INTRODUÇÃO

Biomarcadores tem se tornado uma parte essencial da pesquisa clínica. Um biomarcador deve ser um indicador de doença que possa ser medido de forma acurada, fidedigna, de forma fácil e preferencialmente com técnicas não-invasivas. Idealmente, o biomarcador está associado com a patofisiologia da doença e seus efeitos sobre o paciente (Paulsen, 2009).

Por definição, um biomarcador é uma característica que pode ser objetivamente medida e avaliada como um indicador de um processo biológico normal, processo patológico ou resposta farmacológica a uma intervenção terapêutica. Diferentes marcadores podem ser usados para várias avaliações: diagnosticar uma condição médica, predizer o curso ou desfecho de um individuo com essa condição, predizer se um indivíduo em particular irá se beneficiar de um tratamento em particular ou avaliar a resposta do indivíduo ao tratamento (Singh & Rose. 2009).

No contexto psiquiátrico, diversos biomarcadores têm sido investigados: de variações de condutividade da pele a exames de ressonância funcional magnética e sequenciamento genético. No entanto, devido a fatores como facilidade de acesso e baixo custo, muito foco têm sido dado para marcadores biológicos em sangue periférico (Kapczinski et al., 2009).

No Transtorno Bipolar, inúmeros estudos têm demonstrado alterações de mecanismos neurotróficos, estresse oxidativo e processos inflamatórios em medidas séricas. Tais achados têm contribuído significativamente para a

compreensão da patofisiologia da doença. Dentre eles, as alterações de marcadores inflamatórios têm recebido especial atenção. No entanto, o entendimento dos diferentes padrões de envolvimento desses marcadores em cada doença ainda não foi suficientemente estudado. Também, até o momento, poucos foram os estudos que avaliaram seu papel como marcadores de atividade ou preditores de curso de doença.

Diversos estudos avaliaram medidas de citocinas no Transtorno Bipolar (Goldstein et al., 2009) e na Esquizofrenia (Potvin et al., 2008) separadamente, confirmando a associação de alterações inflamatórias com as duas doenças. No entanto, até o momento, nenhum estudo comparou o padrão dessas alterações nos dois transtornos. Da mesma forma, dada a escassez de estudos longitudinais avaliando marcadores biológicos periféricos no Transtorno Bipolar, até o momento não se pode determinar seu valor como preditores de curso de doença.

O desenvolvimento de biomarcadores objetivos de atividade e curso de doença, assim como de resposta ao tratamento, fariam uma diferença significativa na nossa habilidade de tratar pacientes com transtornos psiquiátricos.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Sistema imunológico e Transtorno Psiquiátrico

Nas últimas décadas o entendimento do sistema imunológico e seu papel nas neurociências têm passado por uma revolução. Estudos iniciais sobre o tema retratavam-no como uma vítima passiva do stress e da ativação de mecanismos neuroendócrinos. Muitos desses estudos se focavam em como o sofrimento mental – freqüentemente em função de estressores psicossociais ou transtornos psiquiátricos – estava associado com supressão da resposta imune. Acreditava-se que essa supressão, por sua vez, levasse a uma maior vulnerabilidade a certas doenças, em especial doenças infecciosas e câncer. Gradualmente, no entanto, o sistema imunológico foi sendo reconhecido como um importante colaborador para as conseqüências patofisiológicas do sofrimento emocional sobre a saúde num aspecto geral. (Raison & Miller. 2003).

Atualmente, existe evidência substancial de que o sistema imunológico desempenha um papel ativo no desenvolvimento e nas consequências dos transtornos psiquiátricos ao invés de ser apenas um recipiente passivo de influências neuroendócrinas. No entanto, apesar de diferentes teorias imunológicas das doenças psiquiátricas terem sido propostas, o estabelecimento de um elo causal definitivo aguarda o desenvolvimento de terapêuticas direcionadas à resposta imunológica. Uma rica literatura básica sobre as interações cérebro-imunidade tem evoluído e alvos relevantes tem sido identificados, em especial as citocinas e suas redes de comunicação intercelular.

No caso da depressão, apesar de alguns dados contraditórios, as evidências tendem a ser consistentes. Em múltiplos estudos com indivíduos com ou sem comorbidade clínica, pacientes com depressão demonstram todas as características de uma ativação inata da resposta imune, incluindo aumento de citocinas pró-inflamatórias, reagentes de fase aguda, quimiocinas e moléculas de adesão (Raison et al 2006). Não apenas os valores médios são diferentes entre grupos de indivíduos deprimidos e não-deprimidos, mas vários estudos demonstraram correlações entre marcadores inflamatórios e a gravidade da depressão.

Mas a relação da ativação da resposta imune no contexto da depressão e outros transtornos relacionados ao estresse é bidirecional. Considerando o papel da inflamação numa série de doenças médicas, um importante elo entre stress, depressão e doença clínica pode ser de fato a associação de estresse e depressão com inflamação (Raison et al., 2006). Assim, por exemplo, a capacidade de citocinas inflamatórias influenciarem a formação de placas ateroscleróticas e alterações cardíacas pode contribuir para o prognóstico relativamente ruim observado em pacientes com doença cardíaca e depressão. Um outro foco para essa interação vem da capacidade de estímulos inflamatórios induzirem uma síndrome referida como "sickness behavior" (Dantzer, 2001). "Sickness behavior" inclui sintomas de anedonia, anorexia, fadiga e disfunção cognitiva, e apesar de inicialmente descrita no contexto de uma variedade de doenças infecciosas, pode ser reproduzida pela administração de citocinas (IFN-α, IL-1, IL-6 e TNF- α) a animais de laboratório ou humanos. A

sobreposição dos sintomas da "sickness behavior" com os de depressão representam a parte mais bem estudada da hipótese do envolvimento de citocinas na depressão, com diversos estudos gradualmente elucidando a capacidade das citocinas de interagir com e influenciar virtualmente todos os domínios patofisiológicos que parecem desempenhar um papel na depressão (Raison et al., 2006).

Mais recentemente, estudos tem examinado o papel da inflamação em outras doenças neuropsiquiátricas como a Esquizofrenia (Potvin et al., 2008) e a Doença de Alzheimer (McGeer et al., 2006).

2.2. Citocinas

Citocinas são proteínas responsáveis pela comunicação intercelular no sistema imune. São reguladores centrais do crescimento e diferenciação de leucócitos, sendo produzidas por uma grande variedade de tipos celulares, tendo por alvo diferentes grupos de células e exibindo inúmeras atividades biológicas.

"Citocinas" é na verdade o nome genérico dado a um diverso grupo de proteínas solúveis e peptídeos que agem como reguladores humorais em concentrações nano a picomolares, e que, em condições normais ou patológicas, modulam as atividades funcionais de células individuais e tecidos. Citocinas agem numa variedade maior de alvos celulares do que hormônios e não são produzidas por células de glândulas especializadas, mas sim por células do próprio sistema imune. No entanto, também são produzidas por

células do sistema nervoso, em particular por células da glia (Goncharova et al. 2007).

Já foram identificadas mais de 100 diferentes citocinas humanas. Esse numero foi simplificado pela divisão de citocinas e seus receptores em superfamílias estruturais. Existe uma relação entre níveis de citocinas em fluidos corporais humanos e patogênese de diferentes doenças. Muitos tipos de câncer tiram proveito do papel regulatório dessas proteínas através da produção de citocinas que induzem a inibição generalizada e específica da resposta imune (Kurzrock, 2001). Outros estudos também evidenciaram que algumas citocinas podem induzir apoptose em células cancerígenas (Wall et al., 2003).

Ao se estudar a relevante base de conhecimento da neuroimunologia, ênfase particular deve ser dada quanto à bidirecionalidade da interação neuro-imune. Esses sistemas complexos interagem em múltiplos níveis, tanto neuroendócrinos (eixo hipotálamo-hipófise-adrenal) e neurais (inervação simpática direta de órgãos linfóides), e estão envolvidos no controle da resposta imunológica tanto humoral como celular (Wrona. 2006). O sistema imune, por sua vez, influencia o sistema nervoso central primariamente através de citocinas. A nível molecular, sinalizadores tanto neuronais quanto imunológicos (hormônios, neurotransmissores, neuropeptídeos, citocinas) fazem parte da mesma superfamília o que possibilita a comunicação neuro-imune.

Citocinas influenciam mecanismos complexos que envolvem uma variedade de circuitos neuronais, como termo-regulação, apetite, padrões de

sono e comportamento. Existe também evidência de que redes de citocinas estejam envolvidas em mecanismos centrais de memória e aprendizado.

Seguindo os achados de que a administração de certas citocinas pode induzir alterações de parâmetros endócrinos que estão sob controle neural, um grande número de estudos demonstrou que receptores para algumas dessas citocinas estão presentes no sistema nervoso central. Receptores funcionais de citocinas (em particular IL-1) são encontrados em astrocitos, microglia, oligodendrocitos e na maioria dos neurônios (Cunningham et al., 1992; Otero & Merrill, 1994; Rothwell & Hopkins, 1995). Diversas citocinas como a IL-1, seu receptor antagonista (IL1Rα), IL-2, IL-3, IL-6, IL-8, IL-12 e IFN-γ foram encontradas no cérebro. A expressão constitutiva de IL-1β, IL-6 e TNF-α, mas não IFN-γ, foi também demonstrada no cérebro. Apesar de nem todos os estudos concordarem quanto à localização de locais específicos de ligação para citocinas no cérebro, parece haver um consenso geral de que, em ratos adultos, receptores de IL-1 estão principalmente concentrados no hipocampo (Dopp et al., 1996).

No início do estudo das interações neuro-imunes havia uma dúvida significativa de que o sistema imunológico poderia de alguma forma sinalizar ao cérebro. Se acreditava então que moléculas grande como as citocinas (proteínas de 15-20-kDa) não pudessem cruzar a barreira hematoencefálica. Dessa forma, inicialmente se acreditava que as citocinas não poderiam entrar facilmente no sistema nervoso central. No entanto, trabalhos recentes demonstraram diferentes rotas pelas quais citocinas periféricas podem cruzar diretamente a

barreira hematoencefálica ou indiretamente sinalizar ao cérebro através de outras substâncias (Licinio & Wong,1999).

