Estevão Scotti Muzzi Marques Leitão

Influência de polimorfismos de genes funcionais (*GAD1* rs1978340, *CACNA1C* rs100737 e *BDNF* rs6265) nos neurometabólitos cerebrais em pacientes com transtorno afetivo bipolar e controles saudáveis.

Tese apresentada à Faculdade de Medicina
da Universidade de São Paulo para obtenção
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Programa de Psiquiatria

Orientador: Prof. Dr. Márcio Gerhardt Soeiro-de-Souza

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RESUMO

O transtorno afetivo bipolar (TAB) é caracterizado por instabilidade do humor entre períodos de (hipo) mania e depressão. O córtex do cíngulo anterior (CCA) é a região cortical mais implicada na neurobiologia do transtrono cujas alterações principais envolvem a desregulação do sistema glutamatérgico e neurotróficas. A espectroscopia de prótons por ressonância magnética (^{1}H-MRS) é uma técnica que permite a mensuração *in vivo* de neurometabólitos cerebrais associados à ciclagem Glutamato-Glutamina-GABA [Glx (Glu + Gln), Glutamato (Glu), Glutamina (Gln)], a vias neurotróficas e de neuroplasticidade [N-acetilaspartato (NAA), compostos contendo colina (Cho), mio-Inositol (mI)] bem como no metabolismo energético celular [Creatina (Cr)]. Estudos genéticos têm associado polimorfismos de nucleotídeo único (SNPs) nos genes *CACNA1*C (rs1006737), *BDNF* (rs6265) e *GAD 1* (rs1978340, rs3749034) com o TAB, os quais estão envolvidos, respectivamente, com a formação de canais de cálcio, fatores neurotróficos e homeostase Glu/GABA. Diante disso, os objetivos deste estudo foram: 1-realizar uma meta-análise sobre alterações neurometabólicas no córtex do cíngulo anterior (CCA) no TAB; 2- avaliar a influência dos SNPs do *CACNA1C* (rs1006737), *BDNF* (rs6265) e *GAD 1* (rs1978340 e rs3749034) na quantificação de metabólitos nesta região cortical. O objetivo 1 revelou que as principais alterações neurometabólicas no CCA reveladas pela meta-análise foram elevação de Glx, Gln e Cho no TAB em relação a controles saudáveis, apontando para alterações no sistema glutamatérgico e na ciclagem de fosfolipídios de membrana. Entretanto, ainda é pouco conhecida a dinâmica desses neurometabólitos nos diversos estados de humor do TAB. O objetivo 2 revelou que o aumento dos neurometabolitos glutamatérgicos no TAB-I mostrou-se associado ao alelo A do polimorfismo *GAD* rs3749034, ao genótipo AA do *CACNA1C* rs100737 e ao alelo *val* do polimorfismo *BDNF* rs6265, já nos controles saudáveis, o alelo *met* do *BDNF* rs6265 pareceu conferir neuroproteção, por estar associado a níveis elevados de NAA/Cr. Os polimorfismos *CACNA1C* rs100737 e *BDNF* rs6265 apresentaram efeito pleiotrófico influenciados pelo diagnóstico e sexo. Portanto, a arquitetura poligênica mediada por esses polimorfismos funcionais parece determinar alterações em canais de cálcio e no sistema glutamatérgico, implicados nos processos de excito-toxicidade e plasticidade neuronal no TAB.

Palavras-chave: Córtex do cíngulo anterior. Espectroscopia de prótons por ressonância magnética (^{1}H-MRS). Genética funcional. Neurometabólitos. Transtorno Afetivo Bipolar.

Scotti-Muzzi, Estêvão. Influence of polymorphisms of functional genes (*GAD1* rs1978340, *CACNA1C* rs100737 e *BDNF* rs6265) on brain neurometabolites in Bipolar Disorder and heathy control subjects. [tese]. São Paulo, Instituto de Psiquiatria; 2023.

ABSTRACT

Bipolar Disorder (BD) is characterized by mood instability from episodes of (hipo) mania to depression. The anterior cingulate cortex (ACC) is the cortical region most implicated in the neurobiology of BD whose alterations include disregulaion of the glutamatergic and neurothophic systems. Proton magnetic resonance spectroscopy (^{1}H-MRS) is a technique that allows the *in vivo* measurement of brain metabolites associated with the glutamate-glutamine-GABA cycling [Glx (Glu + Gln), Glutamate (Glu), Glutamine (Gln)], neurotrophic pathways and neuroplasticity [N-acetilaspartate (NAA), choline containing compaunds (Cho), myo-Inositol (mI)] as well as cellular energetic metabolism [Creatina (Cr)]. Genetic studies have associated single nucleotide polymorphisms (SNPs) in the genes *CACNA1*C (rs1006737), *BDNF* (rs6265) and *GAD 1* (rs1978340, rs3749034) with TB, which are, respectively, involved in the formation of calcium channels, neurophic factors and Glu/GABA homeostasis. Thus, the aims of this study were to: 1-perform a meta-analysis on the neurometabolite changes in the ACC of BD subjestcs; 2- assess the influence of the SNPs *CACNA1C* rs1006737, *BDNF* rs6265 and *GAD 1* (rs1978340 e rs3749034) on the ACC neurometabolites. The meta-analysis revealed increased levels of Glx, Gln and Cho within the ACC of BD in relation to healthy controls, suggesting abnormalities in the glutamatergic system and membrane phospholipid cycling. However, it is still poorly understood the dynamics of such metabolites across the different mood states in BD. The second objective revealed that the increase in glutamatergic metabolites was influenced by the allele A of the *GAD* rs3749034, the AA genotype of the *CACNA1C* rs100737 and the *val* allele of the *BDNF* rs6265 polymorphisms, while the *met* allele of the *BDNF* rs6265 appeared to confer neuroprotection to healthy controls associated with enhanced NAA/Cr levels. The polymorphisms *CACNA1C* rs100737 and *BDNF* rs6265 showed a pleiotropic effect modulated by the diagnosis and sex. Therefore, the genetic architecture of these functional polymorphisms determines alterations in calcium channels, glutamatergic systems, which are implicate in the excito-tocixity and neuroplasticity in BD.

Key-words: Anterior cingulate cortex. Bipolar Disorders. Functional genetics. Neurometabolites. Proton magnetic resonance spectroscopy (^{1}H-MRS).

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SUMÁRIO

1. INTRODUÇÃO

O transtorno afetivo bipolar (TAB) é um transtorno psiquiátrico grave e crônico caracterizado por oscilações no estado de humor entre períodos de mania/hipomania e depressão (Merikanga et al., 2007). Apesar dos avanços significativos do conhecimento acerca da neurobiologia do TAB, ainda estamos distantes de sua total compreensão. Estudos recentes de neuroanatomia estrutural e funcional apontam para uma desintegração dos circuitos fronto-límbicos como um dos prováveis mecanismos fisiopatológicos para o TAB (Maletic; Raison, 2014; Strakowski et al., 2012), com evidências de progressiva perda neuronal durante o curso da doença, sobretudo no córtex do cíngulo anterior (CCA) (Hibar et al., 2017; Maletic; Raison, 2014).

A literatura aponta o CCA como a principal região cortical implicada no TAB. Nesta região, ocorrem conexões neuronais associadas ao controle cognitivo (dorsais) e regulação emocional (ventrais), ligando regiões frontais e límbicas (Maletic; Raison, 2014; Strakowski et al., 2012). No TAB, tem sido descrito redução no volume da substância cinzenta do CCA (Drevets et al., 1997; Haldane; Frangou, 2004; Strakowski et al., 2012; Hanford et al., 2016; Wise et al., 2017; Hibar et al., 2017) assim como anisotropia reduzida em tratos de substância branca (Vederine et al., 2011; Nortje et al., 2013). Além disso, a quetamina, um agente antagonista de receptores glutamatérgicos, tem sido relacionada com a modulação da conectividade funcional entre o CCA e o córtex pré-frontal (PFC) (Lenner et al., 2017).

Além das alterações estruturais, alterações neuroquímicas também têm sido relatadas no CCA em pacientes com TAB, evidenciadas pela técnica de espectroscopia de prótons por ressonância magnética (^{1}H-MRS). Este é um instrumento útil para mensuração *in vivo* de diversos neurometabólitos tais como: Glutamato (Glu), Glutamina (Gln), Glx (Glu + Gln), GABA, N-acetilaspartato (NAA), colina (Cho), mio-inositol (mI) e creatina (Cr) (Buonocore; Maddock, 2015). Em relação às alterações neuroquímicas do CCA registradas em pacientes com TAB, a literatura mostra níveis elevados de metabólitos glutamatérgicos (Dager et al., 2004; Frye et al., 2007; Öngür et al., 2008; Gigante et al., 2012; Soeiro-de-Souza et al., 2013; Soeiro-de-Souza et al., 2018a) e Cho (Yildiz-Yesiloglu; Ankerst, 2006; Galińska-Skok et al., 2016, Soeiro-de-Souza et al., 2018b), assim como níveis reduzidos de NAA (Kraguljac et al., 2012; Soeiro de Souza et al., 2018b).

No TAB, a elevação dos metbólitos glutamatérgicos (Yüksel; Öngür, 2010; Gigante et al., 2012; Chitty et al., 2013) tem sido relacionada a níveis aumentados do cálcio (Ca^{2+}) intracelular e a um possível estado de hiper-excitabilidade neuronal (Mehta et al., 2013), enquanto a redução de NAA tem sido considerada um marcador de comprometimento da viabilidade neuronal e disfunção mitocondrial (Clay, Sillivan; Konradi et al., 2011; Stork; Renshaw, 2005; Moffett et al., 2007). Por outo lado, a elevação de Cho é comumente atribuída a uma ciclagem aumentada de fosfolipídeos de membrana (Stork; Renshaw,

2005). Postula-se, pois, que tais alterações neuroquímicas estariam relacionadas a anormalidades nas cascatas de sinalização intracelular de vias neurotróficas associadas à neuroplasticidade resultando em alterações neuromorfométricas no TAB no longo prazo (Berk et al., 2011). A despeito da abundante literatura acerca de alterações neuroquímicas mensuradas pela técnica de ^{1}H-MRS no CCA no TAB, não havia, até a data de início desse trabalho, nenhum estudo de meta-análise focada nesta região cortical. Os trabalhos prévios de revisão se restringiram a avaliar o perfil neuro-metabólico em diversas regiões corticais (Yildiz-Yesiloglu; Ankerst, 2006; Kraguljac et al., 2012) ou apenas estudar neuro-metabólitos específicos tal como o Glu (Yüksel; Öngür, 2010; Gigante et al., 2012; Chitty et al., 2013). Além disso, poucos estudos levaram em consideração o efeito dos diferentes estados de humor (hipomania, mania, depressão e estado misto) deste complexo transtorno bem como da medicação nos neurometabólitos. Desta forma, faz-se necessária a melhor compreensão acercado perfil neuro-metabólico do CCA mo TAB.

O TAB apresenta um caráter hereditário importante (70–90%) resultante do somatório de múltiplas variações genéticas que, isoladamente, confererm risco baixo ao transtorno. Além disso, muitas dessas variações genéticas apresentam sobreposição com outros transtornos psiquiátricos como esquizofrenia e o transtorno depressivo maior. Entretanto, os aspectos funcionais relacionados a tais variações genéticas permanecem obscuros no TAB (Gordovez; McMahon, 2020). A técnica de associação genômica ampla (GWAS) é particularmente útil para detectar variações funcionais na escala de milhão em todo o genoma, conhecidas como polimorfismos de nucleotídeo único (SNP) (Craddock; Sklar, 2013; Ikeda et al., 2018; Stahl et al., 2019; Li et al., 2022, já tendo sido identificados mais de 30 loci associados ao TAB, sendo alguns deles codificadores de canais iônicos, neurotransmissores e componentes sinápticos (Stahl et al., 2019). Dentre eles, destaca-se a variação funcional no gene *CACNA1*C (rs1006737), a qual tem sido reportada por diversos estudos genéticos de associação ampla (GWAS) (Ferreira et al., 2008; Sklar et al., 2008, Stahl et al., 2019), estudos de associação com genes candidatos (Khalid et al., 2018; Mosheva et al., 2020), análises de risco genético (Croarkin et al., 2018) bem como por revisões (Ou et al., 2015; Gordovez; McMahon, 2020; Harrison et al., 2022, Li et al., 2022) e meta-análises (Liu et al., 2011; Nurnberger et al., 2014). Esse gene, localizado no braço curto do cromossomo 12, codificando a subunidade alfa-1 do canal de Ca^{2+} voltagem dependente, apresenta o alelo A como fator de risco para o TAB. O estudo de genes de risco com funções biológicas conhecidas como o *CACNA1*C é um caminho promissor para melhor compreender a fisiopatologia desse complexo transtorno (Gordovez; McMahon, 2020; Li et al., 2022).

De fato, a função da subunidade alfa-1 do canal de Ca^{2+} voltagem dependente, codificada pelo *CACNA1*C, é regular a entrada de Ca^{2+} no meio intracelular, tendo implicações em processos de transcrição gênica, vias de sinalização intracelular, excitabilidade neuronal, liberação de

neurotransmissores, plasticidade sináptica, formação de memória, aprendizagem e comportamento (Berger; Bartsch, 2014). Em controles saudáveis, o genótipo AA tem sido associado à maior expressão de RNA mensageiro quando comparado aos genótipos AG e GG, o que parece determinar nível aumentado de cálcio intracelular em carreadores do alelo A (Bigos et al., 2010; Uemura; Green; Warsh, 2016). Também em indivíduos saudáveis, o alelo A deste gene tem sido implicado no aumento global de massa cinzenta (Kempton et al., 2009; Wang et al., 2011), bem como da amígdala, hipotálamo (Perrier et al., 2011) e tronco cerebral (Franke et al., 2010), embora alguns autores não confirmaram esses achados (Franke et al., 2010; Soeiro-de-Souza et al., 2012). Além disso, a conectividade frontolímbica (Jogia et al., 2011; Wang et al., 2011; Radua et al., 2013; Dima et al., 2013) e o desempenho cognitivo (Arts; Simons; Os, 2013; Soeiro-de-Souza et al., 2013) parecem ser modulados por tal alelo em indivíduos saudáveis.

No TAB, portadores do alelo de risco A do *CACNA1*C rs1006737 apresentaram redução cortical em regiões parietal, frontal, orbito-frontal e do cíngulo anterior rostral em comparação com o alelo GG (Smedler et al., 2019). Similarmente, Soeiro-de-Souza et al. (2017) observaram redução da espessura cortical do CCA caudal esquerdo em portadores do alelo de risco A em relação aos não carreadores, a qual mostrou-se influenciada pelo tempo de doença. Entretanto, as bases neuroquímicas da influência do *CACNA1C* rs1006737 no TAB permanecem desconhecidas.

Embora o papel modulador do gene *CACNA1*C na plasticidade neuronal ainda seja pouco entendida, ela se dá presumivelmente pela função reguladora do Ca^{2+} (Uemura; Green; Warsh, 2016), através da ativação de vias de sinalização intracelulares dependentes deste íon. Dentre elas, destacam-se aquelas mediadas pela ativação da calmodulina (caM) e por proteínas quinases dependentes de Ca^{2+} bem como pela atividade fosforilativa de proteínas quinase ativadas por mitógeno (MAPK) e quinases regulada por sinal extracelular (ERK) (Berridge, 2014). Estas vias regulam a transcrição de fatores como o CREB (“cyclic AMP-responsive element-binding protein”) (Berger; Bartsch, 2014; Berridge, 2014; Kabir et al., 2017), responsável pela transcrição de genes relevantes para o TAB como o *BDNF* (fator neurotrófico derivado do cérebro) e o *Gad1* (Berridge, 2014), gene responsável pela codificação da proteína GAD 67 que catalisa a descaboxilação do Glu em GABA no SNC.

O BDNF é sintetizado em neurônios e na micróglia e está envolvido em processos de crescimento, sobrevivência, transmissão sináptica e plasticidade neuronal (Parkhurst et al., 2013). É liberado em duas formas: pró-BDNF e BDNF maduro. Enquanto o primeiro inicia cascatas pró-apoptóticas, o segundo induz cascatas anti-apoptóticas, envolvidas nos processos de neuroplasticidade e resiliência (Chen et al., 2004; Nagappan; Lu, 2005). No TAB, estudos *post-mortem* reportam decréscimo do mRNA do BDNF cerebral total (Kim; Rapoport; Rao, 2010) e estudos *in vivo* reportam níveis séricos reduzidos de BDNF nas fases de mania e depressão (Cunha et al., 2006; Machado-Vieira et al., 2007;

Fernandes et al., 2011; Munkholm; Vinberg; Kessing, 2016), mas não na eutimia (Fernandes et al., 2011; Munkholm; Vinberg; Kessing, 2016).

O gene regulador da síntese de BDNF localiza-se no cromossomo 11 (11p14.1) e apresenta um polirfismo (*BDNF*rs6265) com 3 possíveis genótipos: *val66val*, *val66met* e *met66met*. A frequência do alelo *met* varia substancialmente na população mundial (Petryshen et al., 2010), sendo o homozigoto *met/met* mais raro em populações europeias (Shimizu; Hashimoto; Iyo, 2004) e mais frequente em populações asiáticas (Pivac et al., 2009). O alelo *met* do polimorfismo *BDNF* rs6265 tem sido associado a transporte deficitário de mRNA aos dendritos e empacotamento reduzido do BDNF em células hipocampais (Egan et al., 2003; Baj et al., 2011). O alelo *val* do *BDNF* rs6265 – e não o *met* – tem sido consistentemente associado ao TAB (Neves-Pereira et al., 2002; Sklar et al., 2002; Li; Chang; Xiao, 2016; Paul et al., 2021), embora alguns estudos de associação genética não tenham documentado tal relação (Hong et al., 2003; Hong et al., 2022; Kanazawa et al., 2007; Gonzalez-Castro et al., 2015).