De acordo com (Brebner et al. 2000), as interleucinas pró-inflamatórias IL-1, IL-6 e TNF-α influenciam atividade neuroendócrina, promovem alterações de neurotransmissores centrais e induzem uma constelação de sintomas comportamentais. Com essa visão de variações de aminas centrais influenciadas por citocinas, foi sugerido que a ativação imune pode influenciar comportamentos complexos assim como estados de humor.

Em adição à modulação neuroendócrina (Savino & Dardenne, 2000), foi demonstrado que citocinas também governam importantes propriedades de neurônios, incluindo mecanismos de sobrevivência e apoptose. As interações entre citocinas, o eixo HHA e monoaminas são complexas e envolvem múltiplos fatores, incluindo glutamato, cálcio e proteína kinase C, entre outros (Barkhudaryan et al., 1999). O papel da inflamação no dano e degeneração neuronal está bem estabelecido (Aktas et al., 2007; Allan et al., 2001) e parece ser particularmente importante naqueles com distúrbios em outros sistemas metabólicos (McIntyre et al., 2007).

2.3. Inflamação e Transtorno Bipolar

A teoria do envolvimento de macrófagos na depressão foi articulada cerca de 20 anos atrás em um esforço de consolidar diversas observações relacionadas, incluindo um reconhecimento crescente de que citocinas pró-inflamatórias podem precipitar sintomas depressivos em voluntários saudáveis e

que depressão comumente ocorria em doenças associadas com inflamação, tal como doença arterial coronariana e artrite reumatóide (Smith RS. 1991). Desde então, evidência tem se acumulado indicando que alterações inflamatórias são salientes na patogênese e possivelmente no tratamento da depressão maior (Schiepers et al., 2005; Raison et al., 2006).

Até recentemente, no entanto, apenas alguns estudos haviam examinado o papel da inflamação no Transtorno Bipolar. O Transtorno Bipolar é uma doença neuropsiquiátrica grave e incapacitante de início frequentemente precoce e cujo diagnóstico e tratamento adequados por vezes só são feitos após muitos anos de doença (Hirschfeld et al., 2003). Somada à frequente comorbidade psiquiátrica no Transtorno Bipolar (Grant et al. 2005), a co-ocorrência de comorbidades clínicas também é comum (Kupfer DJ. JAMA 2005).

De fato, foi até mesmo hipotetizado que inflamação sistêmica possa estar associada com morte natural prematura no Transtorno Bipolar (Tsai et al. 2005).

2.3.1. Inflamação Durante Mania ou Depressão

Diferentes estudos têm achado evidência de aumento de marcadores pró-inflamatórios durante a mania, particularmente CRP, receptor solúvel da IL-2 (sIL-2R), IL-6 e TNF-α. Resultados quanto a alteração de marcadores anti-inflamatórios (como IL-4, IL-10) têm sido menos consistentes (Goldstein et al., 2009).

Por exemplo, pelo menos dois estudos encontraram níveis elevados de IL-4 em pacientes maníacos comparados com controles (Kim Y et al., 2004;

Ortiz-Domínguez et al. 2007), enquanto um outro estudo mostrou que indivíduos com TB apresentavam níveis significativamente mais altos de IL-6 e TNF-α ao mesmo tempo que tinham níveis significativamente menores de IL-4 em relação a controles (Kim et al., 2007).

Um número menor de estudos examinou inflamação durante a depressão bipolar, no entanto, o aumento de diversos marcadores pró-inflamatórios parece se sobrepor ao observado na mania, incluindo-se aí sIL-2R, IL-6, IL-8, CRP e TNF-α (Kim et al., 2004; Middle et al. 2000). Por fim, existe evidência preliminar de aumentos de IL-1β e IL-6 durante a depressão em relação à mania e aumentos de sIL-2R, IL4 e CRP durante a mania em relação à depressão (Papiol et al., 2004; Rapaport et al., 2001).

2.3.2. Mudanças na inflamação após tratamento e/ou melhora sintomática

A maioria dos estudos que avaliou a associação entre marcadores pró-inflamatórios e tratamento e/ou resolução de sintomas não reportou achados significativos. A natureza dessa associação pode variar entre as diferentes citocinas. Diferentes estudos avaliando sIL-2R e IL-6 sugerem que estes estão associados com tratamento e/ou resolução de sintomas (Breunis et al., 2003; Kim et al. 2004b; Bosetti et al., 2002). Em contraste, apesar de diversos estudos terem encontrado aumento de níveis de TNF-α durante mania e depressão bipolar, associações significativas com tratamento e/ou resolução de sintomas

não foram observadas (Padmos et al., 2008; Bosetti et al. 2003; Rapaport & Manji, 2001).

2.3.3. Inflamação durante eutimia

Poucos estudos publicaram achados referentes a inflamação durante eutimia. Um estudo encontrou aumento de sIL-2R em pacientes com transtorno bipolar eutímicos em relação a controles, de forma similar ao observado durante mania e depressão (Breunis et al., 2003). Apesar de nenhum achado significativo ter sido relatado quanto à citocina IL-10 durante mania ou depressão, um estudo (Boufidou et al., 2004) encontrou diminuição dos níveis de IL-10 em pacientes eutímicos com transtorno bipolar em uso de lítio. O mesmo estudo também encontrou níveis diminuídos de IL-2 e IL-6.

2.3.4 Associação entre Citocinas e Variáveis Demográficas e Clínicas

Diversos estudos (Knijff et al., 2006; Pae et al., 2004; Lee et al., 2008) investigaram se citocinas estão associadas com variáveis clínicas como duração de doença, idade de início, tabagismo e obesidade. Da mesma forma, foi também examinada a associação com variáveis demográficas como idade e sexo. No entanto, nenhum correlato clínico ou demográfico de inflamação em sujeitos com Transtorno Bipolar foi encontrado até o momento. No entanto, vale ressaltar que a literatura apresenta importantes limitações metodológicas, e essas podem explicar em parte a ausência de associações. Em particular, pequenos tamanhos amostrais e heterogeneidade de amostra e métodos contribuem para isso.

Citocina	Mania vs. Controles	Mania vs. Eutimia	Depressão vs. Controles	THB	Mania vs. Depressão	Achados Confirmatórios
IL-1RA	↑	↑				
IL-1-β	↓				D>M	+
IL-2	↓		↓	↓		+
sIL-2R	↑↑↑↑	↑↑	↑	↑	M>D	+++
IL-4	↑↑↓				M>D	
IL-6	↑↑		↑	↓	D>M	+++
sIL-6R	↑			↑		+
IL-8	↑		↑			
IL-10				↓		+
IL-12						+
CRP	↑↑	↑	↑		M>D	+
TNF-α	↑↑↑		↑↑			
IFN-γ	↑↓↓	↓↓		↓		+

Tabela – Sumário dos principais achados relacionados a marcadores inflamatórios no Transtorno Bipolar. Cada seta representa um estudo que encontrou uma diferença estatisticamente significativa entre grupos, representanto aumento o diminuição. Adaptado de Goldstein et al. 2009.

2.3.5 Polimorfismos Genéticos Associados a Inflamação

Um estudo que examinou a região promotora do gene IL1B em pacientes com Esquizofrenia ou Transtorno Bipolar e controles encontrou um excesso significativo da combinação de alelos estudada especialmente nos indivíduos com Transtorno Bipolar que apresentavam história familiar de Transtorno Bipolar, Esquizofrenia ou Transtorno Depressivo (Papiol et al., 2004). Os autores concluíram que o cluster de variabilidade genética da IL1 pode oferecer suscetibilidade genética compartilhada entre Transtorno Bipolar e Esquizofrenia. Em contraste, um outro grupo estudando o mesmo polimorfismo encontrou uma

associação significativa em pacientes com Esquizofrenia mas não com Transtorno Bipolar (Kim et al., 2004c).

Um outro estudo da Coréia examinou o polimorfismo do gene TNFA em pacientes bipolares em relação a controles e encontrou uma freqüência significativamente maior nessa amostra (21.3% contra 7.2%). Em contraste, um grupo brasileiro não encontrou nenhuma associação significativa entre o mesmo polimorfismo e Transtorno Bipolar, apesar de terem achado uma associação com Esquizofrenia (Pae et al., 2004).

Um outro estudo identificou um aumento da expressão de RNAs mensageiros de genes pró-inflamatórios em 52% de uma amostra de adultos com Transtorno Bipolar em comparação a 18% observado em controles. O mesmo estudo examinou adultos jovens filhos de pais com Transtorno Bipolar e observou a mesma alteração pró-inflamatória em 88% dos que também tinham um diagnóstico de transtorno de humor e 45% nos que não tinham qualquer diagnóstico em relação a 19% dos controles com a mesma idade. O gene da IL6 estava entre as variáveis que mais fortemente distinguiram adultos com Transtorno Bipolar dos controles (Padmos et al. 2008).

Em resumo, achados genéticos sugerem que Transtorno Bipolar está possivelmente associado com polimorfismos genéticos de IL1 e IL6 enquanto os achados para polimorfismos de TNFA são contraditórios.

2.3.6 Inflamação e Estabilizadores de Humor

Diversos estudos clínicos e pré-clínicos sugerem que o mecanismo de ação de diferentes estabilizadores de humor (antipsicóticos, carbamazepina, lamotrigina, lítio e valproato) pode incluir a inibição da COX-2 e redução de citocinas inflamatórias (Lee et al., 2008; Rao et al., 2008; Rapaport & Manji, 2001).