Entretanto, pouco se sabe sobre a influência do polimorfismo *BDNF* rs6265 nos neurobetabólitos cerebrais no TAB e controles saudáveis. Gruber et al. (2012) relataram níveis reduzidos de NAA/Cr e Glx/Cr no hipocampo de portadores do alelo *met* em comparação com homozigotos *val/val* em uma população composta por 66 esquizofrênicos, 45 bipolares e 47 indivíduos saudáveis. Similarmente, Stern et al. (2008) observaram níveis baixos de NAA/Cr no hipocampo de 69 indivíduos saudáveis poradores alelo *met* em relação aos homozigotos *val*. Por outro lado, Gallinat et al. (2010) e Martens et al. (2021) encontraram níveis elevados de NAA/Cr no CCA de portadores saudáveis do alelo *met*.

O gene *GAD1*, localizado no cromossomo 2q31.1, é responsável pela codificação da enzima ácido glutâmico descarboxylase (GAD1), cuja isoforma GAD67 é a principal reponsável pela conversão de Glu em GABA no SNC (Geller et al., 2004). Em estudos *post-mortem* com pacientes acometidos pelo TAB, as regiões corticais pré-frontal, cíngulo anterior, hipocampo e cerebelo apresentaram baixa expressão de RNA do gene *GAD*1 (Guidotti et al., 2000; Heckers et al., 2002; Fatemi et al., 2005; Thompson et al., 2009; Volk et al., 2016). Embora polimorfismos funcionais (SNPS) no gene *GAD* 1 (rs1978340, rs872123, rs3749034) têm sido associados ao TAB (Chung et al., 2017; Geller et al., 2004; Lundorf et al., 2005; Arrúe et al., 2019), pouco se sabe sobre seu efeito nos neurometabólitos cerebrais. Em controles saudáveis, Marenco et al. (2010) associaram níveis elevados de GABA/Cr no CCA ao genótipo AA mas não há relato na literatura sobre a influência desse polimorfismo nos níveis corticais de Glu e GABA em pacientes com TAB.

1.1. Objetivos do estudo

a) Realizar estudo de meta-análise visando compreender o perfil neuro-metabólico (^{1}H-MRS) da região do CCA de pacientes com TAB em relação a controles.

b) Avaliar através de um estudo transversal de ^{1}H-MRS no CCA a influência de polimorfismos funcionais nos genes *GAD1* (rs1978340, rs872123, rs3749034), *CACNA1C* (rs1006737) e *BDNF* (rs6265) nos níveis de neurometabólitos em indivíduos com TAB e controles saudáveis.

1.2. Hipóteses do estudo

Para o estudo transversal, as hipóteses nula e alternativa são:

- **Hipótese nula: A** presença dos alelos de risco dos polimorfismos nos genes *GAD1* (rs1978340, rs872123, rs3749034), *CACNA1C* (rs1006737) e *BDNF* (rs6264) não influenciam os níveis de metabólitos do ACC de pacientes com TAB tipo I eutímicos ou em controles saudáveis.
- **H1:** A presença dos respectivos alelos de risco para o TAB dos polimorfismos *GAD1* (rs1978340, rs872123, rs3749034), *CACNA1C* (rs1006737) e *BDNF* (rs6264) modulam os níveis de metabólitos corticais no ACC de pacientes bipolares tipo I eutímicos em relação a controles saudáveis.

1.3. Justificativa

As revisões e meta-análises realizadas até o momento sobre alterações de metabólitos corticais no TAB focaram apenas em determinados neurometabólitos (ex: Glutamato) ou avaliaram de foma generalista tais alterações em diversas regiões corticais. Ademais, a maioria desses trabalhos prévios não avaliaram as possíveis diferenças de neurometabólitos entre as diversas fases da doença bipolar e tampouco levaram em consideração o efeito das medicações. Em face da inexistência de meta-análise sobre neurometabólitos centradas no CCA, faz-se necessário esse estudo antes de se proceder qualquer estudo translacional tal como a influência de fatores genéticos sobre os neurometálitos no CCA.

Da mesma forma, carece na literatura científica informações acerca da influência de fatores genéticos sobre os metabólitos cerebrais no TAB. Assim, não se conhece qual a influência de variantes nos genes o *CACNA1C*, *GAD1* e *BDNF* nos níveis de neurotransmissores cerebrais intimamente associados ao metabolismo do Ca^{2+}, sistema glutamatérgico e neuroplasticidade.

2. MATERIAL E MÉTODOS

2.1. Meta-análise sobre alterações neurometabólicas no CCA

Foi conduzida pesquisa nas principais bases de dados de literatura cientifica (PubMed, EMBASE, Cochrane Library, Medline, Scopus e Google Scholar), seguindo os protocolos do PRISMA ("Preferred Reporting Items for Systematic Reviews and Meta-Analyses") (Moher et al., 2009). Os termos *"Magnetic Resonance Spectroscopy "* e *"bipolar disorder"* foram utilizados para a pesquisa realizada até fevereiro de 2019. Dos artigos encontrados, selecionou-se aqueles focados no cíngulo anterior, seguindo os seguintes critérios: estudos escritos na língua inglesa; estudos que utilizaram a técnica de ^{1}H-MRS para avaliar o CCA no TAB; estudos de desenho transversal comparando populações com TAB e controles; estudos utilizando o DSM como critério diagnóstico.

Médias e desvios-padrão referentes às concentrações de Glx, Glu, Gln, GABA, NAA, mI and Cho assim como o tamanho amostral foram extraídos de cada estudo e incluídos na meta-análise. Análises de sensibilidade e/ou sub-análises foram realizadas de acordo com a técnica mensuração (contrações absolutas e relativas à creatina), técnica de ^{1}H-MRS, estado de humor e status de medicação. As meta-análises foram realizadas utilizando o software *Comprehensive Meta-analysis* desenvolvido pela Biostat (Borenstein et al., 2005). Utilizou-se o "Hedges' g" como tamanho de efeito (Hedges; Olkin., 1985), seguindo um intervalo de confiança de 95%, com a seguinte classificação: 0.2= baixo; 0.5= médio e 0.8= alto (Cohen, 1988). O valor de significância foi de $p < 0.05$ e os níveis de heterogeneidade foram estimados pelo instrumento estatístico I^2, considerando-se I^2 de 25%, 50% e 75% como pequeno, moderado e alto, respectivamente (Higgins et al., 2003). Um modelo de efeito aleatório foi adotado considerando a variação metodológica e clínica entre os estudos selecionados.

2.2. Estudos sobre a influência dos polimorfismos *CACNA1C, GAD1* e *BDNF* nos níveis de neurometabólitos

2.2.1. Amostragem

Este estudo é baseado em um banco de dados concebido e organizado pelo meu orientador Dr. Marcio Gerhardt Soeiro-de-Souza [Certificado de Apresentação para Apreciação Ética (CAAE): 30203420700000068]. As amostras deste estudo são constituídas de 124 pacientes bipolares tipo I eutímicos selecionados para os estudos translacionais sobre a influência das variantes *CACNA1*c (rs 1006737) e *BDNF* (rs 6265) nos neurometabólitos do CCA. Tais pacientes são oriundos de estudos sobre

TAB realizados entre 2008 e 2016 pelos Programa de Transtornos Afetivos (GRUDA), Programa de Transtorno Bipolar (PROMAN) e LIM-27, lotados no Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP). O diagnóstico psiquiátrico foi validado por profissionais experientes através de entrevista clínica estruturada (SCID-I/P) (First; Spitzer; Williams, 1996), seguindo critérios do DSM-IV TR (DSM-IV, 2000), os quais se certificaram o estado de eutimia bem como pelas as escalas de Young para avaliação de mania (YMRS) (Young et al., 1978) e de Hamilton para avaliação de depressão (HDRS) (Hamilton., 1960), as quais certificaram escore inferior a 7 para ambas as escalas por pelo menos 3 meses. Nesse período, não houve alteração de esquema ou dosagem da medicação em uso (combinações diversas de lítio, anticonvulsivantes, antipsicóticos e antidepressivos). Foram excluídos da amostra indivíduos com histórico de transtornos neurológicos ou clínicos, traumatismo craniano, uso prévio de substâncias ilícitas ou abuso de drogas nos últimos 3 meses e aqueles submetidos a eletroconvulsoterapia nos últimos 6 meses. Além disso, para avaliar sintomas maníacos e depressivos residuais, foram utilizadas as escalas de Young para avaliação de mania (YMRS) (Young et al., 1978) e de Hamilton para avaliação de depressão (HDRS) (Hamilton., 1960).

As amostras de controles saudáveis constituíram-se de 74 indivíduos entre 18 e 40 anos (estudantes de medicina da USP recrutados no centro acadêmico) as quais foram submetidas ao "Mini International Neuropsychiatric Interview" (MINI) (Sheehan et al, 1998), visando certificar ausência de história psiquiátrica atual ou pretérica, presença de parentes de 1 grau afetados por transtornos psiquiátricos bem como uso de substâncias nos últimos 3 meses.

Esta pesquisa foi devidamente aprovada pelo Comitê de Ética do HC-FMUSP [Certificado de Apresentação para Apreciação Ética (CAAE): 30203420700000068] e obteve-se a assinatura do Termo de Consentimento Livre e Esclarecido de todos os participantes do estudo.

2.2.2. Espectroscopia por Ressonância Magnética (^{1}H-MRS)

Os dados de espectroscopia foram adquiridos no Setor de Ressonância Magnética do Instituto de Radiologia (InRad) do HC-FMUSP, usando o aparelho Philips Achieva 3,0 Tesla e bobina de cabeça de 8 canais. Realizou-se aquisição de imagens sagitais volumétricas 3D ponderadas em T1 (3DT1-FFE), com TE/TR=2,8/6,2 ms e resolução espacial de 1mm^3, para correta localização do vóxel de interesse, no caso o CCA. Foram realizadas então aquisições de espectroscopia de vóxel único com sequência de pulso PRESS, com TE/TR=80/1500ms, englobando 2x2x2cm^3 na região do cíngulo anterior.

O número total de repetições foi 128, com *phasecycle*=8 e duração total de aproximadamente 4 minutos. Para quantificação do espectro, utilizou-se o programa LC Model (Provencher, 1993), sendo quantificados os seguintes metabólitos: Glx, Glu, NAA, Cr, Cho, mI, Glu, os quais foram analisados em

relação à Cr e ao conteúdo da água no vóxel, como descrito por Soeiro-de Souza et al. (2018a,b). Parte da amostragem destes estudos prévios foram submetidas à análise genética para o presente estudo. As concentrações dos neurometabólitos foram apresentados em relação à creatina.

2.2.3. Estudos genéticos: extração de DNA e genotipagem

Cerca de 7-10 mL de sangue periférico dos participantes acima mencionados foram coletados via punção venosa do antebraço no dia dos exames de imagem. Procedeu-se a extração do DNA genômico, obtido a partir dos leucócitos, utilizando-se o protocolo baseado em *salting-out* (Laitinen; Samarut; Hölttä, 1994). O DNA foi re-suspendido em tampão TE [Tris- HCla10 mM e EDTAa1mM (pH8,0)] e armazenado a -20ºC. Determinou-se a concentração e pureza do DNA por espectrofotometria à 260 nm, utilizando o espectrofotômetro NanoDrop® ND-1000UV-Vis*r* e eletroforese em gel de agarose 1%. O DNA obtido foi genotipado para o gene *CACNA1C* (rs1006737) e *BDNF* (rs6265) utilizando a técnica de discriminação alélica por PCR *Real Time* usando equipamento de PCR Real Time 7500 Real-Time System. As análises foram realizadas e o material armazenado no laboratório LIM/23 da Faculdade de Medicina da Universidade de São Paulo (FMUSP). Certificou-se se a distribuição genotípica seguia a distribuição de equilíbrio de Hardy-Weinberg.

2.2.4. Análise estatística

Para os estudos transversais, realizou-se inicialmente teste de χ^2 para variáveis categóricas e teste *t* para variáveis contínuas com distribuição normal. Verificou-se diferenças significativas entre os grupos em relação a idade, sexo e massa cinzenta e as análises subsequentes foram corrigidas por estes fatores através do modelo linear generalizado (MLG) univariado. Neste modelo, os metabólitos foram considerados variáveis dependentes e as variáveis grupo (TAB e C), SNP, idade, sexo e fGM (correção pela massa cinzenta e líquido céfalo-raquidiano) como covariáveis. As análises foram realizadas utilizando-se o software IBM SPSS versão 20.

3. RESULTADOS

3.1. Capítulo I – Anterior cingulate cortex neurometabolites in bipolar disorder are influenced by mood state and medication: A meta-analysis of 1H-MRS studies

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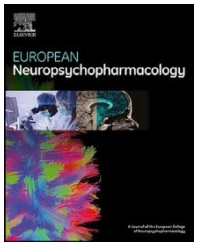

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REVIEW

Anterior cingulate cortex neurometabolites in bipolar disorder are influenced by mood state and medication: A meta-analysis of ^{1}H-MRS studies

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The anterior cingulate cortex (ACC), a brain region that mediates affect and cognition by connecting the frontal cortex to limbic structures, has been consistently implicated in the neurobiology of Bipolar Disorder (BD). Proton magnetic resonance spectroscopy (^{1}H-MRS) studies have extensively compared *in vivo* neurometabolite levels of BD patients and healthy controls (HC) in the ACC. However, these studies have not been analyzed in a systematic review or meta-analysis and nor has the influence of mood state and medication on neurometabolites been examined in this cortical region. A systematic review and a meta-analysis of ^{1}H-MRS studies comparing ACC neurometabolite profiles of adult BD patients and HC subjects was conducted, retrieving 27 articles published between 2000 and 2018. Overall increased ACC levels of Glx [glutamine (Gln) + glutamate)/Creatine], Gln, choline (Cho) and Cho/Creatine were found in BD compared to HC. Bipolar depression was associated with higher Cho levels, while euthymia correlated with higher glutamine (Gln) and Cho. Mood stabilizers appeared to affect ACC Glu and Gln metabolites. Increased ACC Cho observed in euthymia, depression and in medication-free groups could be considered a trait marker in BD and attributed to increased cell membrane phospholipid turnover. Overall increased ACC Glx was associated with elevated Gln levels, particularly influenced by euthymia, but no abnormality in Glu was detected. Further ^{1}H-MRS studies, on other voxels, should assess more homogeneous (mood state-specific), larger BD samples and account for medication status using more sensitive ^{1}H-MRS techniques.

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KEYWORDS
Choline; Glutamate; Glutamine; Glx;Myo-inositol; N-acetylaspartate

1. Introduction

Bipolar disorder (BD) is a severe, chronic illness characterized by significant oscillations in mood ranging from depression to mania (Merikangas et al., 2007). Although little is known about the neurobiology of BD, recent structural and functional neuroanatomical studies have reported disintegration in fronto-subcortical circuitry (Strakowski et al., 2012; Maletic and Raison., 2014), particularly circuits mediated by the anterior cingulate cortex (ACC), a key center integrating cognitive and affective neuronal connections.

The ACC (Supplementary Material, Figure S1) is one of the most studied brain region by neuroimaging in BD. Alterations involving the ACC in BD range from abnormal morphology and brain connectivity to neurochemistry. Reduction in ACC gray matter volume has been one of the most consistent findings reported in BD (Drevets et al., 1997; Haldane and Frangou., 2004; Strakowski et al., 2012). In fact, the largest structural magnetic resonance image (MRI) study to date (Hibar et al., 2017), as well as a recent systematic review (Hanford et al., 2016) and a meta-analysis (Wise et al., 2017), have confirmed this cortical thinning. A voxel-based quantitative meta-analysis of functional MRI (fMRI) studies has also suggested a fronto-limbic dysfunction in the ACC of BD patients relative to HC subjects (Chen et al., 2011), whilst systematic reviews and meta- analyses of diffusion tensor imaging (DTI) studies in BD report alterations in white matter tracts (decreased fractional anisotropy) connecting the ACC to subcortical limbic structures (Vederine et al., 2011; Nortje et al., 2013). Additionally, ketamine, a glutamatergic receptor antagonist agent that promotes transient antidepressant effects, has been shown to increase glutamine levels in the ACC (Rowland et al., 2005) and also to modulate the functional connectivity between the ACC and pre-frontal cortex (PFC) (Lenner et al., 2017). Therefore, alterations in the morphology, connectivity and neurochemistry of the ACC appear to be implicated in BD neurobiology.

The neurochemical alterations in BD can be assessed by proton magnetic resonance spectroscopy (^{1}H-MRS), a non-invasive technique based on the signal intensity arising from protons in a given metabolite as a function of their frequency displayed in a spectrum whose peaks are proportional to the concentrations of the correspondent metabolite (Buonocore and Maddock., 2015). Thus, ^{1}H-MRS provides *in vivo* absolute or relative to creatine (/Cr) measurements of several brain neurometabolites, such as Glutamate (Glu), Glutamine (Gln), Glx (Glu + Gln), Gamma-Aminobutyric Acid (GABA), N-acetylaspartate (NAA), Choline (Cho), and Myo-inositol (mI). Precise measurements of Glu and Gln require the use of more sensitive techniques with higher field strengths ($\geq$ 3 T) and the sum of these metabolites are commonly referred as Glx (Buonocore and Maddock, 2015).

While Glu and GABA are the most common excitatory and inhibitory neurotransmitters, respectively, in the cen-tral nervous system, Gln is a "non-excitatory" form of stored Glu (Walls et al., 2015). NAA, in turn, is an amino acid synthesized in neuronal mitochondria and has been considered a marker of neuronal energy metabolism, viability and health (Stork and Renshaw., 2005). Myo-inositol (mI) is an organic osmolyte located in astrocytes and considered a glial cell marker (Brand et al., 1993), whilst Cho corresponds to phosphoryl and glycerol phosphoryl choline and is an indicator of cell membrane turnover (synthesis or breakdown) (Stork and Renshaw., 2005; Moffett et al., 2007). Creatine (Cr) has been widely used as an internal standard for ^{1}H- MRS because it is assumed that there is very little variation in the cr-phosphocreatine (P-Cr) equilibrium, resulting in a stable concentration of Cr (Buonocore and Maddock., 2015). Previous ^{1}H-MRS studies in BD have reported increased Glx (Glutamate+Glutamine) and/or Glu in cortical frontal regions in BD (Yildiz-Yesiloglu and Ankerst., 2006; Yüksel and Öngür., 2010; Gigante et al., 2012; Chitty et al., 2013), commonly interpreted as an increased glutamatergic neurotransmission or excitatory state. Although Glx corresponds to not only a greater proportion of Glu, but also Gln, GABA and glutathione (GSH), its major contributors are Glu and Gln. Regarding NAA, previous systematic reviews and meta-analyses (Yildiz-Yesiloglu and Ankerst., 2006; Kraguljac et al., 2012) have found reduced levels in frontal regions, the hippocampus and basal ganglia, and has been interpreted as an indicator of mitochondrial dysfunction and impartment in energy production, resulting in increased lactate (Stork and Renshaw, 2005). Neuronal damage has also been suggested by previous systematic reviews that pointed to higher levels of Cho (Yildiz-Yesiloglu and Ankerst., 2006) and mI (Silverstone et al., 2005) in BD, a finding not confirmed by a meta-analysis (Kraguljac et al., 2012). In contrast, GABA has been consistently reported as unaltered in BD (Schur et al., 2016; Chiapponi et al., 2016; Romeo et al., 2018).

However, ^{1}H-MRS studies in BD are especially challeng- ing because BD subjects can present any of at least three mood states (depression, mania and euthymia) at time of scan. Therefore, it has been hard to interpret the relationship between neurometabolite profile and clinical presentation since most studies have not taken into account the influences of both mood state and medication on neurometabolite dynamics. Indeed, very few ^{1}H-MRS systematic reviews and meta-analyses performed thus far have taken into consideration the effect of mood state or medication on metabolites levels, while none have focused specifically on a specific brain region such as the ACC. Since understanding the neurometabolic profile of the ACC in BD and how it is affected by mood states and medications is critical to elucidating the neurobiology of this disorder, the aim of this systematic review and meta-analysis was to examine, for the first time, ACC-specific cross-sectional ^{1}H-MRS studies in BD

patients compared to healthy controls, and to investigate the influence of mood state and medication on results.

2. Experimental procedures

2.1 Literature Search and inclusion citeria

We conducted a search of the literature on the PubMed, EMBASE, Cochrane Library, Medline, Scopus and Google Scholar platforms following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009), as depicted in Supplementary Material, Figure S2. Using the search strings "proton magnetic resonance spectroscopy or 1 H-MRS"AND "bipolar disorder"AND "anterior cingulate cortex"from database inception until February 2019, a total of 53 articles published (2000 - 2018) were identified.Of the 53 manuscripts considered eligible for full-text search, only 27 cross-sectional studies met the following study inclusion criteria: articles written in English; studies performed in subjects with mean age ≥ 18 years; ACC as the region of interest; cross-sectional studies comparing BD and HC groups; longitudinal studies (2 studies) were included only if they provided baseline data comparing BD and HC groups; magnetic field ≥1.5 Tesla (except for Glx, Glu and Gln ≥3 Tesla); and studies using the DSM diagnostic criteria for BD and mood states (mania, depression or euthymia). Besides DSM-criteria, the use of mood-state classification by a symptoms scale was accepted.

The quality of each individual study was assessed in relation to clinical and demographic aspects (e.g., age, sex, education, ill- ness duration and severity, exclusion of systemic diseases, neurodevelopmental disorder, history of drug or alcohol abuse), sample sizes, and ^{1}H-MRS acquisition and analysis methodology (e.g., voxel placement, use of Cramer-Rao lower bound (CRLB) or equivalent filtering for spectral quality assessment).

2.2 Meta analytic procedures

Means and standard deviations (SD) of Glx, Glu, Gln, GABA, NAA, mI and Cho concentrations, as well as sample sizes for both BD and HC groups were extracted from each study or acquired by contacting the respective corresponding author if the data was presented as graphs or median/quartile range (e.g., Prisciandaro et al., 2017; Wise et al., 2018). In cases of overlapping samples involving two publications, only the study with the larger sample size was included in the meta-analysis. Absolute and over creatine (/Cr) concentrations were considered, but sensitivity and/or subgroup meta-analyses stratified by mood state (hypomania/mania, depression, or euthymia) and medication status (medication-free) were performed. Meanifull meta-analyses were considered if there were a minimum ≥3 studies available. The meta-analyses were performed using Comprehensive Meta-analysis software version 3.3 developed by Biostat (Borenstein et al., 2005). The effect size used was Hedges' g (Hedges and Olkin., 1985) using 95% confi-dence intervals, adopting the following classification: 0.2=small; 0.5=medium; and 0.8=large (Cohen, 1988). Random effects modeling was used because considerable clinical and method- ological heterogeneity among the selected studies was assumed. Meta-regressions were performed to assess the relationship be- tween moderators (subject age and publication year) and effect size of neurometabolites between patients and controls. Publica- tion bias (meta-analysis ≥ 10 studies) was evaluated using a funnel plot and Egger's linear regression method test (Egger et al., 1997). The p-value was set to 0.05 and heterogeneity was estimated using I^2 statistics, consid- ering I^2 of 25%, 50% and 75% as small, moderate, and high levels of heterogeneity, respectively (Higgins et al., 2003).

3. Results

3.1 Glutamatergic metabolites

Table 1 shows twelve studies published between 2007 and 2018 that compared ACC concentrations of Glx, Glu and Gln in adult BD patients relative to HC by ^{1} H-MRS at a magnetic field ≥3T.

3.1.1 Glx

Overview. A meta-analysis of four studies for which data were available involving a total of 354 subjects (197 BD, 157 HC), showed significantly overall higher ACC Glx/Cr in BD relative to HC (g: 0.47, 95% CI: 0.18 to 0.75, Z: 3.22, p = 0.001; tau 2 = 0.01; Q:3.51, df:3; p = 0.31, I 2 = 14.5%), independently of the quantification method used (Fig.1). Meta-regression revealed no influence of subject age [co- efficient: −0.074; 95% CI: (−0.169, 0.020); p = 0.12] or year of publication [coefficient: 0.0135; 95% CI: (−0.071, 0.058); p = 0.75] on the results.

Mood states. There were not enough studies (n<3) to perform meaninfull meta-analyses in depressive, manic and euthymic mood states.

Medication status. There were not enough studies (n<3) to perform a meaningful meta-analysis in medication-free and medicated samples.

3.1.2. Glutamate (Glu)

Overview. Table 1 shows ten studies that measured Glu or Glu/Cr in the ACC of BD subjects as compared to HC using both conventional techniques (e.g. PRESS) with a magnetic field ≥3T or more sensitive techniques (e.g. JPRESS). The pooled meta-analysis of 9 studies (592 subjects: 303 BD; 289 HC) revealed no significant differences between groups (g: 0.08, 95% CI: −0.36 to −0.19, Z:0.58, p=0.55; tau^2=0.18; Q:32.8,df:8; p< 0.0001; I^2=75.6%; Supplementary Material, **Fig. S3A**).

Mood states. There were not enough studies (n < 3) to perform meaninfull meta-analyses in depressive and manic mood states (Supplementary Material, **Fig. S3A**). The pooled meta-analysis of 5 studies that assessed ACC Glu/Cr or Glu in euthymic mood state found also no significant differences between groups (g: 0.17, 95% CI: −0.30 to −0.65, Z: 0.7; p =0.70; tau^2= 0.27;Q: 21.82, df:3 p<0.0001, I^2=86.2%; Supplementary Material, **Fig. S3A**)

Medication status. There were not enough studies (n< 3) to perform meaninfull meta-analysis in the medication-free group (Supplementary Material, **Fig.S3B**). The meta-analysis of eight studies performed in medicated subjects revealed no differences between groups (Supplementary Material, **Fig. S3B**). However, some studies provided evidence of lower levels of Glu/Cr in euthymic subjects taking anticonvulsants (Soeiro-de-Souza et al., 2013,

2015,2018a).

3.1.3. Glutamine (Gln)

Overview. Four studies (Table 1) measured ACC Gln using higher magnetic fields and/or 2D MRS sequences. Three of these studies reported increased Gln/Glu or decreased Glu/Gln in BD patients relative to controls (Öngür et al., 2008 ; Soeiro-de-Souza et al., 2015 ; Kubo et al., 2017). The meta-analysis of these studies (227 subjects: 115 BD; 112 64 HC) revealed significantly increased Gln levels in BD relative to HC groups (Fig. 2): g:0.57, 95% CI: 0.18 to 0.96, Z: 2.8, p = 0.004; tau^2 = 0.23; Q = 14.51, df = 4, p = 0.006; I 2 = 72.4%. Meta-regression revealed no influence of subject age [coefficient: 0.06; 95% CI: (−0.0046, 0.12); p = 0.06] or year of publication [coefficient: 0.048; 95% CI: (−0.11, 0.21); p = 0.21] on the results.

Mood states. There were not enough studies (n < 3) to perform meaninfull meta-analyses in depressive and manic mood states (Fig. 2). The sensitivity meta-analysis of three studies performed in euthymic state (194 subjects; 100 BD; 94 HC) revealed increased ACC Gln levels in BD (Fig. 2): g: 0.94, 95% CI: 0.12 to 1.77, Z: 2.26, p = 0.024; tau^2 = 0.44; Q = 12.68, df = 2, p = 0.002; I 2 = 84.24%. Furthermore, decreased Glu/Gln (Soeiro-de-Souza et al., 2015) or increased Gln/Glu (Kubo et al., 2017) were found in euthymic BD samples.

Medication status. No study was performed in medication-free sample. In one study performed on medicated subjets, higher Gln was observed among anticonvulsant users com- pared to non-users (Soeiro-de-Souza et al., 2015).

3.2. N - acetylaspartate (NAA)

3.2.1. ***Overview***. Eighteen studies provided NAA data for the ACC (Table 1). The pooled meta-analysis of the sixteen of studies that measured ACC NAA, comprising 1028 subjects (502 BD and 526 HC), revealed no significant differences between BD and HC: g: - 0.07, 95% CI: −0.31 to 0.16, Z: −0.16, p = 0.52; tau^2 = 0.76; Q= 207.5, df = 19, p < 0.0001; I^2 = 90.84% (Supplementary Material, Fig. S4A). Although visual inspection of the funnel plot showed some asymmetry (Supplementary Material, Fig. S4B), no publication bias was detected by Egger's linear regression method [Supplementary Material, Fig. S 4B ; Egger: bias = −1.88 (95% CI = −7.2 to 3.5) p = 0.47].

3.2.2. *Mood states*. It was possible to obtain meaningful meta-analyses for the three different mood states regarding NAA (Supplementary Material, Fig. S4A). A pooled meta-analysis of four studies performed in subjects under depression comprising 263 individuals (159 BD and 104 HC) revealed no significant differences in ACC NAA levels between groups: g: 0.48, 95% CI: −1.39 to −0.43, Z: −1.03, p = 0.3; tau^2 = 0.60; Q = 26.8, df = 4, p < 0.0001; I^2 =85% (Supplementary Material, Fig. S4A). Similarly, the sensitivity meta-analysis of three studies performed in manic/hypomanic states revealed also no differences between BD and HC (g: −0.07, 95% CI: −0.31 to 0.16, Z: - 0.63, p = 0.52; tau^2 = 0; Q = 1.63, df = 2, p = 0.44; I^2=0%) and for the euthymic sub-group, a pooled meta-analysis of 7 studies revealed similar results (g: −0.16, 95% CI: −0.22 to 0.33, Z: 0.39, p = 0.69; tau^2= 0.12; Q =13.7, df=5, p= 0.017; I^2= 63.7%; Supplementary Material, Fig. S 4A).

3.2.3. *Medication status*: The meta-analysis of three studies performed in medication-free individuals totaling 207 subjects (132 BD, 75 HC) revealed no differences in ACC levels of NAA in BD relative to HC (g: 0.38, 95% CI:- 0.60 to −1.37, Z: 0.76, p = 0.44; tau 2 = 0.84; Q = 21.2, df: 2, p < 0.001; I 2 = 90%; Supplementary Material, Fig. S4C), although all were performed in patients during a depressive state. Similalry, no between-group was recorded in the medicated sub-group regarding NAA (Supplementary Material, Fig. S4C). However, some studies provided evidence of increased levels of NAA in euthymic subjects on lithium (Soeiro-de-Souza et al., 2018b) and after anticonvulsant treatment (Croarkin et al., 2015).

3.3. Choline-containing compounds (Cho)

3.3.1 Overview. Sixteen studies evaluated Cho in the ACC (Table 1), comprising 982 subjects (538 BD and 444 HC). The pooled meta-analysis of fifteen of these studies (959 subjects: 529 BD and 430 HC) showed significantly increased levels of Cho in BD compared to HC (Fig. 3A; g: 0.43, 95% CI: 0.28 to 0.58, Z: 5.8, p < 0.0001; tau^2 = 0.49, Q = 113.6, df = 16, p < 0.000, I^2 = 85.9%). Meta-regression revealed no influence of subject age [coefficient:0.013; 95% CI: (−0.05, 0.08); p = 0.7] on Cho, but a positive influence of year of publication on these results was observed [coefficient: 0.007; 95% CI: (0.006, 0.14); p = 0.03], as shown in Fig. 3 **B** . Although visual inspection of the funnel plot showed some asymmetry (Supplementary Material, **Fig. S5**), no publication bias was detected by Egger's linear regression method (Egger: bias = 0.8 (95% CI = 4.1 to 5.7)p=0.73).

3.3.2. ***Mood states*.** Sensitivity meta-analyses were performed according to the different mood states. A pooled meta-analysis of three studies (199 subjects :124 BD, 75 HC) performed in the depressive state showed significantly increased ACC Cho in the BD group (Fig. 3 **A**; g: 0.40; 95% CI: 0.11 to 0.69, Z: 2.69, p = 0.007; tau 2 = 0; Q = 1.22, df:2, p = 0.54; I^2 = 0%). Meta-regression revealed no influence of subject age [coefficient: −0.028; 95% CI: (−0.09, 0.042); p = 0.43] or year of publication [coefficient: 0.04; 95% CI: (−0.02, 0.1); p = 0.25] on the results. Similarly, a pooled meta- analysis of six studies performed under euthymia, comprising 441 subjects (236 BD; 205 HC), revealed increased lev els of ACC Cho in BD (Fig. 3 A; g:0.48, 95% CI: 0.20 to 0.66, Z = 5.01, p < 0.0001; tau^2 = 0, Q = 2.51, df = 6, p = 0.77; I^2 = 0%). Meta-regression revealed no influence of subject age [coefficient: −0.02; 95% CI: (−0.05, 0.014); p = 0.25] or year of publication [coefficient: 0.02; 95% CI: (−0.01, 0.06); p = 0.22] on the results. However, for the manic/hypomanic sub-group, a pooled meta-analysis of three studies showed no significant differences between groups (g: 0.12, 95% CI: −0.18 to 0.54, Z = 0.60, p = 0.54; tau 2 = 0; Q = 1.33, df = 2, p = 0.5; I 2 = 0%).

Table 1 Cross-sectional ^{1}HMRS studies comparing ACC neurometabolites concentration between bipolar disorder and healthy controls subjects.

Reference	BD type	Sample Size (P/C)	Mean Age	Mood State	Medication	Field Strength (T)/ Echo Time (ms)	MRS sequence	Metabolites reported	Significant Results
Moore et al., 2000	I	9/14	37.9/36	DMS	Li, Ac,Ad	1.5/30	STEAM	Cho/Cr, mI/Cr	Cho/Cr↑
Dager et al., 2004	I, II	23/26	30.3/31	DMS, D	MF	1.5/Variable	PEPSI	NAA, Cho, mI	None
Amaral et al., 2006	I	13/15	34.5/34	E	Li, Ac,Ad, Ap	1.5/144	PRESS	NAA/Cr, Cho/Cr	None
Frye et al., 2007a	I, II	23/12	35.6/32	D	MF	1.5/30	PRESS	Cho, Cho/cr	None
Frye et al., 2007b	I	16/17	37.5/32.9	M/H	Li, Ac, Ap	3/20	STEAM	Glx/Cr, NAA/Cr, Cho/Cr, mI/Cr	None
Malhi et al., 2007	I	9/9	40.7/41	H	Li, Ac, MF	1.5/30	FSPGR	NAA, Cho, mI	NAA ↓, Cho ↓
Malhi et al., 2007	I	9/9	40.7/41	E	Li, Ac, MF	1.5/30	FSPGR	NAA, Cho	None
Öngür et al., 2008	I	15/21	36.3/34.3	M	Li, Ac Ap	4/Variable	J-PRESS	Glu, Gln, NAA, Cho, mI	Gln/Glu ↑
Port et al., 2008	I, II	21/21	30.8/31	DMS	MF	3/ 30	PRESSCI	Cho	None
Scherk et al., 2009	I	33/29	33/29	E	Li	1.5/30	PRESS	NAA/Cr, Cho/Cr, mI/Cr	None
Brady et al., 2012	I	14/21	37.6/35	M	Li,Ac,Ap	4/ Variable	JPRESS	NAA/Cr	None
Xu et al., 2013	I, II	24/20	34/31	DMS	MF	3/30	2D MRSI	Glx/Cr, Glu/Cr,	None
Soeiro-de-Souza et al., 2013	I	40/40	29/29	E	Li,Ac,Ad,Ap	3/80	PRESS	Glx/Cr, Glu/C, NAA/Cr, Cho/Cr, mI/Cr	Glx/Cr ↑, Glu/Cr ↑, Cho/Cr ↑
Zhong et al., 2014	NM	20/13	30.5/28	D	MF	1.5/ 144	PRESS	NAA/Cr, Cho/Cr	None
Ehrlich et al., 2015	I	21/42	45.9/39.3	E	Li, Ac,Ad,Ap	3/80	PRESS	Glu/Cr, Gln/Cr, NAA/Cr, Cho/Cr	Glu/Cr ↑, Gln/Cr ↑, NAA/Cr ↓
Croarkin et al., 2015	I, II	15/9	NM	D	Li,Ac,Ad,Ap	1.5/30	L-COZY	NAA/Cr	NAA/Cr ↓
Soeiro-de-Souza et al., 2015	I	40/44	31.7/25.7	E	Li,Ac,Ad,Ap	3/Variable	JPRESS	Glu, Gln	Glu↓, Gln ↑, Glu/Gln ↓
Cao et al., 2016	I	50/44	35.7/35.4	DMS	Li, Ac, Ad, MF	3/80	PRESS	Glu/Cr, NAA/Cr, Cho/Cr, mI/Cr	Cho/Cr ↑
Li et al., 2016	NM	13/20	31/31.7	D	MF	3/30	2D MRS-PRESS	Glx, NAA, Cho, mI	Glx ↑
Galin´ska-Skok et al., 2016	I	27/10	43/40.2	DMS	Li, Ac, Ap, Ad	1.5/35	PRESS	NAA/Cr, Cho/Cr, mI/Cr	None
Kubo et al., 2017	I, II	20/23	45.0/46.4	DMS, E	Li,Ac,Ap	3/18	STEAM	Glu, Gln, NAA, Cho	Gln ↑, Gln/Glu ↑, NAA ↑, Cho ↑
Prisciandaro et al., 2017	I, II	20/19	36.8/38.0	D	Li,Ac,Ad,Ap	3/ variable	JPRESS	Glu/Cr	None
Soeiro-de-Souza et al., 2018a	I	128/80	32.04/28.1	E	Li,Ac,Ap	3/80	PRESS	Glx/Cr, Glu/Cr	Glx/Cr ↑, Glu/Cr ↑
Soeiro-de-Souza et al., 2018b	I	129/79	32/28.4	E	Li,Ac,Ap	3/80	PRESS	NAA, Cho, mI	NAA ↑, Cho↑

(*continued on next page*)

Table 1 (*continued*)

Reference	BD type	Sample Size (P/C)	Mean Age	Mood State	Medication	Field Strength (T)/ Echo Time (ms)	MRS sequence	Metabolites reported	Significant Results
Wise et al., 2018	NM	9/20	31.44/30	D	MF	3/30	PRESS	Glu/Cr	None
Zhong et al., 2018	NM	92/42	25.4/25.	D	MF	3/ 144	PRESS	NAA/Cr, Cho/Cr, mI/Cr	None
Huber et al., 2018	I, II	19/10	18/19	D	NM	3/ variable	JPRESS	NAA	None

Abbreviations: Ac: Anticonvulsants; **Ad:** Antidepressants; **Ap:** Antipsychotics; **C**: Healthy Controls; **D:** Depression; **DMS:** Different mood states; **E:** Euthymia; **H:** Hypomania; **Li**: Lithium; **M:** Mania; **MF**: Medication-free; **MS**: Mood stabilizers; **N**: Number of subjects; **NM:** Not Mentioned; **P**: Patients.