2.4. Inflamação e Esquizofrenia

A maioria da citocinas exerce efeitos pleiotrópicos e sobrepostos através da interação com receptores específicos expressados em diferentes células-alvo. Receptores de citocinas também existem em forma solúvel, como o receptor solúvel da IL-2 (sIL-2R), que é liberado da superfície da membrana de células imunes ativadas e pode inibir a atividade biológica da IL-2, um importante fator de crescimento para células-T, por impedir sua ligação com receptores ancorados de membrana. Assim, sIL-2R é visto como marcador de ativação imunológica. Em contraste, a ligação do receptor solúvel da IL-6 (sIL-6R) com a IL-6 forma um complexo que aumenta a atividade biológica da própria IL-6. A ação de citocinas pode também ser inibida também por antagonistas de receptores que ocorrem naturalmente, como o antagonista da IL-1 (IL-RA), que compete com o ligante fisiológico pelos receptores de membrana da IL-1. O IL-RA é produzido em resposta a vários estímulos inflamatórios, incluindo IL-1 e IL-6 (Raison & Miller, 2004). IL-1, IL-6, TNF-α e IFN-γ são considerados pró-inflamatórios, no sentido de que aumentam a resposta imune a infecções e

inflamação promovendo o recrutamento leucocitário aos locais de inflamação e através da ativação de células inflamatórias. IL-1RA, IL-4 e IL-10 são citocinas anti-inflamatórias que contribuem para a redução da resposta inflamatória.

Dentre diferentes explicações etiológicas para a Esquizofrenia, diversas hipóteses foram propostas envolvendo alterações imunológicas tais como infecções e disfunção auto-imune (Brown et al., 2002; Eaton et al., 2006). Uma das principais propostas para a investigação dessas idéias tem sido o estudo de citocinas. No entanto, evidências que suportem a teoria de disfunção imune têm sido inconsistentes. As citocinas mais frequentemente estudadas na Esquizofrenia tem sido a IL-2 e IL-6. Apesar de alguns estudos evidenciarem uma diminuição de níveis de IL-2 na Esquizofrenia (Arolt et al., 2000; Bessler et al., 1995), outros estudos não confirmam essa tendência (Kaminska et al. 2001), enquanto outros mostram uma relação inversa (Ebrinç et al., 2002). Quanto ao aumento de IL-6, os resultados tem sido mais consistentes (Maes et al., 2000; Shintani et al., 1991). Uma questão importante nessa avaliação foi a descoberta do impacto dos antipsicóticos nos parâmetros imunológicos (Ghezzi et al., 1996; Maes et al., 1994).

Recentemente, uma metanálise incluiu 62 estudos que avaliaram citocinas em Esquizofrenia (Potvin et al. 2008). Aumentos significativos foram confirmados para IL-1RA, sIL-2R e IL-6, corroborando a existência de um processo inflamatório contínuo na Esquizofrenia. Nos casos da IL-6 e IL-1RA em especial, as alterações observadas não parecem relacionadas ao uso de medicações antipsicóticas.

3. OBJETIVOS

3.1. Objetivo Geral

Estudar as alterações de biomarcadores inflamatórios no Transtorno Bipolar em comparação à Esquizofrenia e como preditores de episódios de humor no curso do Transtorno Bipolar.

3.2. Objetivos Específicos

- Comparar níveis séricos de citocinas pró-inflamatórias (IL-6 e TNF-α) e anti-inflamatórias (IL-10) em pacientes com Transtorno Bipolar e na Esquizofrenia em relação a controles saudáveis.

- Avaliar níveis séricos de citocinas (IL-6, IL-10 e TNF-α) e BDNF de pacientes eutímicos com Transtorno Bipolar relacionando as alterações com a ocorrência prospectiva de episódios de humor ao longo de um ano de seguimento.

4. CONSIDERAÇÕES ÉTICAS

Todos os pacientes e controles assinaram termo de consentimento informado previamente ao início dos estudos.

Foi assegurada a ausência de vinculação da concordância em participar do estudo com a continuidade do atendimento no programa de atendimento de transtorno de humor bipolar (PROTAHBI) ou ambulatório de esquizofrenia (PRODESQ), no Hospital de Clínicas de Porto Alegre.

Ambos os estudos foram aprovados pelos Comitês de Ética em Pesquisa das instituições envolvidas – o Hospital de Clínicas de Porto Alegre e o University of British Columbia Hospital.

5. ARTIGOS

5.1. ARTIGO 1 – Aceito para publicação – Revista Brasileira de Psiquiatria

SERUM LEVELS OF IL-6, IL-10 AND TNF-α IN PATIENTS WITH BIPOLAR DISORDER AND WITH SCHIZOPHRENIA: DIFFERENCES IN PRO AND ANTI-INFLAMMATORY BALANCE

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ABSTRACT

Objective: Previous reports suggest cytokines as potential mediators of the interaction between immune and neuroendocrine systems, and a proinflammatory state associated with BD and SZ. The aim is to compare cytokine levels between the two disorders.

Methods: Twenty euthymic BD patients, 53 chronic stabilized SZ patients and 80 healthy controls were recruited. Subjects were all non-smokers and non-obese. Cytokines TNF-α, IL-6, and IL-10 were examined by sandwich ELISA.

Results: IL-6 was increased in SZ when compared to controls ($p<0.0001$) and euthymic BD patients ($p<0.0001$). IL-6 levels were not different in controls compared to euthymic BD patients ($p=0.357$). IL-10 was lower in controls compared to SZ ($p=0.001$) or BD ($p=0.004$). There was no significant difference in TNF-α serum levels among the groups ($p=0.284$). Separation by gender did not change significantly and no correlation between antipsychotic dose and cytokine levels in patients with SZ were found.

Discussion: These findings evidence a chronic immune activation in SZ. BD seems to present an episode-related inflammatory syndrome. Increased anti-inflammatory factor IL-10 in BD and SZ suggest different patterns of inflammatory balance between these two disorders. Results provide further support to

investigate cytokines as possible biomarkers for disease activity or treatment response.

Key words: Bipolar Disorder; Schizophrenia; IL-6; IL-10; TNF-α

Abbreviations: BD, Bipolar Disorder; BDNF, Brain-derived neurotrophic factor; BPRS, Brief Psychiatric Rating Scale; CGI, Clinical Global Impression; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, fourth Edition; HAMD-21, Hamilton Depression Rating Scale, 21-item version; IL-6, Interleukin-6; IL-10, Interleukin-10; PBS, phosphate-buffered saline; SD, Standard deviation; SZ, Schizophrenia; Tumor necrosis factor alpha, TNF-α; YMRS, Young Mania Rating Scale.

INTRODUCTION

Bipolar disorder and schizophrenia share many epidemiologic and clinical features[1]. Increasing data suggests an overlap in genetic susceptibility across the two diagnoses[2]. Although their pathophysiology remains unclear, mechanisms such as oxidative stress and neuroplasticity have recently been implicated[3-6]. However, these mechanisms seem to be differently involved in each disorder[7, 8].

It is well recognized that people with severe mental illnesses, such as SZ or BD have worse physical health outcomes and reduced life expectancy compared to the general population[9]. The excessive cardiovascular mortality associated with SZ and BD is attributed in part to an increased risk of the modifiable atherosclerotic risk factors; obesity, smoking, diabetes, hypertension and dyslipidaemia[10-12]. Inflammation is a mechanism that takes part not only in atherosclerosis but also in different neuropsychiatric disorders, where it is involved in complex and reciprocal interactions with neurotrophins and oxidative stress. Considering these interactions, a Systemic Toxicity Index for mood episodes has been recently proposed that assesses neurotrophins, oxidative stress markers and inflammatory markers[13].

Cytokines action on brain cells has been shown to be an important component of the neuroimmunological and neuroinflammatory response. Cytokines and related secretory products from immune cells communicate with the endocrine and central nervous system to modulate their collective functions[14]. In the field of mood disorders, the immune system seems to play a particularly

important role. Over the years a significant body of evidence has been accumulating suggesting that major depression is associated with dysfunction of inflammatory mediators[15]. Patients with unipolar depression that are otherwise clinically healthy have been shown to present activated inflammatory pathways, with increased pro-inflammatory cytokines, acute-phase proteins, and increased expression of chemokines and adhesion molecules[16-18].

More recently, available evidence indicates that BD and inflammation are linked through shared genetic polymorphisms and gene expression as well as altered cytokine levels[19] during acute episodes[20] and euthymia[21]. It has been suggested that inflammatory cytokines, particularly TNF-α may play a critical role in the process of changes in neuroplasticity, cell resilience and neuronal survival[22, 23]. Additionally, BDNF and TNF-α serum levels combined, have been proposed as staging biomarkers for BD[24].

Inflammatory processes have also been implicated with the pathophysiology of SZ, and different studies have focused on the role of inflammation in SZ[25]. Many observations of immune deregulation in SZ overlap with central pathophysiological mechanisms as well as with clinical manifestations of the illness. This is supported by a growing database of investigations, some of which even suggest that a subgroup of patients with SZ demonstrate features of an autoimmune process[26]. Even if not yet completely understood, the general evidence of a pro-inflammatory state in SZ is consistent[23].

So far no other study has compared inflammatory markers in BD and SZ. Examining the different expression of physiopathological mechanisms may provide better understanding of the etiological processes underlying both disorders as well as new possibilities of treatment. Therefore, this study was designed to examine the cytokine levels of euthymic patients with BD and chronic stabilized patients with SZ, comparing with levels from healthy controls.

METHODS

Subjects

This study protocol was approved by the local ethics committee of the Hospital de Clínicas de Porto Alegre, RS, Brazil (HCPA). All subjects provided written informed consent before inclusion in the study.