Fig. 1 Forest Plot for meta-analysis of ACC Glx/Cr in overall BD.

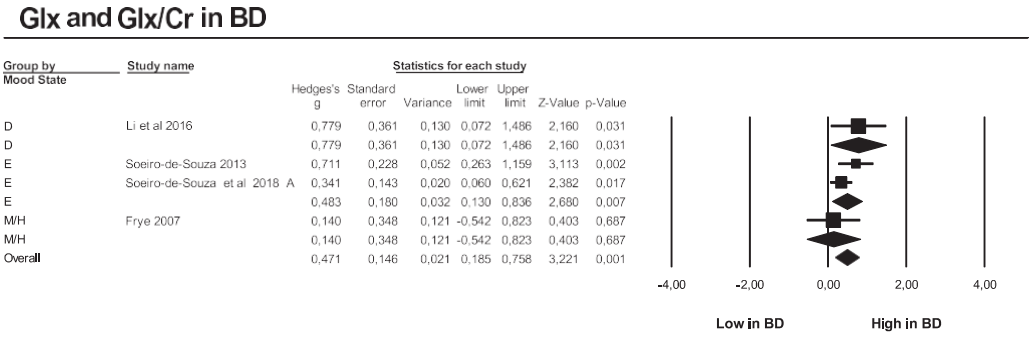
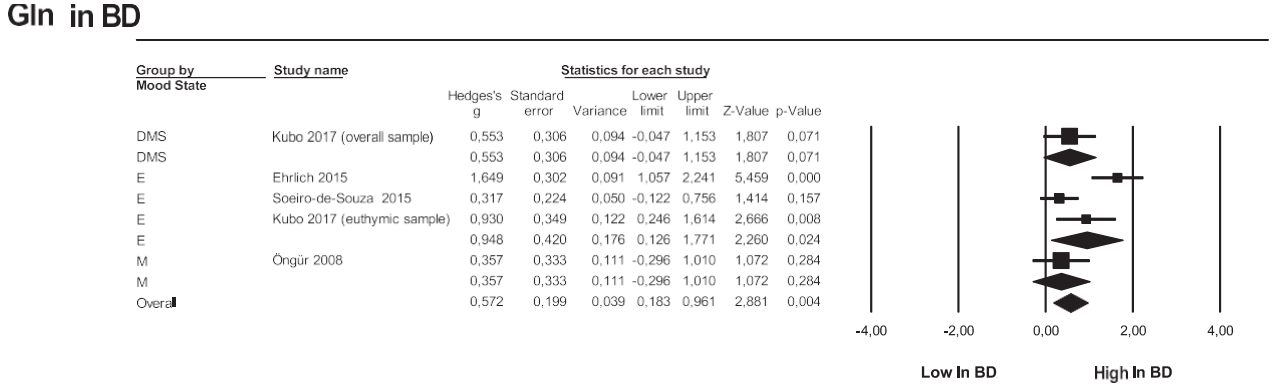
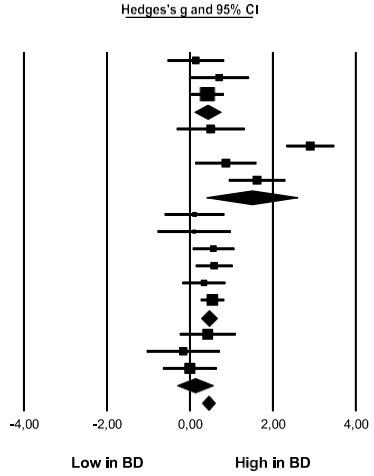


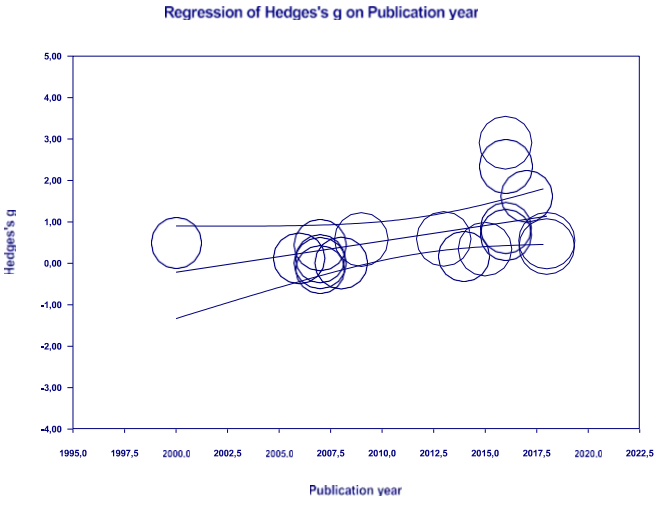
Fig. 2 Forest Plot for meta-analysis of ACC Gln-in overall BD as influenced by the mood states. DSM: Different mood states; E: Euthymia; M: Mania/hypomania



Cho and Cho/Cr in BD

Group by Mood State	Study name	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
D	Zhong 2014	0,145	0,348	0,121	-0,538	0,827	0,415	0,678
D	Li 2016	0,704	0,367	0,134	-0,014	1,423	1,921	0,055
D	Zhong 2018	0,401	0,187	0,035	0,035	0,767	2,145	0,032
D		0,404	0,150	0,023	0,110	0,698	2,690	0,007
DMS	Moore 2000	0,505	0,418	0,175	-0,315	1,325	1,207	0,227
DMS	Cao 2016 (overall sample)	2,905	0,295	0,087	2,327	3,483	9,854	0,000
DMS	Galiska-Skok 2016	0,869	0,376	0,141	0,132	1,606	2,310	0,021
DMS	Kubo 2017	1,623	0,347	0,121	0,942	2,303	4,671	0,000
DMS		1,498	0,560	0,313	0,401	2,595	2,677	0,007
E	Amaral 2006	0,113	0,368	0,136	-0,608	0,835	0,308	0,758
E	Malhi 2007 (euthymic sample)	0,103	0,449	0,202	-0,777	0,984	0,230	0,818
E	Scherk 2009	0,570	0,256	0,066	0,067	1,073	2,223	0,026
E	Soeiro-de-Souza 2013	0,586	0,226	0,051	0,143	1,030	2,592	0,010
E	Ehrlich 2015	0,341	0,266	0,071	-0,180	0,861	1,282	0,200
E	Soeiro-de-Souza 2018B	0,545	0,145	0,021	0,262	0,829	3,766	0,000
E		0,480	0,096	0,009	0,292	0,668	5,011	0,000
M	Frye 2007	0,432	0,344	0,118	-0,242	1,107	1,257	0,209
M	Malhi 2007 (manic sample)	-0,158	0,450	0,202	-1,040	0,723	-0,352	0,725
M	Öngür 2008	0,000	0,331	0,109	-0,648	0,648	0,000	1,000
M		0,127	0,211	0,044	-0,285	0,540	0,605	0,545
Overall		0,435	0,075	0,006	0,289	0,582	5,820	0,000





Cho and Cho/Cr in BD: medication effect

Group by Medication status	Study name	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
M	Moore 2000	0,505	0,418	0,175	-0,315	1,325	1,207	0,227
M	Amaral 2006	0,113	0,368	0,136	-0,608	0,835	0,308	0,758
M	Frye 2007	0,432	0,344	0,118	-0,242	1,107	1,257	0,209
M	Malhi 2007 (manic sample)	-0,158	0,450	0,202	-1,040	0,723	-0,352	0,725
M	Malhi 2007 (euthymic sample)	0,103	0,449	0,202	-0,777	0,984	0,230	0,818
M	Öngür 2008	0,000	0,331	0,109	-0,648	0,648	0,000	1,000
M	Scherk 2009	0,570	0,256	0,066	0,067	1,073	2,223	0,026
M	Soeiro-de-Souza 2013	0,586	0,226	0,051	0,143	1,030	2,592	0,010
M	Ehrlich 2015	0,341	0,266	0,071	-0,180	0,861	1,282	0,200
M	Cao 2016 (overall sample)	2,905	0,295	0,087	2,327	3,483	9,854	0,000
M	Galiska-Skok 2016	0,869	0,376	0,141	0,132	1,606	2,310	0,021
M	Kubo 2017	1,623	0,347	0,121	0,942	2,303	4,671	0,000
M	Soeiro-de-Souza 2018B	0,545	0,145	0,021	0,262	0,829	3,766	0,000
M		0,667	0,213	0,045	0,250	1,084	3,133	0,002
MF	Zhong 2014	0,145	0,348	0,121	-0,538	0,827	0,415	0,678
MF	Li 2016	0,704	0,367	0,134	-0,014	1,423	1,921	0,055
MF	Zhong 2018	0,401	0,187	0,035	0,035	0,767	2,145	0,032
MF		0,404	0,150	0,023	0,110	0,698	2,690	0,007
Overall		0,491	0,123	0,015	0,251	0,732	4,005	0,000

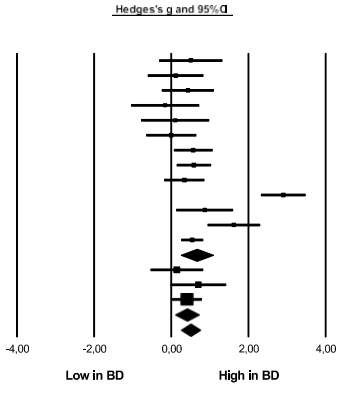


Fig. 3 **A** - Forest Plot for meta-analysis of ACC Cho/Cr and Cho in overall BD. **B**- Meta-regression analyses of the influences of the publication year on the effect size of studies assessing ACC Cho/Cr and Cho in overall BD. **C**- Forest Plot for meta-analysis of ACC Cho and Cho/Cr in overall BD as influenced by the medication status.

3.3.3. ***Medication status***. A pooled meta-analysis of three studies performed in medication-free individuals, comprising 199 subjects (124 BD, 75 HC) revealed significantly increased Cho in BD (Fig. 3 C; g: 0.40; 95% CI: 0.11 to 0.69, Z: 4.0, $p < 0.001$; $tau^2 = 0$; $Q = 1.22$, df:2, $p = 0.54$; I^2=0%), although this involved the same sample as the depressive state analysis. Meta-regression demonstrated no influence of subject age or year of publication on Cho (see depressive mood state). Similalry, the meta-analysis of medicated subjects also showed increased Cho and Cho/Cr levels in medicated BD (Fig. 3 **C** ; g: 0.66; 95% CI: 0.25 to 1.08, Z: 3.1, $p = 0.002$; $tau^2 = 0.49$; $Q = 79$, df: 12, $p < 0.001$; $I^2 = 87\%$).

3.4. Myo-inositol

3.4.1. Overview. Twelve studies measured mI in the ACC (Table 1). The pooled meta-analysis of eleven of these studies assessing mI (757 subjects: 432 BD and 325 HC) found no significant difference (Supplementary Material, **Fig. S 6A**) (g: 0.04, 95% CI: −0.11 to 0.19, Z: 0.31, $p = 0.60$; $tau^2 = 0.13$; $Q = 34.8$, df: 12, $p < 0.001$; $I^2 = 65\%$). Although some asymmetry was ob- served in the funnel plot (Supplementary Material, **Fig. S 6B**), no publication bias was detected by Egger's linear re-gression method [Supplementary Material, Fig. S6B; Egger: bias = −1.60 (95% CI = −5.06 to 1.85); $p = 0.33$].

3.4.2. *Mood states*. Sensitivity meta-analyses were conducted considering the different mood-states (Supplementary Material, Fig. S 6A). There were too few studies ($n < 3$) to conduct a meaningful meta-analysis in the depressive mood state. A pooled meta-analysis of three studies performed in manic/hypomanic state encompassing 87 subjects (40 BD and 47 HC) found no differences in ACC mI in BD compared to controls (g: −0.05, 95% CI: −0.46 to 0.35; Z: −0.27, $p = 0.78$; $tau^2 = 0$, $Q = 0.50$, df:2, $p = 0.92$; $I^2 = 0\%$) and a pooled meta-analysis of four studies in the euthymic state also revealed no between-group differences (368 subjects: 211 BD; 157 HC): g: 0.11, 95% CI: −0.08 to 0.32, Z: 1.12, $p = 0.26$; $tau^2 = 0$; $Q = 0.36$, df = 3, $p = 0.94$; I 2 = 0%.

3.4.3. Medication status. A pooled meta-analysis of three medication-free studies (Supplementary Material, **Fig. S 6C**), totaling 377 sub- jects (271 BD and 106 HC) revealed no differences in ACC mI between patients and controls (g: −0.21, 95% CI: - 1.16 to 0.73, Z: 0.44, $p = 0.65$; $tau^2 = 0.64$; $Q = 20.9$, df = 2, $p < 0.001$, $I^2 = 90\%$). Similarly, the sensitivity meta-analysis including only studies with medicated patients showed a similar result: g: −0.10, 95% CI: - 0.33 to 0.12, Z: −0.88, $p = 0.37$; $tau^2 = 0.0355$; $Q = 13$, df = 9, $p = 0.14$, I = 32%.

3.5. GABA

3.5.1. Overview. Four studies measured ACC GABA in the ACC involving a t tal of 184 subjects (103 BD; 81 HC) (**Table S1**). The pooled meta-analysis of these studies revealed no significant dif- ference between groups: g: 0.18, 95% CI: −0.10 to 0.47, Z: 1.24, $p = 0.21$; $tau^2 = 0$; $Q = 2.72$, df = 3, $p = 0.23$, I 2 = 0% (Supplementary Material, **Fig. S 7**).

3.5.2. *Mood states*. There were too few studies ($n < 3$) to conduct meaningful meta-analyses for the depressive, manic and euthymic mood state (Supplementary Material, **Fig. S 7**).

3.5.3. *Medication status*. No studies measured GABA in a mediation-free sample. In medicated subjects, most authors found no significant dif- ferences between groups (**Table S1**). The overall results of the meta-analyses are shown in Sup- plementary Material, **TableS2**.

Discussion

To the best of our knowledge, this is the first ACC-oriented systematic review and meta-analysis to focus on the neurometabolic profile in BD, taking into account the mood state and medication status. Meta-analyses (Supplementary Material, **Table S2**) revealed overall increased ACC levels of Glx, Gln and Cho in BD compared to HC but no significant differences for Glu, NAA, GABA or mI. While euthymia was associated with increased Cho and Gln, bipolar depression was only associated with increased Cho. No consistent data were available for mania, largely due to the small number of studies. Regarding medication effects on ACC neurometabolites, meta-analyses revealed that both medication-free and medicated BD subjects had increased ACC Cho. It was not possible to perform further meta-analyses exploring the medication effect due to the heterogeneity in medication profiles across studies or lack of detailed medication information. However, some selected studies provided evidence that anticonvulsants may impact ACC balance between Glu/Gln in BD.

The overall increased ACC Glx levels in BD patients relative to HC revealed by a meta-analysis with moderate effect size is in line with previous meta-analyses that simultaneously assessed different voxels and/or mixed adult and child/adolescent populations (Gigante et al., 2012 ; Chitty et al., 2013). However, there was an insufficient number of studies reporting ACC Glx in specific mood states to perform meta-analysis, precluding any conclusion as to whether increased ACC Glx is a general feature in BD or a mood state-dependent phenomenon. Since Glx represents the sum of several metabolites, predominantly Glu and Gln (Buonocore and Maddock, 2015) , increased Glx has been interpreted as an indicator of glutamatergic neurotransmission (Govindaraju et al., 2000). Thus, it has been postu-lated that the putative increased glutamatergic neurotransmission causes supra-activation of glutamatergic receptors, increasing calcium post-synaptic influx, resulting in excito-toxicity, cell

damage or even neuronal death (Berk et al., 2011 ; Mehta et al., 2013). Considering that Glx does not disclose whether Glu or Gln are elevated and that the latter is a non-neuroactive glutamatergic metabolite (Albrecht et al., 2007), increased Glx may not necessarily represent enhanced glutamatergic neurotransmission. Indeed, our meta- analysis failed to confirm any alteration of ACC Glu in BD, and thus did not support the notion of an increased glutamatergic state in the ACC as a general feature in BD.

Conversely, we found increased overall Gln, which was particularly influenced by the euthymic mood state. However, there were too few studies to perform meta-analyses in mania and depression, precluding any conclusion regarding the ACC Gln dynamics across mood states in BD. In the central nervous system, Gln is synthetized in ascrocytes from the extracellular Glu via the Gln synthetase pathway and serves as the precursor for neuronal Glu synthesis in the glutamatergic neurons (Walls et al., 2015). Such Glu-Gln cycling across neurons and astrocytes has been interpreted as an evolutionary acquisition to buffer glutamate-related ex-citotoxicity, since Gln is a "non-excitatory"form of stored Glu (Walls et al., 2015; Cooper and Jeitner, 2016). Since the increased Gln found was associated with the euthymic mood state, we may hypothetise that there might occur a shift in Glu-Gln cycle towards the latter under euthymia. Indeed, anticonvulsants medication has been reported to both decrease Glu (Friedman et al., 2004 ; Strawn et al., 2012) and increase Gln (Soeiro-de-Souza et al., 2015). Furthrmore, some selected studies have reported that euthymic BD subjects taking anticonvulsants had lower Glx/Cr (Soeiro-de-Souza et al., 2013) or Glu/Cr (Soeiro-de-Souza et al., 2013 ; 2018 a), as well as increased levels of Gln (Soeiro-de Souza et al., 2015 ; Kubo et al., 2017). However, our results should be interpreted with caution because only 4 studies have assessed Gln and none were performed unsing a field strength higher than 4 T. Therefore, we highly recommend that further studies investigate the exact composition of increased ACC Glx, using more sensitive techniques and higher magnetic fields that allow more precise measurement of Gln and Glu, as well as in different mood states. The overall increases in ACC Cho in BD demonstrated by meta-analyses with larger effect sizes and lower levels of heterogeneity strongly suggest it is a potential trait marker in BD, given it was observed in euthymia, depression and medication-free subjects, but not in mania. However, this finding conflicts with a previous meta-analysis focused on Cho that mixed data from multiple voxels (Kraguljac et al., 2012), although some previous reports have correlated increased Cho to the severity of both depressive (Moore et al., 2000) and manic (Cecil et al., 2002) states. Elevated Cho has been associated mostly with increased phospholipid cycling or membrane breakdown that results in the release of membrane choline compounds, commonly observed in neurodegenerative (e.g., Alzheimer Disease) and demyelination (e.g., Multiple Sclerosis) processes (Stork and Ren- shaw., 2005). Given that cortical thinning in frontal areas is one of the most consistent neurobiological findings documented in BD, including in the ACC (Hibar et al., 2017), our results corroborate the notion that increased Cho could be a neurochemical trait associated with increased phospholipid turnover and possibly correlated with neuro-morphometric losses. Such a phenomenon appeared to be more influenced by the depressive than manic episodes, although there were only 3 studies performed in both these mood states. Increased Cho was also observed in the pooled meta-analysis of studies that mixed subjets under different mood states and it appeared not to be influenced by the medication status. Additionally, a positive correlation was also noted between publication year and effect size, only for the overall meta-analysis, suggesting the robustness of recent studies (e.g., Soeiro-de-Souza et al., 2018 a and Zhong et al., 2018) has contributed to this positive result since these studies have assessed larger samples in a single mood state. Therefore, further studies assessing homogeneous samples in specific mood state using appropriate techniques (e.g. phos- phorus magnetic resonance spectroscopy) are warranted. Such increased Cho levels, possibly associated with ACC lower cortical volume, appear not to stem from mitochondrial dysfunction since no consistent changes in NAA were observed in overall, depressed, euthymia, manic or medication-free samples. This result contradicts previous systematic review and meta-analysis (Yildiz-Yesiloglu and Ankerst., 2006; Kraguljac et al., 2012) reporting NAA decline in frontal areas, the hippocampus and basal ganglia in BD, considered a surrogate of mitochondrial dysfunction and neuronal loss (Stork and Renshaw, 2005 ; Moffett et al., 2007). Therefore, our data do not corroborate the mitochondrial oxidative metabolism dysfunction theory (Stork and Renshaw., 2005) for neuroprogression in BD (Berk et al., 2011). Such a Cho-NAA discordant result suggests that the increased Cho levels might be associated with abnormalities in white matter microstructures, axonal myelination and white matter tracts disconnectivity (Öngür et al., 2010 Benedetti et al., 2011 ; Nortje et al., 2013) rather than neuronal damage. Alternatively, it may be related to inflammatory and neurotrophic pathways (Berk et al., 2011) instead of energetic metabolism imbalances.

No evidence of alterations in ACC GABA in BD patients compared to HC was found by the present meta-analysis. This finding is in agreement with previous investigations (Chiapponi et al., 2016; Schür et al., 2016) and may potentially be confounded by the medication effect since medications such as anticonvulsants and benzodiazepines are known to modulate GABA (Sanacora et al., 2002). Similaly, meta-analyses showed no differences in mI between

groups, contradicting findings of a previous systematic review that documented higher mI levels in frontal regions (Silverstone et al., 2005).

The main limitation of the present study was the high heterogeneity observed in some overall meta-analyses, largely as a result of the different study designs, stages of illness and absence of detailed medication information, as well as differences among studies in relation to voxel size, magnetic field strength, ^{1}H-MRS editing technique, echo-time, metabolite quantification method, tissue composition correction and software used for quantification. Of these factors, voxel size may be the most significant, since there are specific cytoarchitectural areas with different functions within the ACC (Bush et al., 2000), aside from the fact that in overly small voxels, the signal-to-noise ratio might be poor. Echo-time also varied widely among studies, a factor that has been found to influence the quality of metabolite measurements, in particular Glu (Schubert et al., 2004). Although the high I^2 values found in the present meta-analyses were comparable to those described by previous studies (Gigante et al., 2012 ; Schür et al., 2016 ; Romeo et al., 2018), sub-analyses of some of the metabolites (e.g. Cho) revealed no heterogeneity, highlighting the need for more methodologically homogenous ^{1}H-MRS studies in BD. Additionally, no meaningful publication bias was detected in the present study.

5. Concluding remarks

The results of the present meta-analysis corroborate the relevance of ACC to understanding BD neurobiology across different mood states. Although there is still room for improvement in ACC 1 H-MRS literature, our results strongly support the hypothesis of increased ACC cell membrane phospholipid turnover and increased Glx in BD. However, the nature of Glx increase in the ACC, as well as the Glu-Gln balance across mood states, are still poorly understood, but there is some evidence that medications can modulate the Glu-Gln cycle within the ACC. Further ^{1}H-MRS studies, in the ACC or other voxels, should assess more homogeneous (preferentially mood-state specific), larger BD samples and account for medication status using more sensitive ^{1}H-MRS techniques.

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Author contributions

Study conception: ES-M, KU-R, MG S-S; Study design: ES-M, KU-R, MGS-S; Data search and extraction: ES-M, 70MGS-S; Data analysis and interpretation: ES-M, KU-R, MG S-S; Writing of manuscript: ES-M, KU-R, MGS-S. All authors approved the final version of the manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

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Supplementary materials

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Supplementary Material

Anterior cingulate cortex neurometabolites in Bipolar Disorder are influenced by mood state and medication: a meta-analysis of ^{1}H-MRS studies.

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Running Title: ACC neurometabolite profile in BD

Figure S1- ACC Location in a MRI scan.

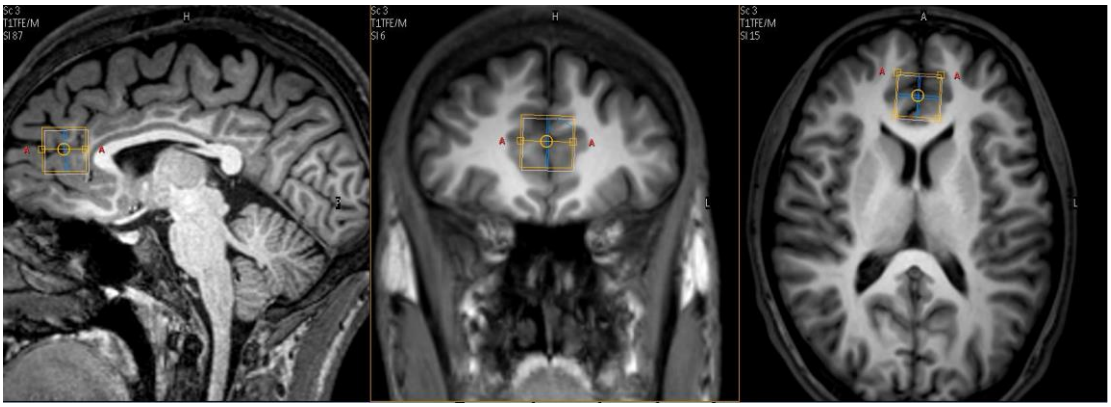


Figure S2 – PRISMA flow chart of selection process for inclusion of studies

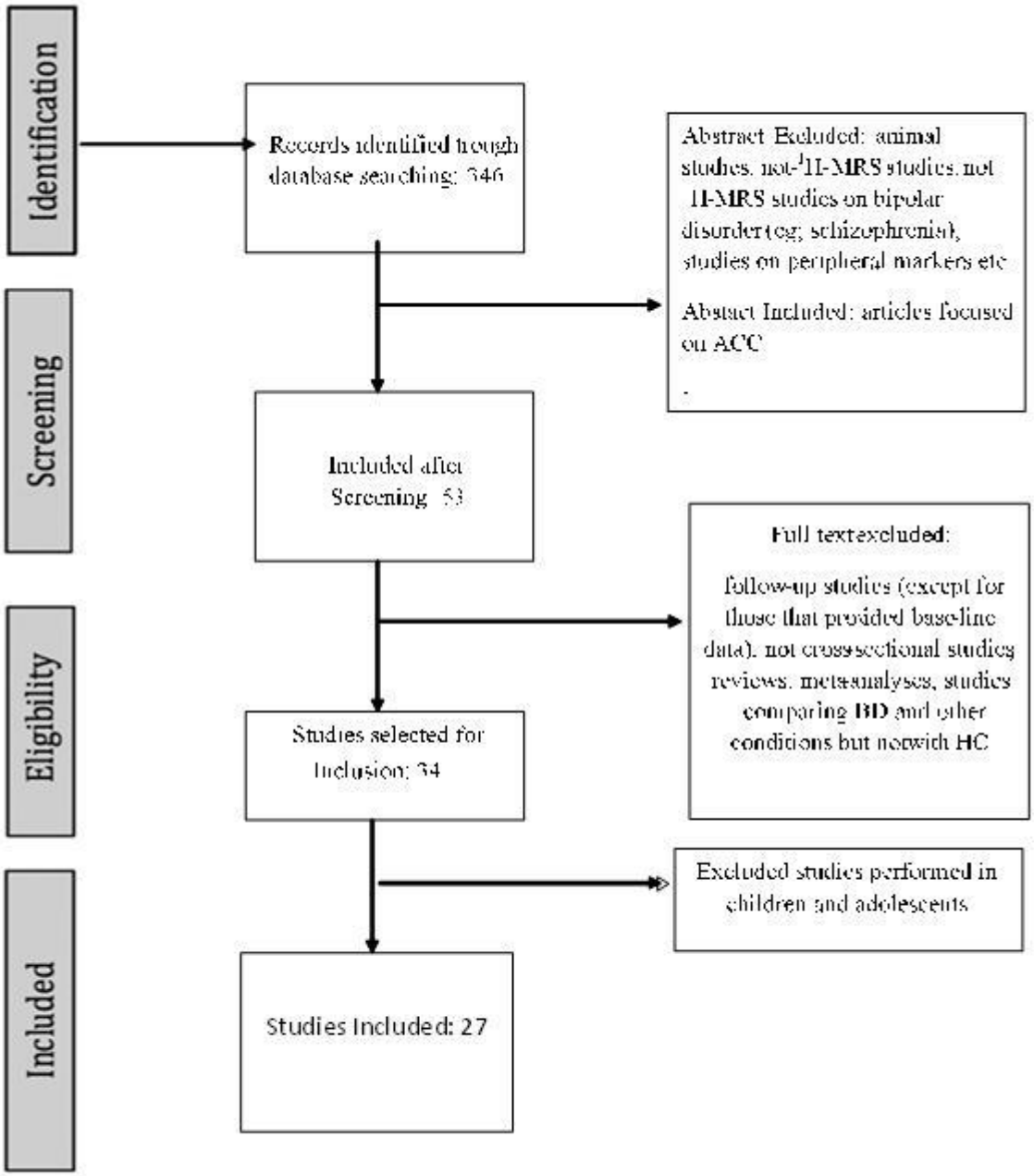
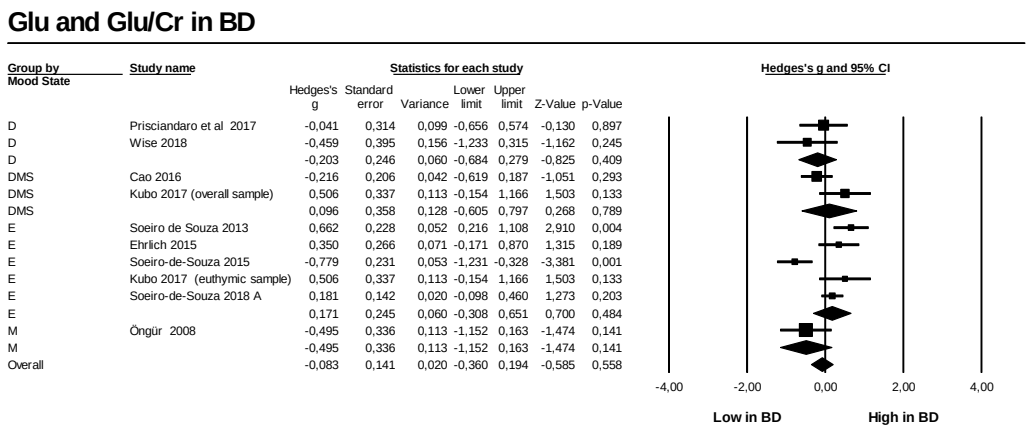
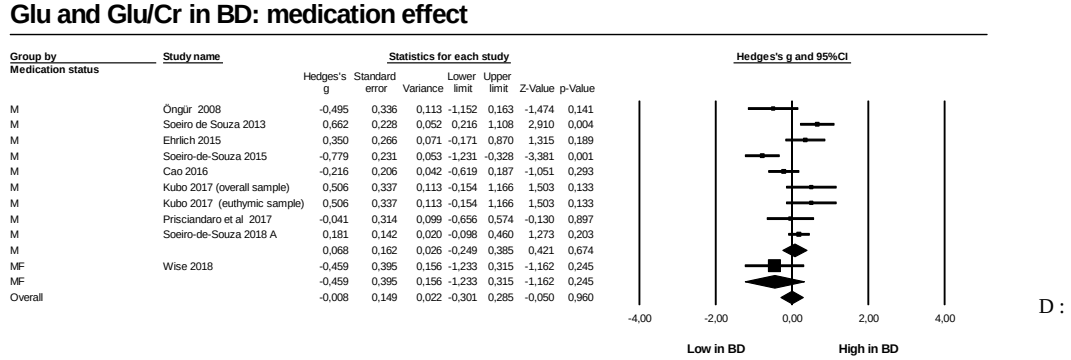


Figure S3 A- Forest Plot for meta-analysis of ACC Glu/Cr and Glu in overall BD and under different mood states.



D Depression; DMS: Different mood states; E: Euthymia; M:mania

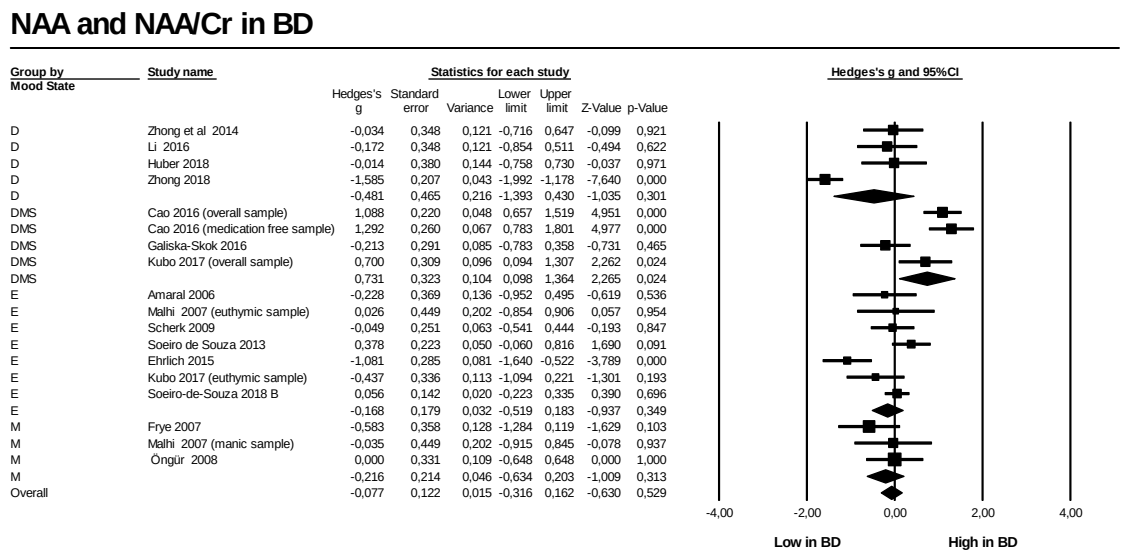
Figure S3 B- Forest Plot for meta-analysis of ACC Glu/Cr and Glu in overall BD and under different medication status.



D :

Depression; DMS: Different mood states; E: Euthymia; M:mania/hypomania

Figure S 4A: Forest plot for meta-analysis of ACC NAA/cr and NAA in overall BD and under different mood states.



D : Depression; DMS: Different mood states; E: Euthymia; M:mania/hypomania

Figure S 4 B- Funnel plot for meta-analysis of ACC NAA and NAA/Cr in overall BD.