We selected fifty-three stabilized outpatients with SZ, all chronically medicated, from the HCPA Schizophrenia Program and twenty euthymic outpatients with BD from the HCPA Bipolar Disorders Program. All subjects had a comprehensive clinical interview by a trained psychiatrist. Only non-smokers, non-obese subjects and those presenting no major medical conditions like atherosclerotic heart disease and diabetes (patients and controls) were included. Obesity was defined as a body mass index (weight in kilograms/height^2 in meters) of 30 or more. The diagnosis of BD and SZ were confirmed according to the DSM-IV[27]. Psychiatric status in patients with SZ was assessed with the BPRS[28] and the CGI[29]. Only patients with BPRS scores ≤ 25 and no history of a psychiatric hospitalization over the last two years were included in the study. Mood symptoms in patients with BD were assessed using the YMRS[30] and the HAMD-21[31]. Patients were considered euthymic, and therefore able to participate in the study, when both YMRS and HAMD scores were lower than seven. The control group consisted of eighty medication-free healthy volunteers who had no current or previous history as well as no first-degree family history of a major psychiatric disorder, including dementia or mental retardation assessed by the non-patient version of the Structured Clinical Interview for DSM-IV (SCID).

Serum cytokines measurement

Each subject had 5 ml of blood collected by venipuncture without anticoagulants at the same day of clinical interview. Serum was obtained by centrifugation at 3,000xg for 5 minutes and kept frozen at -70°C for up to 6 months, until the assay.

Serum cytokines were measured according to the procedures supplied by the manufacturer using sandwich ELISA kits for TNF-α, IL-6 and IL-10 (DuoSet, R&D Systems, Minneapolis, Minn., USA). All samples were assayed in duplicates and on the same plate. The detection limits for these assays were 5 pg/ml. In brief, the capture antibody (concentration provided by the manufacturer) was diluted in PBS, added to each well and left overnight at 4 °C. The plate was washed 4 times in PBS with 0.05% Tween-20 (Sigma, St. Louis, Mo., USA). The plate was blocked with 1% bovine serum albumin and incubated for 1 h at room temperature before washing 4 times with PBS and 0.05% Tween-20. The samples and standards were added and the plate incubated overnight at 4 °C. After washing the plate, detection antibody (concentration provided by the manufacturer) diluted in PBS was added. The plate was incubated for 2 h at room temperature. After washing the plate, streptavidin (DuoSet, R&D Systems) was added and the plate incubated for 30 min. At last, color reagent *o*-phenylenediamine (Sigma) was added to each well, and the reaction was allowed to develop in the dark for 15 min. The reaction was stopped with the addition of 1 M H_2SO_4 to each well. The absorbance was read on a plate

reader at 492 nm wave length (Emax, Molecular Devices, Minneapolis, Minn., USA).

Statistical Analysis

Analysis was performed using SPSS Version 16.0. Demographic and clinical characteristics were analyzed using chi-square and ANOVA test as indicated. Descriptive analyses are presented as mean ± SD or median (interquartile range) and p-values < 0.05 were considered significant. Cytokine levels showed a non-Gaussian distribution and were analyzed with appropriate tests as indicated in tables; Kruskal–Wallis test and Mann–Whitney test with Bonferroni correction. Relationships between variables were assessed with Spearman rank correlation coefficient.

RESULTS

The subjects' demographics and clinical characteristics are summarized in table 1. The three groups were not significantly different for age (p=0.115). Length of illness was higher in the group with SZ than in the group with BD (18.0±16.7 vs. 12.0±16.3, p=0.026). Patients with SZ were predominantly male (76%), while the BD and control groups had lower frequency of male subjects (40% for both groups). In the SZ group, 12 patients were on atypical antipsychotics, 13 on typical antipsychotics and 28 on clozapine. The BD group had 15 patients on lithium, 8 on other mood stabilizers, 10 on antipsychotics, 3 on antidepressants and 6 on benzodiazepines.

IL-6 was significantly increased in patients with SZ when compared to either controls (p<0.0001) or euthymic patients with BD (p<0.0001). IL-6 levels were not different in controls compared to euthymic BD patients (p=0.357). IL-10 was significantly lower in controls compared to SZ (p=0.001) or BD (p=0.004). There was no significant difference in TNF-α serum levels among the three groups (p=0.284) (figure 1). The significance did not change when patients were separated by gender (p<0.001 and p=0.008 for IL-6, p=0.003 and p=0.048 for IL-10 and p=0.165 and p=0.159 for TNF-α, for males and females, respectively). No correlation was found in patients with SZ between antipsychotic dose (in milligrams of chlorpromazine equivalents) and IL-6 (rho = - 0.267, p = 0.084), IL-10 (rho = 0.031, p = 0.845) or TNF-α (rho = - 0.145, p = 0.363).

DISCUSSION

Our results are in line with previous reports of increased inflammatory markers in BD and SZ. However, we found significantly higher levels of pro-inflammatory cytokine IL-6 in patients with SZ compared to either BD or healthy controls. Previous reports have found an association with IL-6 and acute symptomatology in BD[20] and SZ[32]. Considering that our comparison involved patients in non-acute phases of illness (euthymic BD vs. chronic SZ), our finding of increased IL-6 in SZ provides further evidence of a chronic immune activation and inflammatory syndrome in SZ[23]. While increased IL-6 seems to be state-dependent in BD, it is a trait in patients with SZ. Nevertheless, this finding could be also be explained by longer duration of illness in the SZ group[33].

Increased levels of TNF-α have also been previously reported in BD and SZ, and it was shown to be associated with symptomatology in both disorders[20, 34]. Therefore, the fact TNF-α levels were not different from controls in our sample can be explained by the non-acute profile of our subjects.

Even though inflammation plays a role in both disorders, the overall balance of pro and anti-inflammatory components is differently expressed. The increased levels of anti-inflammatory IL-10 in both SZ and BD groups if compared to controls should be looked at in this context. While it could be explained as a direct response to the elevation of IL-6 in SZ, it could reflect a compensatory mechanism as a result of previous episodes in BD. As levels of IL-10 seem to decline with the disease progression[35], the general pro-inflammatory

balance in BD worsens in the later stages of the illness as the anti-inflammatory response abates.

We believe the comparative assessment of pathophysiological mechanisms in BD and SZ may provide interesting insights about different roles they play in each disorder. Such comparisons have previously suggested different profiles of oxidative stress[8], neurotrophins[7] and different patterns of correlation between oxidative stress markers and neurotrophins in SZ[36] and BD[37]. While different lines of evidence suggest an episode-related pattern of deterioration in BD, SZ seems to have a chronic deteriorating course from the beginning. This observation brings us back to the classic differentiation of the two diagnosis based on either an episodic or a chronic-deteriorating course. In SZ, however, difficulties in defining remission in a chronically ill and stable population may not allow clear differentiation of state and trait effects.

Our report must be interpreted in light of its limitations, such as the lack of control for potential confounders. Factors such as diet and sedentariness can contribute substantially to alterations in inflammatory markers[11, 38, 39]. The excessive burden of medical conditions in BD and SZ is increasingly recognized[9]. The majority of these conditions are related to inflammation, especially cardiovascular illness, obesity and insulin resistance. Even though we excluded patients with significant medical problems, controlling for medical conditions, metabolic alterations and life style factors was not possible in our study.

Another potential confounder is different medications used by patients with BP and SZ. It appears that mood stabilizers in general, and especially lithium, attenuate the pro-inflammatory milieu in BD[19]. Similarly, antipsychotics may also impact the production of cytokines[40]. Therefore, even in the absence of any correlation between cytokine levels and the dose of antipsychotic medications in our sample, the confounder effect of medications cannot be ruled out.

Our findings support the involvement of inflammatory mechanisms as a component of the pathophysiology of both BD and SZ and provide insight into different patterns for this association. We believe studies looking at comparative analysis between disorders may provide further understanding of the inflammatory component of psychiatric illnesses and hopefully provide grounds for better interventions.

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Table 1

Characteristics of healthy controls and patients with Bipolar Disorder (BD) and Schizophrenia (SZ).

	SZ (n=53)	BD euthymic (n=20)	Control group (n=80)	p-value
Gender (M/F proportion)	75.5/24.5	40/60	40/60	<0.0001*
Age, years[a]	39.9 (9.4)	46.6 (12.6)	40.7 (12.5)	0.115**
Years of illness[b]	18.0(16.7)	12.0(16.3)	-	0.026***
YMRS score[b]	-	1.5 (6.5)	-	-
HAMD score[b]	-	4.0 (4.5)	-	-
BPRS score[b]	12.0 (11.0)	-	-	-
CGI score[b]	2.0 (1.0)	-	-	-
Antipsychotic daily dose, In mg of chlorpromazine equivalents[a]	501.9 (218.1)	-	-	-

YMRS, Young Mania Rating Scale; HAMD, Hamilton Depression Rating Scale;
BPRS, Brief Psychiatry Rating Scale; CGI, clinical global impression.
*Chi-Square
**One-way ANOVA
*** Mann-Whitney
[a]Shown as mean ± standard deviation
[b] Shown as median (interquartile range)
control/euthymic vs. SZ, p<0.0001 (Mann-Whitney)
SZ/euthymic vs. control, p=0.004 (Mann-Whitney)