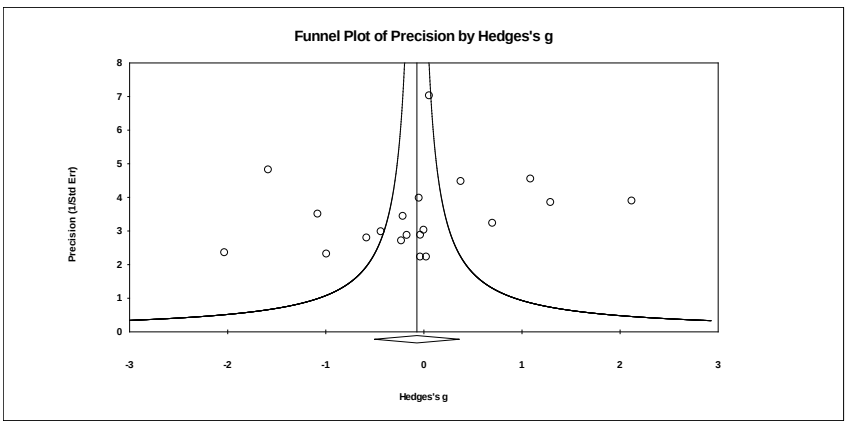
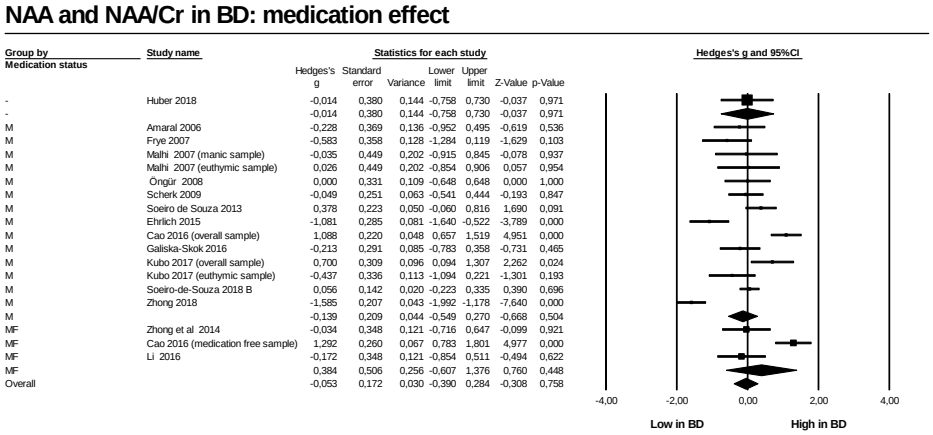


Figure S 4C Forest Plot for meta-analysis of ACC NAA and NAA/Cr in overall BD as influenced by the medication status



M: Medicated; MF: medication-free

Figure S 5- Funnel plot for meta-analysis of ACC Cho and Cho/Cr in overall BD.

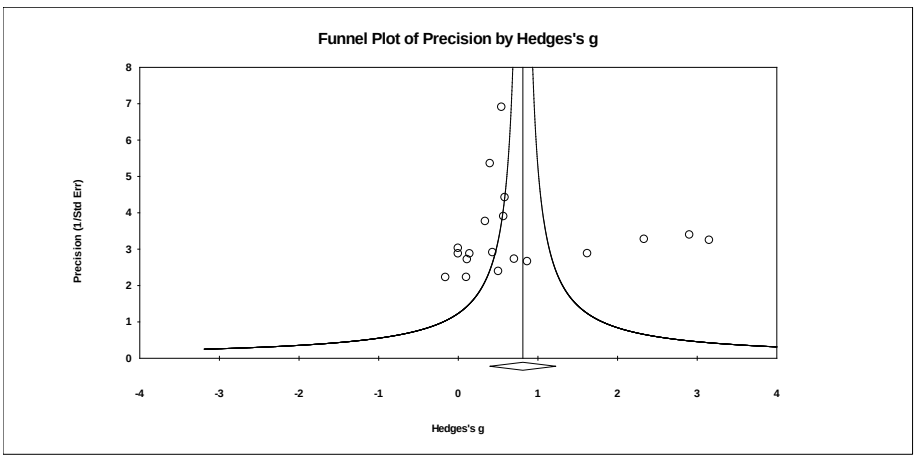
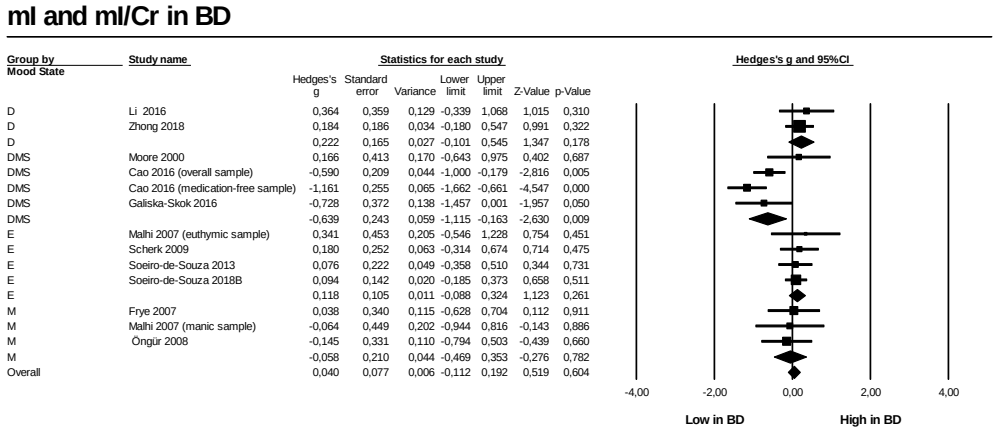


Figure S 6A- Forest plot for meta-analysis of ACC mI/cr and mI in overall BD and under different mood states.



D : Depression; DMS: Different mood states; E: Euthymia; M:mania/hypomania

Figure S 6B- Funnel plot for meta-analysis of ACC mI and mI/Cr in overall BD.

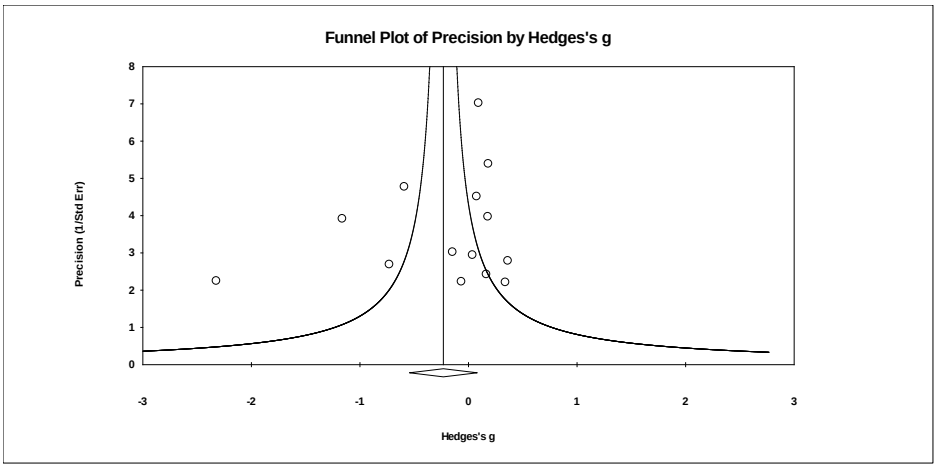
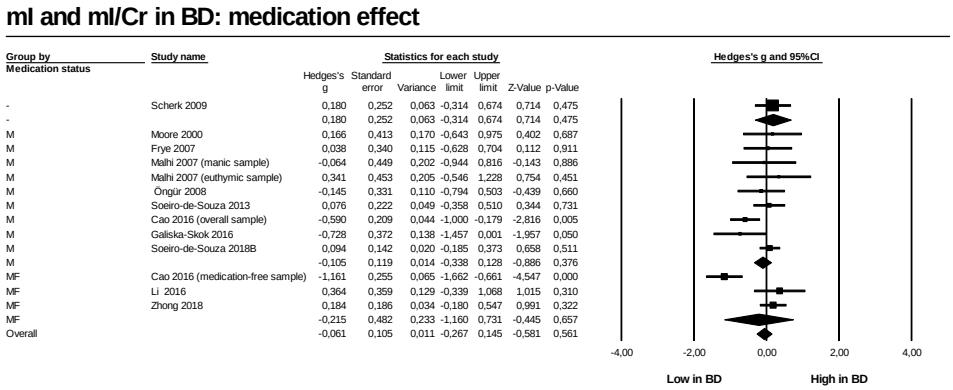
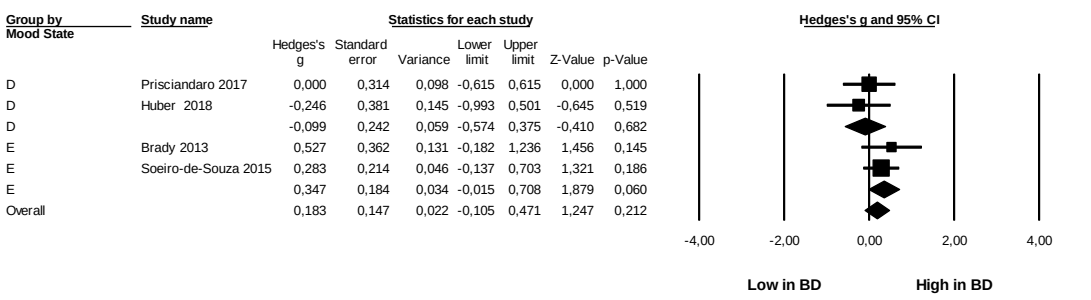


Figure S 6C- Forest Plot for meta-analysis of ACC mI and mI/Cr in overall BD as influenced by the medication status.



M: Medicated; MF: medication-free

Figure S 7- Forest Plot for meta-analysis of ACC GABA in overall BD and under different mood states.



D : Depression; E: Euthymia

Table S1 -Cross-sectional ACC [1]HMRS studies comparing GABA concentration between Bipolar Disorders and Healthy controls subjects

Reference	BD Type	P	C	Mean Age (P/C)	Mood State	Voxel Size (cm^3)	Field Strengh (Tesla)	Echo Time (ms)	MRS sequence	Quantifi-cation	Medication	Result	Direction
Brady 2013	I	14	14	32.6/36.9	E	16.7	4	68	MEGA PRESS	GABA/cr	Li, Ac,Ad, Ap, Benz	S	Inc
Soeiro-de-Souza 2015	I	50	38	31.7/25.7	E	18	3	variable	JPRESS	GABA	Li, Ac, Ad,Ap,Benz	NS	-
Prisciandaro 2017[1]	I,II	20	19	36.8/38.0	D	7	3	variable	JPRESS	GABA/cr	Li, Ac,Ad, Ap	NS	-
Huber 2018	I,II	19	10	18/19	D	18.7	3	variable	JPRESS	GABA	NM	NS	-

Abbreviations: Ac: Anticonvulsants; **Ad:** Antidepressants; **Ap:** Antipsychotics; **C**: Healthy Controls; **Dec**: Decreased; **D:**depression; **DMS:** different mood states; **E:** Euthymia; **H:** hypomania; **Inc:** Increased; **Li**: Lithium; **M:** mania; **MF**:medication-free; **MS**: mood stabilizers; **N**: number of subjects; **NM:** not mentioned; **NS**: non-significant; **P**: Patients; **S**:significant**.**

[1]Authors did not mention the mood state which was assumed as Depressive based on Montgomery-Asberg Depression Rating Scale data.

TableS2- Summary of the main meta-analytic results regarding ACC neurometabolites in BD.

Metabolite/group	BD-Overall	Mania	BD Depression	Euthymia	Medication-free
Glx	Inc	-	-	-	-
Glu	NA	-	-	NA	-
Gln	Inc	-	-	Inc	-
GABA	NA	-	-	-	-
NAA	NA	NA	NA	NA	NA
Cho	Inc	NA	Inc	Inc	Inc
mI	NA	NA	-	NA	NA

Legend:

Inc: Increased; NA: Not altered; -: Not known

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3.2. Capítulo II – ACC Glu/GABA ratio is decreased in euthymic bipolar disorder I patients: possible *in vivo* neurometabolite explanation for mood stabilization

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ORIGINAL PAPER

ACC Glu/GABA ratio is decreased in euthymic bipolar disorder I patients: possible in vivo neurometabolite explanation for mood stabilization

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Abstract

Bipolar disorder (BD) is characterized by unstable mood states ranging from mania to depression. Although there is some evidence that mood instability may result from an imbalance between excitatory glutamatergic and inhibitory GABA-ergic neurotransmission, few proton magnetic resonance spectroscopy (^{1}H-MRS) studies have measured these two neurometabolites simultaneously in BD. The enzyme glutamic acid decarboxylase (GAD1) catalyzes the decarboxylation of glutamate (Glu) to GABA, and its single nucleotide polymorphisms (SNPs) might influence Glu/GABA ratio. Thus, we investigated Glu/GABA ratio in the dorsal anterior cingulate cortex (dACC) of euthymic BD type I patients and healthy controls (HC), and assessed the influence of both mood stabilizers and *GAD1* SNPs on this ratio. Eighty-eight subjects (50 euthymic BD type I patients and 38 HC) underwent 3T ^{1}H-MRS in the dACC (2 × 2 × 4.5 cm^3) using a two-dimensional JPRESS sequence and all subjects were genotyped for 4 SNPs in the *GAD1* gene. BD patients had lower dACC Glu/GABA ratio compared to HC, where this was influenced by anticonvulsant and antipsychotic medications, but not lithium. The presence of *GAD*1 rs1978340 allele A was associated with higher Glu/GABA ratio in BD, while patients without this allele taking mood stabilizers had a lower Glu/GABA ratio. The lowering of dACC Glu/GABA could be one explanation for the mood stabilizing action of anticonvulsants and antipsychotics in BD type I euthymia. Therefore, this putative role of Glu/GABA ratio and the influence of GAD1 genotype interacting with mood stabilization medication should be confirmed by further studies involving larger samples and other mood states.

ClincalTrials.gov registration: NCT01237158.

Keywords GABA · Glutamate · Bipolar · Anticonvulsants · *GAD1*

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Introduction

Bipolar disorder (BD) is a severe chronic psychiatric dis- order characterized by unstable mood and psychomotor energy, promoting mood states of hypomania, mania, and depression [1]. Glutamate (Glu) and Gamma-aminobutyric acid (GABA) are the main excitatory and inhibitory neuro- transmitters, respectively, in the brain. These neurotrans- mitters play a major role in neural migration, differentia- tion, and synaptic plasticity [2, 3]. One of the hypotheses regarding the neurobiology of BD is that mood instability may result from an imbalance between excitatory Glu [4] and inhibitory GABA [5] neurometabolites in key brain regions involved in affect regulation, such as the anterior cingulate cortex (ACC). In fact, this hypothesis of an imbalance in neuronal excitation/inhibition is supported by reports of alterations in GABA and Glu levels in both plasma [6, 7] and cerebral spinal fluid (CSF) [8, 9] of BD patients.

Proton magnetic resonance spectroscopy (^{1}H-MRS) is a technique that measures in vivo brain metabolites such as Glu and GABA. While the systematic reviews and meta-analyses on both these metabolites have shown increased individual levels of Glu in different brain regions of BD subjects as compared to controls [4,10,11], no consist-ent differences compared to healthy subjects have been reported for GABA [12,13]. Thus far, no studies have examined Glu/GABA ratio in BD, a putative measure of excitatory/inhibitory balance [14,15]. Understanding Glu/ GABA balance during euthymia and how mood stabilizers influence this ratio can yield important information about the neurometabolite mechanisms of mood balance in BD. The ACC has been considered a key region in the neurobiology of BD, having been implicated in mood regulation. This region plays a major role in fronto-limbic connectivity, exhibiting functional and structural abnor- malities in patients with BD [16]. In fact, ACC is the most studied voxel in BD and the largest ^{1}H-MRS study on euthymic BD type I has confirmed reports of higher Glx/ Cre and Glu/Cre in this region among patients compared to HC [17].

Recent meta-analyses of ^{1}H-MRS studies in BD [4,10,11] have reported increased levels of Glu or Glx (sum of Glu, glutamine and GABA) in patients relative to controls in many different brain voxels. In euthymia, elevated levels of Glu and/or Glx have been documented in the hippocampus [18], occipital cortex (OCC) [19,20], and ACC of patients compared to healthy controls [17,21,22]. GABA levels are more difficult to determine by ^{1}H-MRS because extracting data from spectra with coinciding resonant signals is still a complex task [23] which requires a special MRS acquisition technique. Consequently, there are fewer studies on GABA than on Glu, with the majority showing no significant differences in brain GABA levels between BD and HC [24]. Moreover, the few ^{1}H-MRS GABA studies in BD reveal substantial sample (mood state) and voxel (ACC,OCC, basal ganglia, prefrontal, and temporal cortices) heterogeneity [5,19,22,25–28]. Results for these voxels range from increased [29] to decreased [19] GABA in the OCC compared to controls, increased [5] to no GABA difference [22,28] in the ACC, and no difference in GABA at all for the whole brain [26] or prefrontal cortex [27].

Glu/GABA ratio has been considered a measure of the excitatory/inhibitory balance, playing a major role in brain neurodevelopment and plasticity [2,14]. The ratio has also been found to be elevated in some pathological conditions [14], such as autism and schizophrenia spectrum disorders [30,31], suggesting it may serve as a putative proxy of excitation/inhibition balance associated with altered cor tical function [14]. Although Glu and GABA are closely associated in normal human brain because most GABA is synthesized from glutamate, very little is known about Glu–Gln–GABA balance under normal or pathological conditions such as BD. Only five studies have measured Glu and GABA levels simultaneously, while none have investigated Glu/GABA ratio [19,22, 26–28]. Bhagwagar et al. found a lower GABA/Cre ratio and higher Glx in the OCC region in a sample of 16 euthymic medication-free BD patients compared to 18 HC subjects [19]. The other four studies that measured both GABA and Glu levels in BD patients reported no differences in levels of the two metabolites. None of these studies measured Glu/GABA ratio in patients versus HC in the ACC [22,28].