Figure 1. **A)** Box-plot of serum IL6 levels in SZ patients, BD euthymic and. controls Median levels are indicated by horizontal lines, outliers are indicated by O. Kruskal-Wallis test was performed to analyze variance between groups ($p<0.0001$) and multiple comparisons were assessed by Mann-Whitney test (control vs. SZ, $p<0.0001$; euthymic vs. SZ, $p<0.0001$). **B)** Box-plot of serum IL10 levels in SZ patients, BD euthymic and controls. Median levels are indicated by horizontal lines, outliers are indicated by O or *. Kruskal-Wallis test was performed to analyze variance between groups ($p=0.001$) and m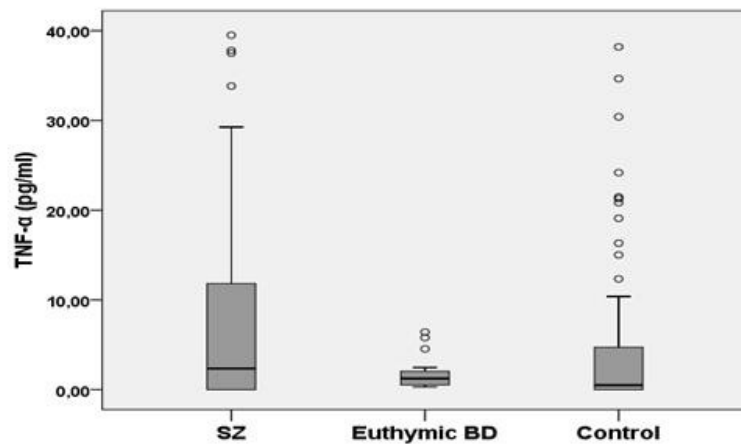ultiple comparisons were assessed by Mann-Whitney test (euthymic vs. control, $p=0.004$; SZ vs. Control, $p=0.001$). **C)** Box-plot of serum TNF-α levels in SZ patients, BD euthymic and controls. Median levels are indicated by horizontal lines, outliers are indicated by O or *. Kruskal-Wallis test was performed to analyze variance between groups ($p=0.284$).
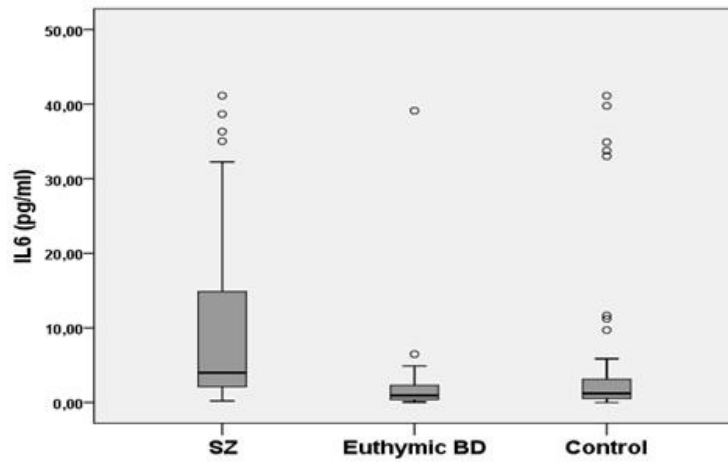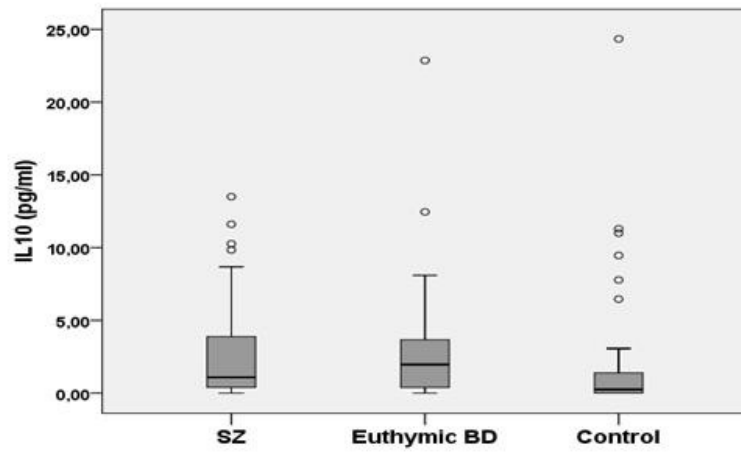
5.2. ARTIGO 2

Artigo submetido para publicação – Bipolar Disorders

Brain-derived neurotrophic factor and inflammatory markers as predictors of outcome in bipolar disorder: prospective data from the Systematic Treatment Optimization Program for Early Mania (STOP-EM).

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These data were presented at the 4th Biennial Conference of the International Society for Bipolar Disorders, March 17-20, 2010, São Paulo, Brazil.

Dr. Kunz received the 2010 Samuel Gershon Award for Junior Investigators from the International Society for Bipolar Disorders for an earlier version of this report.

Abstract

Background: Recently, increasing attention has been directed towards assessing the role of biomarkers in psychiatric disorders. Bipolar Disorder (BD) has been associated with dysfunction of neuroplasticity and inflammation. However, the impact of these factors on disease progression and as potential predictors of outcome remains unclear. Objectives: To assess the roles of brain-derived neurotrophic factor (BDNF) levels and the cytokines TNF-alpha, IL-6 and IL-10 as predictors of outcome in BD. Methods: Serum levels of BDNF and the cytokines TNF-α, IL-6, and IL-10 were measured in 25 patients with BD at the time of recovery from their first manic episode. Patients were followed prospectively for one year, and the number of mood episodes and the number of days spent with mood symptoms were assessed using NIMH Life Charts. Results: Baseline IL-6 levels were greater in patients who experienced at least one depressive episode during follow-up than in patients who did not (F=6.759, p=0.017). No other biomarker was differently expressed in patients with and without depressive relapse. Also, IL-6 (r=0.46, p=0.021) and Body Mass Index (r=-0.433, p<0.031) were the only variables correlated with number of days depressed during the one year follow-up. In a linear regression model, IL-6 significantly predicted number of days depressed during follow-up (R^2=0.211; F=6.161; p=0.021). Conclusions: The pro-inflammatory cytokine IL-6 is positively correlated with the number of days depressed during a one-year follow up after a first manic episode in patients with BD. To our knowledge this is the first report to

prospectively assess a serum biomarker as a potential predictor of outcome in BD.

Introduction

Bipolar Disorder (BD) is a chronic and highly disabling mood disorder characterized by high rates of relapse, persistent subsyndromal symptomatology, significant psychosocial dysfunction, elevated mortality owing to suicide and an increased incidence of serious medical disorders (Yatham LN et al. 2005).

Even with access to modern medications, most patients with BD experience high levels of morbidity, spending almost one-half of their lives with mood symptoms, most of which are depressive (Judd LL et al. 2002). Our group has previously shown that more than 40% of patients who experience their first manic episode will present at least one depressive episode during the first year after diagnosis (Yatham LN et al. 2009a).

Since improving the prediction of illness course in BD is key to better guiding treatment planning, recent attention has focused on clinical characteristics associated with mood recurrence (Tohen M et al. 2003) and functional outcomes (Rosa AR et al. 2009). To date, however, no studies in BD have examined the utility of biological markers in predicting clinical outcomes.

Neurotrophic and inflammatory markers have been shown to be associated with both symptomatology and disease progression in BD. Therefore, they are promising tools for prediction of treatment response and staging. A staging model has recently been proposed that emphasizes the potential use of biomarkers for differentiating early and late-stage BD patients in the inter-episodic period

(Kapczinski et al. 2009). However the ability of this model to predict outcomes has not yet been prospectively validated.

We recently reported cross-sectional data comparing levels of brain-derived neurotrophic factor (BDNF) and inflammatory cytokines (TNF-alpha, IL-6 and IL-10) in first-episode patients with BD and healthy subjects with no personal or family history of BD. Our results demonstrated that alterations in IL-6 and IL-10 can already be observed in an early stage of illness when compared with healthy subjects, while more prominent changes including other markers can be seen in patients at later stages (Kauer-Sant'Anna et al. 2009)

The current report examines prospective data from a cohort of patients in the 12-months following their first mania with the objective of investigating the properties of these biomarkers in predicting recurrence of mood episodes.

Patients and Methods

Twenty-five patients with BD type 1 were recruited from the University of British Columbia Hospital and Vancouver General Hospital through the Systematic Treatment Optimization Program for Early Mania (STOP-EM).

STOP-EM is a prospective naturalistic study of clinical outcomes, functional status, quality of life, cognitive functioning, brain morphology, and neurochemistry in patients who recently recovered from their first DSM-IV-TR-defined manic or mixed episode. STOP-EM and the procedures described here were approved by the University of British Columbia Clinical Research Ethics Board, and written informed consent was obtained from all patients and healthy subjects prior to any study procedures taking place (Yatham LN et al. 2009a).

Patients aged 14 to 35 years who experienced their first manic or mixed episode within the three months preceding recruitment were eligible to be enrolled in the program. Maintenance treatment for BD was provided by physicians with expertise in the management of mood disorders, according to clinical practice guidelines from the Canadian Network for Mood and Anxiety Treatments (CANMAT) (Yatham LN et al. 2009b).

Assessment of Demographic and Clinical Outcomes

At enrolment into STOP-EM, the diagnoses of BD and first manic episode were confirmed using the Mini International Neuropsychiatric Interview (MINI) (Sheehan DV et al. 1998). Sociodemographic information and data regarding prior depressive and hypomanic episodes were collected using a standardized protocol. Details of the study protocol have been published in a previous report (Yatham LN et al. 2009a). As part of the clinical assessment, patients underwent a physical examination, including height and weight at enrolment and every 6 months. Body Mass Index (BMI) was calculated using the formula BMI=weight (kg)/height (m)^2.

Psychiatric status at enrolment was assessed by clinical interview and using clinical rating scales, including the Young Mania Rating Scale (YMRS) (Young RC et al. 1978), Hamilton Depression Rating Scale, 29-item version (HAM-D-29) (Williams JBW et al. 1988). For inclusion in this study, all patients had to be euthymic at baseline according to DSM-IV criteria. Functioning was assessed with the Global Assessment of Functioning scale (GAF; APA, 2000). At each 6-month visit patients completed an NIMH Life Chart (Roy-Byrne P et al. 1985). We used data from the life chart, supplemented by clinical interview and collateral information, to determine if the patient experienced a relapse during the preceding 6 months and to quantify the number of days spent with depression, mania, and hypomania. Most of the patients had blood samples collected for biochemical analyses at the time they were enrolled into STOP-EM. Patients that had samples collected after initial enrolment, had their follow up periods

considered from the moment of blood sampling and then followed naturalistically for one year.

Biochemical assays

Ten milliliters of blood were drawn from each subject by venepuncture into a free-anticoagulant vacuum tube. The blood was immediately centrifuged at 3000g for 5 min, and serum was kept frozen at -80 $^{\circ}$C until assayed.

BDNF assay

BDNF serum levels were measured with sandwich-ELISA, using a commercial kit according to the manufacturer's instructions (Chemicon, Temecula, CA, USA). Briefly, 96-well, flat-bottomed microtitre plates were coated for 24 h with the samples diluted 1:2 in sample diluents and the standard curve ranged from 7.8 to 500 pg BDNF. Plates were then washed four times with wash buffer, monoclonal anti-BNDF rabbit antibody was added (diluted 1:1000 with sample diluents) and incubated for 3 h at room temperature. After washing, a second incubation with peroxidase conjugated anti-rabbit antibody (diluted 1:1000) for 1 h at room temperature was performed. After the addition of streptavidin enzyme, substrate and stop solution, the amount of BDNF was determined (absorbance set at 450 nm). The standard curve demonstrates a direct relationship between optical

density (OD) and BDNF concentration. Total protein was measured by Lowry's method using bovine serum albumin (BSA) as a standard. The assay sensitivity for BDNF was 7.8 pg/ml (range 7.8–500 pg/ml) and the intra-assay coefficient of variation (CV) was 26%.

Cytokine assay

The serum concentration of cytokines was measured using enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene high-binding, 96-well microtitre plates (Nunc-Immuno Plate; Maxisorp, Rochester, NY, USA) were coated with capture antibody. After overnight incubation at 4 ^{o}C, the plates were washed (as in subsequent steps) with PBS containing 0.05% Tween-20 and 0.4 M NaCl and then incubated in dilution buffer (PBS containing 1.0% BSA; 100 ml per well) for 2 h at room temperature to block non-specific binding. After washing, the diluted (1:10 in PBS buffer ; pH 7.4) samples (100 ml per well), or the serially diluted standards of each cytokine were added to the plates and incubated overnight at 4 ^{o}C. After washing the plates, the 100 ml of peroxidase-conjugated antirabbit IgG (Abcam, Cambridge, UK) was added to each well and the plates were incubated for 1 h at room temperature. After further washing, colour development was initiated by the addition of substrate and was allowed to develop for up to 30 min at room temperature and terminated by the addition of 4 M sulphuric acid. Antibody binding was determined from absorbance at 490 nm,

and the concentration of cytokines was calculated from a standard curve. The sensitivity and intra-assay CV for cytokine assays, respectively, were: TNF-a (<2 pg/ml, 2.08–60 pg/ml; CV=33%), IL-6 (<10 pg/ml, 15.6–250 pg/ml, CV=17%), and IL-10 (<1.30 pg/ml, 1.56–50 pg/ml, CV=32%). Laboratory procedures were performed in a double-blind fashion. All assays were performed in duplicate.

Statistics

Statistical analyses were performed using PASW Statistics software version 17.0 (SPSS Inc., Chicago, IL, USA). Our primary outcome of interest was the baseline serum levels of each of the biomarkers in patients with and without a depressive relapse. Sociodemographic and clinical characteristics were compared between patients with and without depressive relapse using χ^2 tests or Fisher's exact test for dichotomous variables, and t-tests for continuous variables. The main comparisons of serum levels of BDNF and cytokines were performed including the two groups (patient who experienced a depressive recurrence during one year of follow up and patients who did not) in a model with age, gender, education, BMI and type of mood stabilizer as covariates (ANCOVA).

A correlation (Pearson coefficient) matrix was performed to examine the relationship of BDNF levels, TNF-alpha, IL-6 and IL-10 with age, age of onset, length of illness, education, number of mood episodes and number of days in a mood episode during follow-up. Based on these correlations, significant

associations were entered as covariates in a linear regression model to examine the role of the serum biomarkers as predictors of number of days in a mood episode. All statistical tests were two-tailed and were performed using a significance level of 0.05.

Results

Of the 25 patients who completed one year of follow up after having their blood samples collected at baseline, 15 experienced at least one depressive episode during this period. Two patients had one new manic episode and three patients reported hypomanic episodes. However, these five patients presented depressive episodes prior to their manic or hypomanic periods. Therefore, we decided to divide our sample between patients with or without a depressive recurrence during follow up.

Baseline sociodemographic and clinical characteristics of patients with and without depressive recurrences are enumerated in table 1. Data is presented as *mean (SD)* for continuous variables and as *% (N)* for categorical variables. Patients with and without depressive recurrences did not significantly differ with respect to age, sex, ethnicity and education. Different frequencies of use of medications were not statistically significant. Clinical characteristics including duration of first manic episode, number of previous depressions, age of onset as well as baseline scores for HAMD, YMRS and GAF were not significantly different between the two groups. However, patients who did not experience a depressive recurrence (and therefore no recurrence of any mood episode) had a greater baseline BMI (25.6 Kg/m^2) than patients who did (22.6 Kg/m^2; F=2.894, df=23, p=0.019).

Figure 1 shows the results for baseline serum levels of BDNF, IL-6, IL-10 and TNF-alpha. There was no significant difference in BDNF (0.9±0.2 vs. 0.8±0.2 pg/uL, df=1, F=0.009, p=0.925), IL-10 (11.0±2.2 vs. 11.0±2.5 pg/mL, df=1, F=0.263, p=0.614) or TNF-alpha (12.1±2.6 vs. 14.1±5.3 pg/mL, df=1, F=10.841, p=0.478) levels between patients with and without depressive recurrence in ANCOVA models with age, gender, education and BMI as covariates.

IL-6 was the only biomarker to present significantly different levels between the to groups, being higher on patients who experienced a depressive recurrence during follow up (10.7±1.6 vs. 12.4±2.3 pg/mL, df=1, F=6.309, p=0.021).

Next, we examined the correlation between BDNF and cytokines with the number of days depressed as assessed using NIMH Life Charts. There was a significant positive correlation of number of days depressed and IL-6 (r=0.46, p=0.021). There was also a negative correlation between BMI and number of days depressed (r=-0.433, p=0.031). There was no correlation of these clinical variables with IL-10, IL-6 and TNF-alpha. Age and BMI were not correlated with any biochemical measure in our sample.

Finally, we performed a linear regression model, using the forced entry method, with number of days depressed as the dependent variable and IL-6 and BMI as the two predictors. This analysis indicated that both IL-6 (Beta=0.444; t=2.651; p=0.015) and BMI (Beta=-0.416; t=-2.486; p=0.021) were significant predictors of the number of days depressed (R^2=0.384; F=6.864; p=0.005).

If considered alone, IL-6 predicted 21% of the variation in number of days depressed during follow up (R^2=0.211; F=6.161; p=0.21)

Discussion

Our results demonstrate that baseline IL-6 levels are greater in patients with BD1 who experience at least one depressive recurrence during one year of naturalistic follow up, when compared with those who do not experience any mood recurrence. Moreover, IL-6 levels significantly predicted the number of days depressed during follow up. Interestingly, baseline BMI significantly improved our regression model when entered as a covariate. IL-6 and BMI levels were not correlated in our sample, thus no multicollinearity was observed. BDNF, TNF-alpha and IL-10 were not associated with depressive recurrence in our sample.

Our findings extend those of previous research into the differences of inflammatory markers and BDNF in patients at early and late stages of BD (Kauer-Sant'Anna et al. 2009). In that study, the pro-inflammatory cytokines TNF-alpha and IL-6 were shown to be increased in the early and late stages of the illness compared to controls, whereas BDNF levels were decreased in the late but not the early stage, and levels of the anti-inflammatory cytokine IL-10 declined with disease progression. These and other findings indicate that patients with BD are likely to be in a pro-inflammatory state (Brietzke & Kapczinski 2008; Brietzke et al. 2009a), which worsens in the later stages of the illness as the anti-inflammatory response abates. Furthermore, greater age-

related decreases in BDNF levels are found in patients with BD when compared to controls (Yatham et al. 2009d).

Neurotrophins and the inflammatory system can interact in a number of ways. BDNF and cytokines appear to cooperate in intracellular signaling (Brietzke and Kapczinski, 2008). It is conceivable that the increase in cytokines and a decrease in BDNF may synergistically function in favor of neuronal toxicity in BD, as BDNF levels decrease (Cunha et al., 2006) and pro-inflammatory cytokines increase (Brietzke et al. 2009b) during mood episodes. If particularly focusing on IL-6, it was found to be increased in acutely depressed patients when compared with control subjects (Brietzke et al. 2009b). In fact, increased IL-6 has been one of the most consistent findings in BD (Goldstein BI et al. 2009). This raises the question of whether the predictive value of IL-6 in our sample was in fact a reflection of underlying inflammatory process that will culminate in clinical depression but are at that time associated with minimal or at most subsyndromic symptomatology. The majority of previous studies have been cross-sectional, which prevents us from clarifying the matter of causality. However, a recent 6-year prospective cohort study focused on the directionality of the depression-inflammation relationship, by measuring C-reactive protein and IL-6 levels in healthy, older adults. They interestingly found that depression may lead to inflammation and that inflammation may be one of the mechanisms which depression contributes to cardiovascular risk (Stewart JC et al. 2009). The same study also demonstrated BMI as a predictor of IL-6 change.

There is a well-established association between behavioral alterations, including depression, and metabolic syndrome. Moreover, recent findings suggest that chronic activation of inflammatory response might be involved (Capuron L et al. 2008). Most of the previous studies examining the role of inflammatory cytokines in BD have not controlled for BMI. Studies that did include known confounding variables such as obesity and smoking did not find that they were significantly associated with inflammation (Goldstein BI et al. 2009). Interestingly, in our sample, BMI was the only statistically significant clinical variable between groups, with patients that did not experience recurrence having a greater BMI at baseline that the ones who did. Moreover, BMI was negatively correlated with number of days depressed and did not correlate with IL-6 levels, which suggest a dissociation of the two mechanisms, with higher inflammatory response not explained by higher BMI. The effects of medication might explain this association of BMI and number of days depressed in our sample; however, our study design precludes any conclusion. We have recently reported data addressing the issue of weight gain after a first manic episode (Bond DJ et al. [Submitted]). What we found was that patients with clinically significant weight gain spent more days depressed, though this did not reach significance. Patients with a high baseline BMI were less likely to experience significant weight gain and so this might also explain the negative association between the baseline BMI and depression in our sample.