Glu–GABA cycling may also be evaluated by assessingthe glutamic acid decarboxylase (GAD1) enzyme found in somata and dendrites of GABA cells [32] that catalyzes the decarboxylation of Glu to GABA. It is encoded by the *GAD1* and *GAD2* genes [33,34], and GABA synthesis in the human brain depends greatly on the *GAD1* gene, whose expression and protein levels have been reported to be reduced in different brain regions of BD and schizophrenia patients, including in the ACC [35–39]. Genetic studies have also demonstrated an association between two single nucleotide polymorphisms (SNPs) in the promoter region of *GAD1* (rs1978340, rs872123) and BD [40], while Chung et al. (2017) found an association between the SNP rs3749034 and an increased risk of developing BD type II [41]. Moreover, reports indicate that genetic variants in the *GAD-1* are associated with higher GABA/Cre ratios in the prefrontal cortex of HC on ^{1}H-MRS [42]. In a study by Marenco et al. of healthy subjects, the rs1978340 genotype AA was associated with higher GABA/Cre levels than the genotypes GG and AG [42]. However, thus far, no study has investigated whether these *GAD-1* SNPs influence Glu/ GABA ratio under normal or pathological conditions.

Although the mechanism of action of mood stabilizers is not fully understood, they are known to exert their effect, partially, by decreasing glutamatergic activity through multiple mechanisms. These mechanisms involve the regulation of synaptic Glu uptake, receptor activity, and intracellular signaling cascade functions [43], as well as the enhancement of neural GABA activity [44]. Therefore, it is not known whether mood stabilizers influence in vivo

Glu/GABA ratio or whether their clinical effect is linked with this ratio. Curiously, mood stabilizers have been reported to exert epigenetic effects [45], especially the effect of valproic acid on *GAD1* gene expression in BD patients [46].

The primary aim of this study was to investigate Glu/GABA ratio in the dorsal ACC (dACC) of BD I patients during euthymia, as compared to HC, and to determine the influence of mood stabilizers on this ratio and possible clinical implications. Finally, as a secondary objective, the association of *GAD-1* SNPS [allele A (rs3749034, rs1978340) or allele C (rs769390, rs11542313)] with Glu/GABA ratio was investigated. We hypothesized that BD patients during euthymia have lower Glu/GABA levels and that individuals with genotypes previously associated with higher GABA (AA rs1978340) [42] have a lower Glu/GABA ratio.

Materials and methods

88 subjects were included in this study. Of these, 50 (31 F, 18–45 years old) were euthymic BD I patients and 38 (15 F, 18–45 years old) were HC subjects. Diagnoses were established by trained psychiatrists based on the Structured Clinical Interview (SCID-I/P) [47] for DSM-IV TR [48]. The patients had been on stable medication regimens for at least 2 months prior to the ^{1}H-MRS scanning session. All included subjects have participated in all analysis in this study. We did not include in this study subjects with neurological disorders or medical disorders, head trauma, or current/past (3 months) substance abuse (including illegal substances), as well as individuals treated with electroconvulsive therapy in the last six months or reporting heavy episodic drinking (consuming ≥ five standard drinks (male), or ≥ four drinks (female), over a 2-h period [49] over the past 3 months. The Young Mania Rating Scale (YMRS) [50] and the Hamilton Depression Rating Scale (HDRS-21) [51] were used to assess residual sub-threshold depressive and manic symptoms. Euthymia was defined as having a YMRS and HDRS < 7 for at least 3 months and with no change to their pharmacological prescription. The patients also fulfilled the DSM-IV criteria for remission.

All HC subjects had no current or past history of psychiatric disorders according to the evaluation conducted by trained psychiatrists using the Mini International Neuropsychiatric Interview (MINI) [52]. In addition, HC subjects had no family history of mood or psychotic disorders among first-degree relatives based on a semi-structured interview. The studied population was not stratified by race/ethnicity due to the heterogeneity of the Brazilian admixed population. The local research ethics committee approved the study. Written informed consent was obtained from all study participants.

Image acquisition

All MRI exams were performed on a Philips 3T Achieva scanner (Philips Healthcare, Best, The Netherlands) using an eight-channel head coil. Spectroscopy measurements were performed using the maximum echo sampled JPRESS sequence proposed by Schulte et al. [53]. The JPRESS sequence is based on the conventional PRESS spin-echo technique used for selection of a single voxel. By varying the echo time of the acquisition, the J coupling evolution is encoded in an additional dimension. This technique is therefore also known as two-dimensional spectroscopy, whereby the signal is measured as a function of chemical shift expressed by the Larmor frequency (as in conventional one-dimensional spectroscopy), but also as a function of the coupling constant J in Hz. With the information of the cou- pling constant J, it is possible to resolve the signals from overlapping multiplets, such as Lac and GSH. In this study, the JPRESS sequence was used to evaluate a voxel of 2 (L–R) × 2 (I–S) × 4.5 cm (A-P) (total voxel size 18 cm^3) in the dACC region, as shown in Fig. 1. The minimum echo time (TE) used was 31 ms, and TE was incremented in 100 steps of 2 ms. For each time increment ΔTE, the maximum- echo sampling started the acquisition ΔTE/2 earlier with respect to the echo top [53]. The repetition time (TR) was 1600 ms, and 8 averages were acquired for each TE step. One non-water suppressed spectrum was also acquired at each TE. The number of points per spectrum was 1024, and the spectral band width was 2000 Hz. An automatic second order B_0 shimming routine was used and water suppression was achieved by VAPOR [54]. Spectroscopy acquisition took 24 min, and the total exam duration, including volumetric imaging and voxel planning, was about 45 min. Metabolite quantification was determined using ProFit (PRiOr knowledge FITting) version 2.0 running on Matlab R2011b [55]. The first version of ProFit was developed by Shulte et al. [56] to fit 2D JPRESS data by extending LC Model [57] principles to 2D data sets. In ProFit, as in the LC Model approach, the prior knowledge is derived from a known metabolite basis set (experimentally acquired or calculated) used in the fitting process, and the VARPRO approach [58] is used to separate the optimization of non-linear and linear parameters for faster convergence. Fuchs et al. improved the quantification program (ProFit version 2.0) by introducing an experimentally-acquired 2D macromolecular baseline into the fitting model and allowing for a more accurate and precise fit by accounting for the actual line shape and additional baseline distortions by self-deconvolution and spline modeling approaches [55].

The metabolite basis set used by ProFit includes spectrafrom a total of 18 brain metabolites including the metabolites of interest in this study: Glu and GABA. Isolated measures of GABA, Glu and glutamine have been the focus of a publication by Soeiro-de-Souza et al. [22]. Basis set metabolite spectra were calculated with the GAMMA

library [59] using the chemical shift and J-coupling values from the literature [60, 61]. Quantitative results in ProFit are given in the form of ratios to Cr signal (met/Cr). These ratios are already corrected for T2 relaxation effects, since ProFit automatically calculates T2 relaxation times for each metabolite from the signal obtained at the different TEs. "Pseudo" absolute metabolite values [met] were obtained by assuming a white matter (WM) Cre concentration of 4.83 mM and a grey matter (GM) Cre concentration of 9.59 mM, as expressed in the equation below. These Cre values in mM were calculated from previously reported Cre concentrations in units of mM/kg [62],

$$[\text{met}] = (f_{\text{GM}} \times 9.59\text{mM} + f_{\text{WM}} \times 4.83\text{mM}) \times \frac{\text{met}}{\text{Cre}},$$

where

$$f_{\text{GM}} = \frac{\text{GM\%}}{\text{GM\%} + \text{WM\%}} \quad \text{and}$$
$$f_{\text{WM}} = \frac{\text{WM\%}}{\text{GM\%} + \text{WM\%}},$$

fractions of Cre signal attributable to GM and WM, respectively. To determine the brain tissue composition contained in the MRS voxel of interest, three-dimensional volumet- ric images were obtained using the 3D-T1- FFE (fast field echo) technique (FA = 8°; TE/TR/TI = 3.2/7/900 ms) with an isotropic voxel size of 1 mm^3. Briefly, the brain tissue was extracted using the brain extraction tool (BET), and seg- mentation into WM, GM, and CSF was achieved using the automated brain segmentation tool FAST [64]. Both tools are part of the FSL suite (https://www.fmrib.ox.ac.uk/fsl). Finally, the MRS voxel was overlaid on the segmented image **using a**

$$f_{\text{GM}} = \frac{\text{GM\%}}{\text{GM\%} + \text{WM\%}} \quad \text{and}$$
$$f_{\text{WM}} = \frac{\text{WM\%}}{\text{GM\%} + \text{WM\%}}.$$

T1 relaxation effects were corrected for, assuming a mono-exponential T1 relaxation with GM T1 of 1.46 s and a WM T1 of 1.24 s [63]. GM % and WM % represent grey and white matter volume percentages, respectively, in the selected MRS voxel, while f_{GM} and f_{WM} represent the Python-based script developed in-house and percent ages of WM, GM, and CSF were calculated for each voxel. The GM fraction (f_{GM}) contributing to the observed MRS signal was calculated as: f_{GM} = GM%/(GM% + WM%).

The ProFit program also provides a Cramér-Rao lower bound (CRLB) [65], a measure of the quality of the metabolite quantification, for each metabolite. CRLB were noted for each metabolite (Glu mean 1.7%; GABA mean 10.8%). Note that we only included in this study ^{1}H-MRS data with CRLB < 20. Supplement Figs. 1 and 2 represents an example of spectra quality from our results.

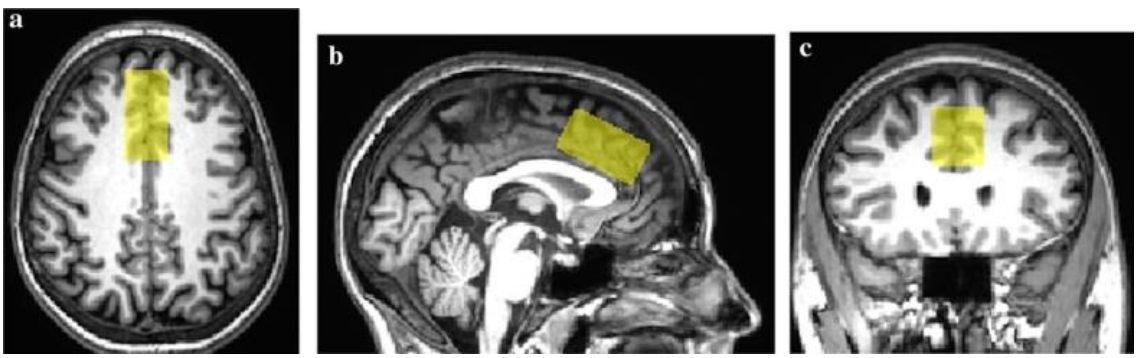
Blood collection and genotyping

DNA was extracted from peripheral blood according to the salting-out protocol [66] on the same day as the MRS exam and was then genotyped for *GAD1* rs3749034 (5'UTR), *rs1978340 (5'flanking promoter), rs11542313 (exon 3), and rs769390 (intron variant)* using real-time PCR allelic dis- crimination. SNP selection was based on previously pub- lished studies on GAD1 in SZ and BD. PCR amplification of all the SNPs was performed in 5 µl reactions with 5 ng of template DNA, 1 × TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA), 1 × each primer and probe assay, and H_2O. Thermal cycling consisted of initial dena- turation for 10 min at 95 °C followed by 40 cycles of dena- turation at 95 °C for 15 s and annealing at 60 °C for 1 min. Fluorescence detection occurred in the annealing step. Amplification and allelic discrimination were performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Quality control of Real-Time PCR results was conducted with direct sequencing using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City).

Analysis was performed by dividing groups according to the presence of allele A (rs3749034, rs1978340) [AA and AG genotypes (A+) vs. GG genotype (A−)] or allele C (rs769390 and rs11542313) [CC and AC genotypes (C+) vs. AA genotype (C−)] due to the small numbers of homozygous AA and CC participants.

None of the *GAD1* SNPs (described below) deviated from Hardy-Weinberg equilibrium [rs3749034 (χ^2 = 0.24, p = 0.6), rs1978340 (χ^2 = 1.6, p = 0.2), rs11542313 (χ^2 = 2.4, p = 0.1), rs769390 (χ^2 = 0.15, p = 0.6)].

Figure 1 MRS voxel location in axial (**a**), sagittal (**b**), and coronal (**c**) planes.



Satistical analysis

First, the sample was tested for homogeneity. Categorical variables were analyzed using x^2 tests and continuous variables were analyzed using *t* tests. Significant differences in age and gender were observed in the sample and to prevent this potential bias from influencing results, age, and gender correction was included in all analyses. Normality was checked using the Kolmogorov–Smirnov test. Normally distributed variables were compared between the two groups using ANCOVAs in which Glu/GABA ratio was entered as a dependent variable and age, gender, and f_{GM} were covariates. To investigate the influence of medication use on Glu/GABA ratio, an ANCOVA test was performed in which medication use was entered as a dependent variable and medication type (lithium, atypical antipsychotics or anticonvulsants), age, gender, and f_{GM} were covariates. To assess the effect of *GAD*1 polymorphisms on Glu/GABA, the variable allele A [A + vs. A−] or allele C [C + vs. C−] was added to the ANCOVA model according to each SNP (as described in Blood collection and Genotyping section). For this analysis, Glu/GABA was entered as the main interest variable along with age, gender and f_{GM} as covariates. The regression coef- ficients (coef) are expressed together with their 95% confi- dence intervals (CI). As a measure of effect size partial eta square values (η^2p) are reported. All statistical analyses were carried out using IBM SPSS version 20.

Results

Sociodemographic data and clinical features are presented in Table 1. After correcting for age and gender, significant differences were found between BD patients and HC subjects in voxel content in terms of GM% (BD 52% vs. HC 54%) ($F = 2.4$, $df = 77$, $p = 0.02$) and CSF% (BD 24% vs. HC 22%) (F = 2.1, $df = 77$, $p = 0.02$), but no differences in WM content (BD 23% vs. HC 23%) ($F = 0.2$, $df = 77$, $p = 0.77$) were evident. BD subjects had lower GM volume and higher CSF volume. These differences were compensated for in the statistical analysis by entering f_{GM} as a variable in the statistical model.

Genetic distribution

Differences in allelic frequency between the BD and HC groups were found for only two *GAD1* SNPs. The rs1978340 allele A was more prevalent in the HC subjects (65%) than in the BD patients (44%) ($\chi^2 = 4.1$ $p = 0.04$) and the rs769390 allele C was also more prevalent in the HC subjects (55%) than in the BD patients (18%) ($\chi^2 = 13.1$ $p < 0.001$). Genotype distribution is reported in Table 1.

Metabolite differences between groups

No influence of tobacco use, age at onset, illness dura- tion or lifetime psychotic symptoms on dACC metabolite concentrations was found. Significant main effects of diagnostic group on metabolite levels were observed after correcting for gender, age, and fGM.

The Glu level ($\eta^2p = 0.062$, coef. 58, 95% CI 0.081, $p = 0.021$) was lower in BD patients (mean 5.73, SD 1.2) than in HC (mean 6.63, SD 0.95). GABA levels ($\eta^2p = 0.063$ coef − 0.07 95% CI −0.16 to 0.02 $p = 0.1$) did not differ between BD patients (mean 0.75, SD 0.22) and controls (mean 0.70, SD 0.17) Table 2.

Glu/GABA ratio was higher in the HC group (mean 9.98, SD 1.01) ($\eta^2p = 0.064$, coef 1.6, 95% CI 0.27–3.0, $p = 0.020$) than in the BD group (mean 8.31, SD 0.88), as shown in Fig. 2.

Influence of mood stabilizers on Glu/GABA

Regarding the use of lithium (monotherapy or combination therapy) ($n = 29$), Glu/GABA ratio in lithium users (mean 8.71 SD 1.12) presented no statistic difference compared to HC, but it was lower in those not using lithium (mean 7.65 SD 1.38) compared to HC (mean 9.96 SD 1.03, $\eta^2p = 0.07$ coef − 2.3, 95% CI 6.2–9.0, $p = 0.013$). On the other hand, subjects on lithium monotherapy presented similar Glu/GABA ratio (mean 9.70, SD 3.10) compared to controls (mean 10.11, SD 3.30). Furthermore, those patients receiving lithium monotherapy ($n = 7$) did not have different isolated Glu (mean 6.27, SD 1.41) or GABA (mean 0.70, SD 0.13) levels within dACC compared to those not taking lithium [Glu (mean 6.63, SD 0.95) GABA (mean 0.70, SD 0.17)] or controls [Glu (mean 6.63, SD 0.95) GABA (mean 0.70, SD 0.17)] (Supp. Table 1).