There were some limitations of this study. First, the sample size may have restricted the ability to detect smaller effect sizes of other predictors of

recurrence. In the same line, due to our limited number of patients, we included even the ones who did not present a depressive episode during follow up in the regression analysis, thus the scatter observed in levels about the lower number of days depressed in the regression line. Second, the study design does not allow an assessment of the impact of different treatments on outcome. And also, even though the NIMH Life Chart is a validated instrument shown to be highly correlated with both depressive and manic symptomatology rating scales (Denicoff KD et al. 2000), the subjectivity involved raises the matter of reliability of a self-rating assessment, which is a potential bias of any self-rated instrument.

Nonetheless, the current study has several strengths. STOP-EM has broad inclusion criteria and few exclusion criteria, and the patients in our sample are thus highly reflective of those seen in routine clinical practice. A particular strength of this study is its prospective design. Prospective studies starting near illness onset are especially valuable for being less confounded by prolonged illness and comorbidity. To our knowledge, this is the first report of a serum biomarker as a predictor of clinical outcome in BD.

The recognition and understanding of underlying neurotrophic and inflammatory mechanisms of disease susceptibility and progression in BD will certainly provide better insight into preventive treatments and interventions.

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Table 1. Baseline Sociodemographic and Clinical Variables

	No Recurrance (N=10)	Recurrance (N=15)	p-value
Age	25 (4.0)	21 (3.6)	0.17[b]
Sex	20% (2)	53.3% (8)	.211[a]
Education	14.8 (2.5)	13.2 (1.5)	.074[b]
Ethnicity	60% (6)	73.3 % (11)	.783[c]
BMI	25.6 (3.7)	22.6 (2.3)	.019[b]
Lithium	30% (3)	53.3% (8)	.414[a]
Valproate	60% (6)	40% (6)	.428[a]
Antipsychotic	70% (7)	80% (12)	.653[a]
Antidepressant	0%	13.3% (2)	.500[a]
Duration of first mania	90.5 (81.3)	94.7 (90.0)	.90[b]
Number of previous depression	0.9 (1.9)	0.8 (0.9)	.95[b]
Age of onset	22.6 (3.7)	19.4 (3.8)	.058[b]
HAMD	7.3 (9.4)	12.2 (11.7)	.275[b]
YMRS	2.6 (4.5)	6.2 (8.3)	.226[b]
GAF	67.5 (13.3)	58.1 (12.4)	.087[b]

Data is presented as *mean (SD)* for continuous variables and *% (N)* for categorical variables.
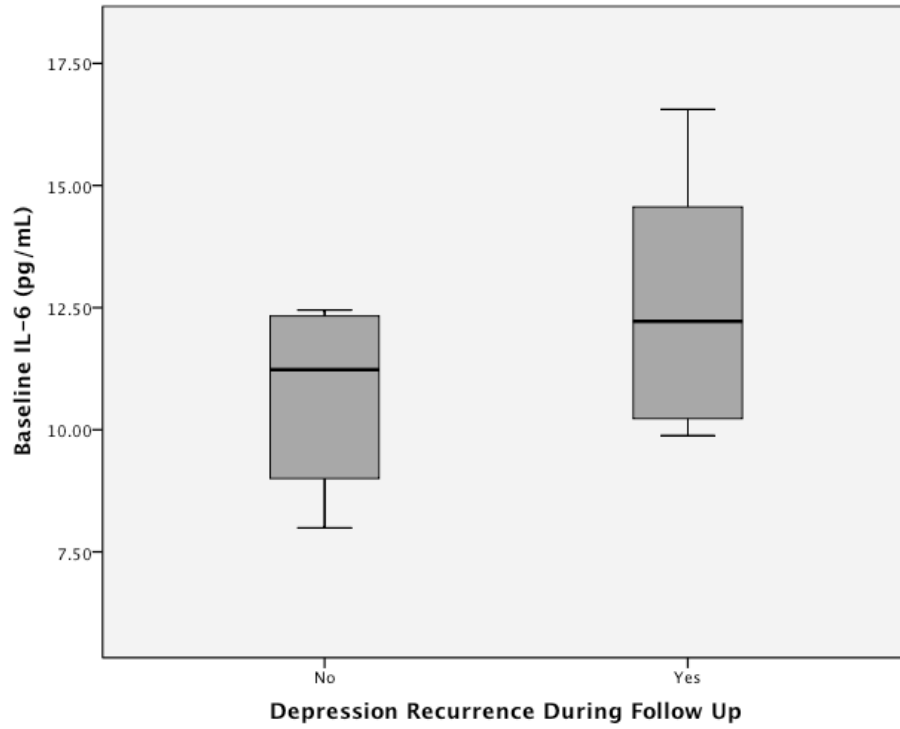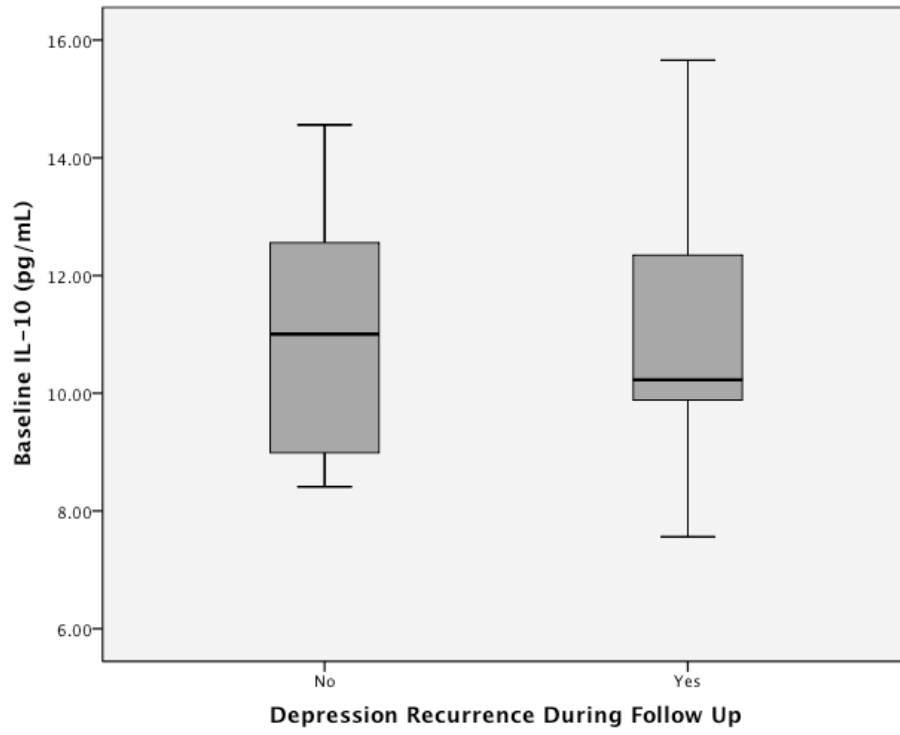
[a] Fisher's exact test.

[b] *t* test.

[c] X^2 test.

Figure 1. Mean serum levels of IL-6, IL-10, TNF-alpha and BDNF for patients who did and did not present a depressive recurrence during one-year follow up. ANCOVA model with age, gender, education, BMI and type of mood stabilizer as covariates.
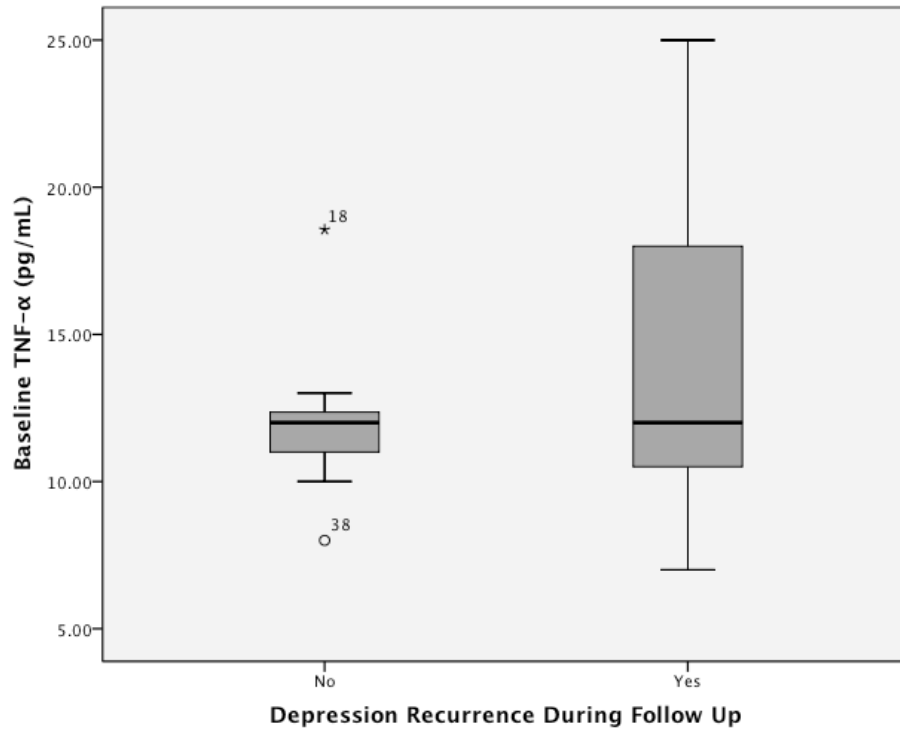
(a) F=6.759, p=0.017

(b) F=1.007, p=0.328

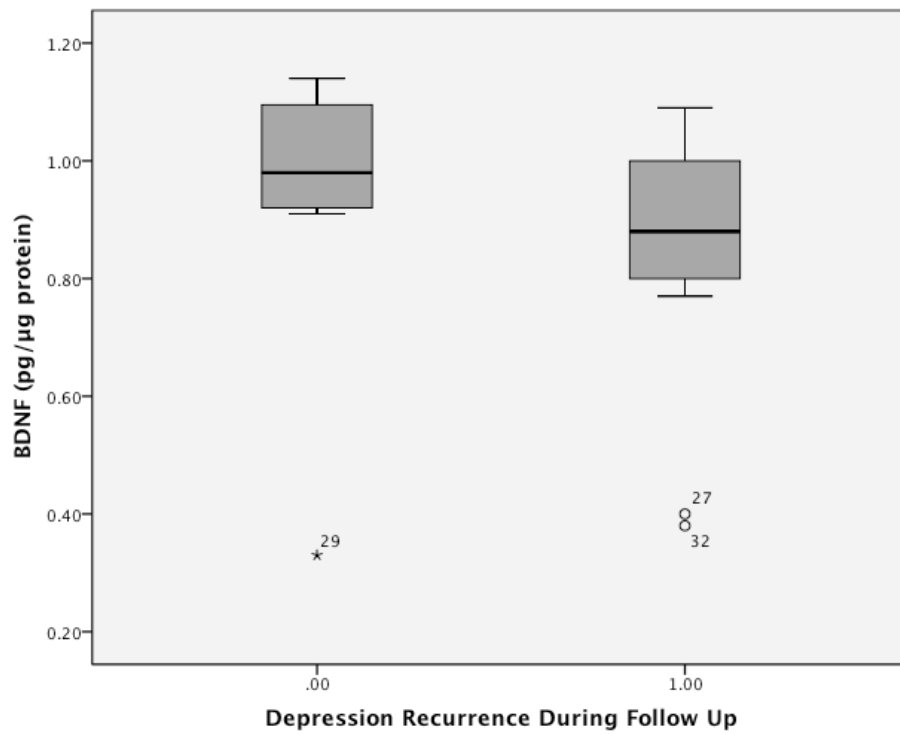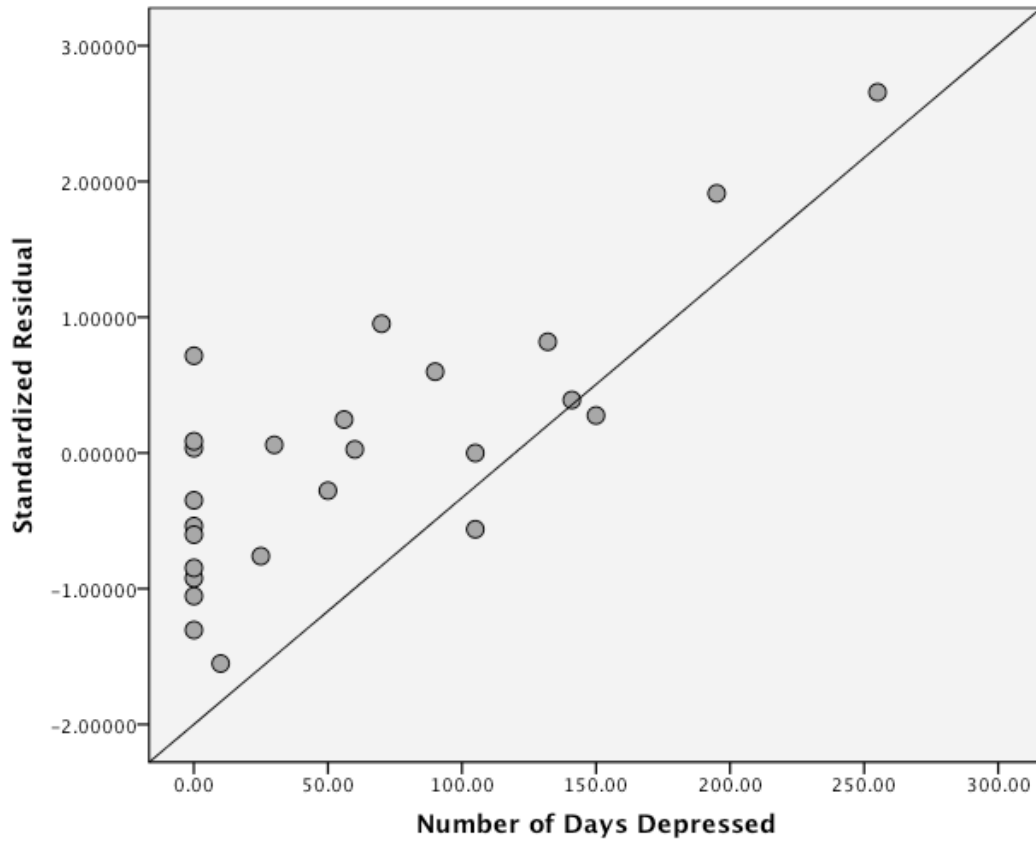(c) F=1.023, p=0.324

(d) F=0.749, p=0.40

Figure 2. Correlation between IL-6 levels at baseline and number or days in a mood episode during follow up. Linear regression with BMI as covariate: Beta=0.46, p=0.005, R^2=0.384, Adjusted R^2=0.328)

6. CONSIDERAÇÕES FINAIS

A pesquisa de biomarcadores segue um "pipeline" semelhante à pesquisa de medicações: da descoberta, passando pela avaliação inicial, pesquisa exploratória em diferentes condições e estágios de doença, seguindo para a validação clínica (Paulsen, 2009). Os estudos apresentados nessa tese colaboram para a pesquisa exploratória do papel das citocinas como potenciais biomarcadores.

Numa avaliação global, o primeiro estudo corrobora a idéia de que existe um estado pró-inflamatório associado com Transtorno Bipolar e com Esquizofrenia. Dentro do objetivo de comparar os níveis de citocinas entre os dois diagnósticos, observamos que esse estado pró-inflamatório apresenta-se de forma diferente em cada transtorno.

Tanto pacientes com Transtorno Bipolar quanto com Esquizofrenia apresentaram níveis aumentados de IL-10 em relação a controles. No entanto, os dois se diferenciam pelo aumento de L-6 encontrado na Esquizofrenia mas não no Transtorno Bipolar. Estes resultados evidenciam uma ativação imune crônica na Esquizofrenia enquanto o Transtorno Bipolar parece apresentar um aumento da atividade inflamatória relacionado ao episódio de humor.

Nesse sentido, esses achados parecem fortalecer a diferenciação clássica dos dois transtornos quanto ao curso crônico e deteriorante da Esquizofrenia e episódico porém com períodos de recuperação no Transtorno Bipolar.

Interessante observar também que nenhum dos grupos apresentou alterações de TNF-α em relação a controles. Em estudos realizados anteriormente, o TNF-α destacou-se sobretudo como um marcador de sintomatologia aguda (Brietzke & Kapczinski, 2008). No entanto, a comparação entre pacientes com Transtorno Bipolar em estágios iniciais de doença com pacientes em estágios mais avançados encontrou um aumento de níveis de TNF-α nos pacientes com maior tempo de doença (Kauer-Sant'Anna et al., 2009). Inclusive, as alterações de marcadores inflamatórios ao longo da progressão da doença foram sugeridos como ferramentas adicionais para o estadiamento clínico do Transtorno Bipolar (Kapczinski et al., 2009).

O aumento de IL-6, também previamente relacionado com sintomatologia aguda, durante períodos relativamente assintomáticos, como no caso dos dois grupos de pacientes estudados, pode representar atividade de doença sem expressividade clínica atual. Tal hipótese foi examinada no segundo estudo.

A avaliação de IL-6, IL-10, TNF-α e BDNF no "baseline" de pacientes que recentemente experimentaram seu primeiro episódio maníaco revelou que apesar de assintomáticos no momento da coleta, pacientes que viriam a apresentar um ou mais episódios depressivos ao longo de um ano de seguimento tinham níveis de IL-6 mais elevados em relação aos pacientes que permaneceram eutímicos durante o seguimento. Assim também, o aumento de IL-6 correlacionou-se positivamente com os números de dias com sintomas depressivos referidos pelos pacientes. Apesar do tamanho amostral pequeno e a questão do uso de um instrumento auto-aplicável para a avaliação do número de

dias deprimido, esse possívelmente o primeiro estudo a avaliar de forma longitudinal prospectiva um grupo de pacientes e correlacionando biomarcadores a variáveis clínicas de seguimento. Assim, outros estudos são necessários para confirmar esse achado e potencialmente estabelecer o uso da IL-6 ou outras citocinas como biomarcadores para predição de episódios depressivos na prática clínica.

Como um todo, esses resultados fornecem apoio adicional para a investigação de citocinas como possíveis biomarcadores para a atividade da doença ou resposta ao tratamento.

O uso de biomarcadores, independentemente do contexto, apresenta muitos desafios. Um biomarcador não é a causa de uma doença. Portanto, o atual interesse em biomarcadores é um sinal de que a psiquiatria tem passado por uma mudança metodológica significativa, se afastando em parte da busca por causas de um transtorno para o desenvolvimento de instrumentos que possam otimizar a prática clínica – por exemplo, estimando a probabilidade que um transtorno esteja presente ou venha a se desenvolver. Uma dificuldade derivada de tal abordagem é que variáveis individuais associadas com um aumento de risco para o desenvolvimento de uma condição ou recorrência, por exemplo, geralmente apresentam tamanho de efeitos pequenos: ou seja, quando usados sozinhos, não são preditores robustos da presença ou ausência de uma condição. No entanto, quando agrupados em um algoritmo que incorpora informação de outros biomarcadores, assim como fatores de risco sociais e ambientais, biomarcadores podem se tornar bons preditores. No

entanto, a informação proveniente de biomarcadores sempre será uma estimativa da probabilidade de que uma condição irá se desenvolver: ou seja, uma estimativa de risco. Assim, mesmo algoritmos poderosos para a estratificação de risco manterão um certo grau de incerteza. A esperança é de que essa incerteza diminua com o tempo, na medida que variáveis com maior poder preditivo sejam identificadas (Singh & Rose, 2009)

A relevância de processos inflamatórios a doenças do cérebro e do corpo pode servir como marco importante para a crescente integração da psiquiatria com outras áreas médicas, e pode melhorar o reconhecimento e tratamento de comorbidades psiquiátricas em pacientes com doenças clínicas.

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