BD patients using anticonvulsants ($n = 23$) (monotherapy or combination therapy) had lower Glu/GABA ratios (mean 8.01 SD 1.26) ($\eta^2p = 0.063$ coef − 1.9, 95% CI − 3.6 to − 0.3, $p = 0.021$) compared to HC subjects (mean 9.97, SD 1.02) but without statistic difference compared to BD patients not using anticonvulsants ($n = 27$) (mean 8.57, SD 1.20). Glu/GABA ratio did not differ comparing anticonvulsants monotherapy ($n = 4$) (mean 7.44, SD 2.27) and HC (mean 10.11 SD 3.30). Moreover, those patients receiving anticonvulsants monotherapy ($n = 4$) did not

Table 1 Subject demographic, clinical, and genetic information

	Healthy controls $n = 38$	Bipolar disorder type I $n = 50$	p
Age (years), mean ± SD	25.7 ± 5.7	33.3 ± 10.6	**0.01***
Gender (male/female)	23/15	19/31	0.05**
Education (years), mean ± SD	14.2 ± 3.1	14.3 ± 3.2	0.39*
Tobacco use (yes/no)	0	5/45	
Age of onset (years) mean ± SD		21 ± 8	
HDRS mean ± SD		3.7 ± 2.1	
YMRS mean ± SD		2.4 ± 2.1	
Lithium use	Monotherapy		$n = 7$
	Combination		$n = 29$
Anticonvulsants	Monotherapy		$n = 4$
	Combination		$n = 23$
Antipsychotics	Monotherapy		$n = 12$
	Combination therapy		$n = 23$
rs1978340 (AA/AG/GG)	03/22/13	04/17/29	0.83**
rs3749034 (AA/AG/GG)	01/14/23	01/12/37	0.21**
rs11542313 (AA/AC/CC)	18/14/06	16/20/14	0.09**
rs769390 (AA/AC/CC)	17/16/05	41/06/03	**0.001****

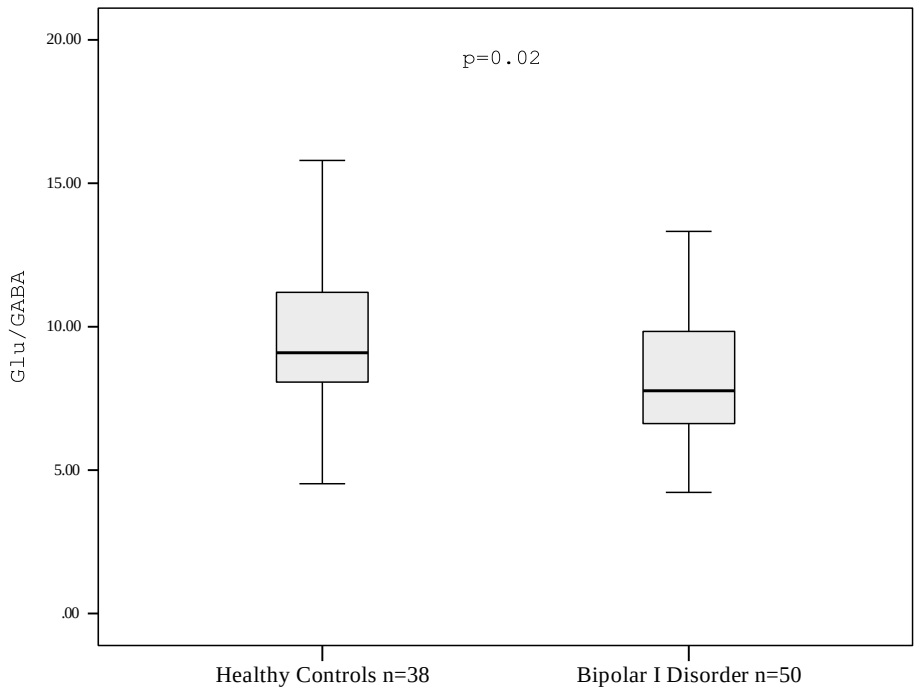
Hamilton depression rating scale HDRS; Young mania rating scale YMRS

*t test, **χ^2 test. Significance level $p < 0.05$

Table 2 DACC glutamate and GABA levels in BD and controls

Metabolite	*n*	Group	Mean	SD	*p*
Glu	38	HC	6.63	0.95	**0.02**
	50	BD	5.73	1.22	
	88	Total	6.12	1.20	
GABA	38	HC	0.70	0.17	0.17
	50	BD	0.75	0.22	
	88	Total	0.73	0.21	
Glu/GABA	38	HC	9.98	1.01	**0.02**
	50	BD	8.31	0.88	
	88	Total	9.03	3.11	

Figure 2 dACC Glu/GABA in BD and HC. Mean values ± SEM (standard error of the mean) according to the Kolmogorov- Smirnov test at 5% confidence level ($p \leq 0.05$)



have different isolated Glu (mean 5.57, SD 1.62) or GABA (mean 0.83, SD 0.41) levels within dACC compared to those not taking anticonvulsants [Glu (mean 5.74, SD 1.2) GABA (mean 0.74, SD 0.20)] or controls [Glu (mean 6.63 SD 0.95) GABA (mean 0.70, SD 0.17)] (Supp. Table 1).

The use of atypical antipsychotics (monotherapy or combination therapy) (n = 23) was associated with lower Glu/GABA ratios (mean 8.13, SD 1.32) compared to HC (mean 10.09, SD 1.04) (η^2p = 0.06 coef − 1.9, 95% CI − 3.7 to − 0.02, p = 0.029). Interestingly, Glu/GABA ratio did not differ between antipsychotic monotherapy (n = 12) (mean 8.49, SD 2.02) and HC (mean 10.11, SD 3.30). Furthermore, atypical antipsychotic monotherapy (n = 12) presented similar levels of Glu (mean 5.79, SD 0.99) and GABA (mean 0.71, SD 0.18) compared to BD not using these drugs [Glu (mean 5.71, SD 1.06), GABA (mean 0.76, SD 0.23)] or healthy controls [Glu (mean 6.63, SD 0.95) GABA (mean 0.70 SD 0.17)] (Supp. Table 1).

Glu/GABA genetic modulation by *GAD* SNPs

BD subjects with allele A rs1978340 presented higher Glu (mean 6.20 SD 0.45) compared to BD non carriers (mean 5.6 SD 0.42) (η^2p = 0.11 coef. 66, 95% CI − 1.1 to − 0.09 p = 0.01). GABA levels were not affected by allele A rs1978340.

For the overall sample (BD plus HC) (n = 88), subjects not carrying the rs1978340 allele A (genotype GG) (n = 42) had lower Glu/GABA ratio (mean 8.19, SD 0.93) compared to allele A carriers (n = 43) (mean 9.77, SD 0.87) (η^2p = 0.68, coef − 1.5, 95% CI − 2.8 to − 0.3, p = 0.016).

When the sample was stratified according to diagnosis, an rs1978340 effect on Glu/GABA was observed only for BD patients, where subjects not carrying the rs1978340 A allele (n = 29) had lower Glu/GABA ratio (mean 7.42, SD 1.04) compared to allele A carriers (mean 9.08, SD 1.16) (η^2p = 0.095 coef − 1.66, 95% CI − 3.1 to − 0.12, p = 0.035), as shown in Fig. 3.

Modulation of Glu/GABA by GAD SNPs and mood stabilizer

Glu/GABA was lower in BD non-carriers of allele Ars1978340 using anticonvulsants (n = 18) (mean 7.5753, SD = 1.19) compared to HC non-carriers of allele A (n = 13) (mean 9.36, SD 1.68) (η^2p = 0.089, coef. − 2.6, 95% CI − 4.4 to − 0.7, p = 0.007).

Glu/GABA in BD patients on lithium (n = 29) not carrying the allele A rs1978340 (n = 7) (mean 7.52, SD 1.57) was lower than HC subjects not carrying allele A (n = 13) (mean 9.46, SD 1.69) (η^2p = 0.07, coef. − 2.6, 95% CI −4.7 to − 0.5, p = 0.01). Glu/GABA in BD patients not on lithium and not carrying the allele A rs1978340 (mean 7.62, SD 1.6) was lower than in HC subjects not carrying allele A (mean 9.46, SD 1.69) (η^2p = 0.08, coef. − 2.7, 95% CI − 4.7 to − 0.7, p = 0.008).

Glu/GABA was lower (mean 7.96, SD 1.51) in subjects on antipsychotics non-carriers of allele A rs1978340

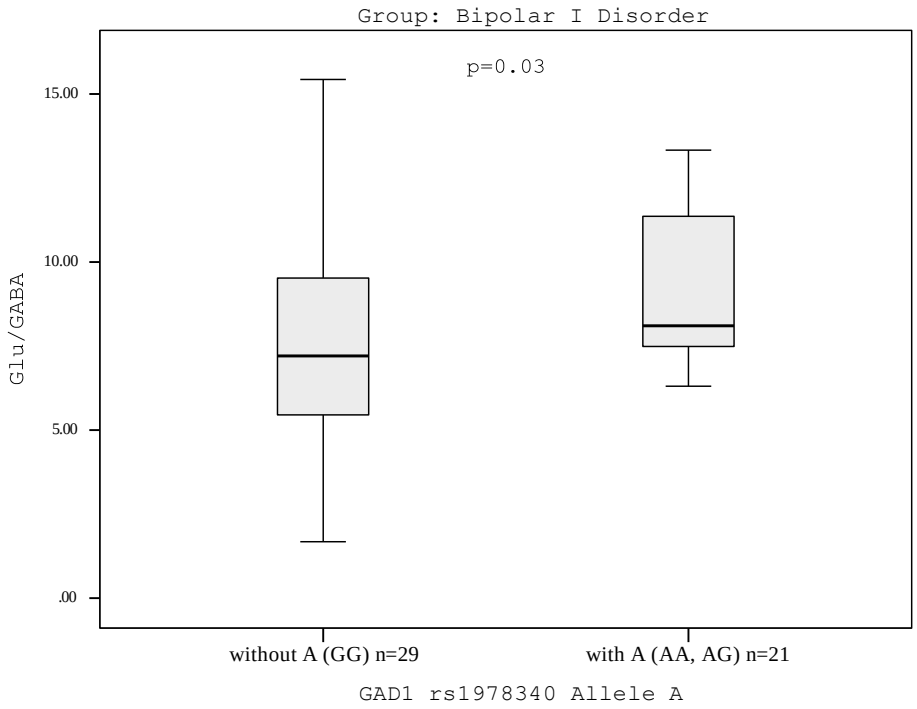


Figure 3 dACC Glu/GABA ratio in BD patients by rs1978340 allele A presence Mean values ± SEM (standard error of the mean) according to the Kolmogorov-Smirnov test at 5% confidence level ($p \leq$ 0.05)

compared to HC non-carriers of allele A rs 1978340 (mean 9.48, SD 1.71) ($\eta^2p = 0.06$, coef. − 2.2, 95% CI − 4.2 to − 0.3, $p = 0.02$).

Rs11542313, rs3749034 and rs769390 had no influence on Glu/GABA.

Discussion

To the best of our knowledge, this is the first study assessing Glu/GABA ratio in BD and determining the effects of genetic background and medication on this ratio. Using a two-dimensional ^{1}H-MRS sequence, we observed a lower Glu/GABA ratio in the dACC of euthymic BD type I patients compared to HC subjects, where this ratio was especially influenced by the use of anticonvulsant and antipsychotic medications. Moreover, we report that not carrying the *GAD*-1 rs1978340 allele A (genotype GG) was associated with lower Glu/GABA ratio in BD patients and that there was an additional lowering effect on Glu/GABA in those using lithium, anticonvulsants or atypical antipsychotics.

Our finding of decreased dACC Glu/GABA ratio in BD is an important first step towards gaining a better understanding of how the balance between these two metabolites is related to achieving euthymia in BD. Imbalances in Glu/GABA ratio may reflect changes in excitatory/inhibitory neurotransmission, one of the main neurobiological hypotheses proposed for mood disorders [13, 43]. Inhibitory GABAergic and excitatory glutamatergic neurotransmissions are closely regulated from both anatomical and functional standpoints [14, 67] and associated with cognition, learning, memory, and emotional behaviors [68]. It therefore follows that the mood and cognitive disturbances observed in BD may stem from excitatory-inhibitory dysregulation in cortical areas involved in the regulation of affect and cognition, such as the dACC [69]. Cortical disrupted excitatory-inhibitory balance has been proposed to underlie neurodevelopmental disorders such as autism spectrum disorders (ASD) and schizophrenia [14, 70]. This imbalance, as measured by the Glu/GABA ratio, has been found to be increased in some cortical areas, including the ACC, of both autism and schizophrenia spec- trum disorder patients [15, 30, 31, 68]. Imbalances in Glu/ GABA ratio in the developing brain may lead to abnormal myelination and delayed synapse maturation [14, 23]. This deleterious effect can result from a hyperexcitable glutamatergic state or deficient GABAergic activity that, in turn, may cause cortical disinhibition and over-xcitation, affecting neuronal growth, and brain connectivity [14].

Thus, it has been hypothesized that increased Glu/GABA ratio in some cortical areas of autism and schizophrenia spectrum disorder patients might explain clinical features of these disorders related to behavior, affective regulation, and social organization [15, 30, 31, 68]. Similarly, decreased excitation/inhibition balance in BD euthymia may be associated with cortical dysfunctions [14] in brain regions that regulate affect and cognition, such as the ACC [69]. Thus, shifts in this balance may explain the structural alterations observed in this region in BD [71] and, ultimately, symptoms such as mood instability and cognitive impairment.

Our finding of lower Glu/GABA in a sample of euthymic medicated BD subjects compared to healthy controls seems to be more influenced by Glu (reduced in BD compared to controls) than GABA levels, as well as modulated by both the pharmacological treatment and genetic characteristics. The greater contribution of Glu than GABA for Glu/GABA ratio modulation is expected in BD since GABA levels have been found unaltered in relation to HC [24]. However, it needs to be noted that it is more challenging to find differences in GABA, due to the larger uncertainty associated to this metabolite, which in our study presented a mean CRLB of 10.8%, while the mean CRLB for Glu was only 1.7%. Additionally, GABA [72] and Glu [17] have proven prone to change in response to pharmacological treatment. Interestingly, those patients taking anticonvulsants and antipsychotics in combination therapy, but not monotherapy, had an even lower Glu/GABA ratio compared to HC subjects. On the other hand, no difference in this ratio was observed between lithium users and HC subjects. Thus, the decrease in Glu/GABA ratio in the BD group was influenced by a combination of pharmacological mechanisms of anticonvulsants and antipsychotics. We hypothesize that the mechanism shared by anticonvulsants and antipsychotics which might explain our results is their pharmacological abilities to lower Glu [73]. In fact, anticonvulsants are known to modulate GABA [74, 75], and to a greater extent Glu [76, 77], in the brain, either by increasing the former or decreasing the latter [43, 75]. Furthermore, there is some evidence that antipsychotics may also decrease Glx levels in frontal areas [78]. Similarly, our group recently demonstrated that Glu/Cre ratio in the ACC of a BD-I sample was lower in euthymic individuals taking anticonvulsants than in patients not taking this class of medication [17]. Moreover, animal model studies have reported that Glu/GABA ratio decreases after electro-convulsive therapy (ECT) [79], an effective treatment for mania and depression, most likely by increasing GABA as demonstrated in human studies [80]. Therefore, we propose that mood stabilizers, in particular anticonvulsants and atypical antipsychotics, may exert their mood stabilizing effect by decreasing Glu/GABA ratio in the dACC. Furthermore, given that atypical antipsychotics are commonly used as an augmentation strategy in BD [81], we may infer that this first-line treatment combination with anticonvulsants [82] could facilitate euthymia by boosting the decrease in Glu/GABA.

Additionally, we have reported modulation of dACC Glu/GABA ratio by a SNP in the promoter region of the *GAD1*gene in BD: rs1978340. The rs1978340 allele A was shown to be associated with higher Glu/GABA compared to non-carriers, which may be associated with mood instability, since increased Glu levels lead to higher neuron excitability [1]. Although previous studies involving HC have reported an association between the rs1978340 genotype AA and higher GABA/Cre in medial prefrontal cortex, possibly by increased GAD67 activity [42], our dACC results

failed to show this, with genetic results proving specific to BD subjects. By contrast, our result of lower Glu/GABA in BD non-carriers of the allele A suggests that the presence of allele A may be implicated in higher amounts of Glu in the dACC, but differently from that reported by Marenco et al. [42] in HC subjects. Since we found that the allele A rs1978340 increased Glu in BD patients but not in controls, we speculate that *GAD*1 polymorphisms rs1978340 work differently in BD. This may occur by altering the GAD67 enzyme activity, resulting in lower rates of Glu decarboxylation to GABA and consequent accumulation of Glu. Therefore, BD carriers of the rs1978340 A allele had higher Glu/GABA compared to BD non-carriers (GG genotype). Moreover, all mood stabilizers (lithium, atypical antipsychotics, and anticonvulsants) had an additional effect of lowering Glu/ GABA in the rs1978340 GG genotype. Curiously, mood stabilizers have been shown to exert an epigenetic effect [45], especially valproic acid. This medication was reported to increase GAD67 mRNA expression in the lymphocytes of BD patients by altering its chromatin structure [46] which, in long-term treatment, could also influence Glu/GABA. We speculate that BD carriers of the rs1978340 GG genotype might be better responders to mood stabilizers than those with genotypes AA and AG, due to a potentially lower dACC Glu/GABA ratio.

This study has some strengths and limitations. Strengths of this study are its large sample of homogeneous BD type I subjects in the same mood state, its pioneering approach to investigating a metabolite ratio little assessed in BD, and the use of an accurate technique that simultaneously measures Glu and GABA. Limitations of this study include age and gender disparities (although results were corrected for age and gender) between the BD and HC groups and the small sample size for analyzing medication (combination versus monotherapy) and genetics (genotype analysis).

This is the first study to investigate Glu/GABA ratio in euthymic BD I subjects and determine how mood stabilizers and GAD 1 modulate this ratio. We found that dACC Glu/ GABA ratio is decreased during euthymia in BD I and that it is mainly influenced by prescription anticonvulsants and antipsychotics. Moreover, the SNP rs1978340 genotype GG (without allele A) was associated with lower Glu/GABA ratio in BD compared to GG controls. This SNP genotype GG seemed to enhance the anti-glutamatergic effect of all mood stabilizers in our BD sample. The role of Glu/GABA as a putative marker for mood stability should be investi- gated in future studies comparing other mood states. Finally, the influence of the GAD1 genotype on Glu/GABA ratio in BD, potentiating drug action should also be investigated in studies involving larger sample sizes.

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Compliance with ethical standards

Conflict of interest None of the authors report biomedical financial interests or potential conflicts of interest.

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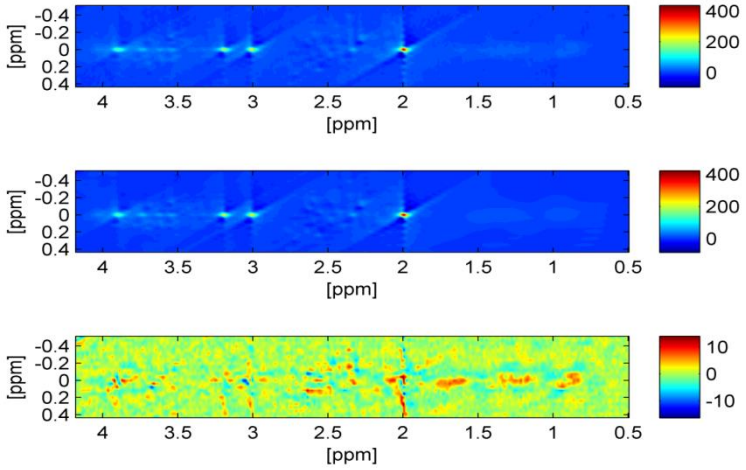
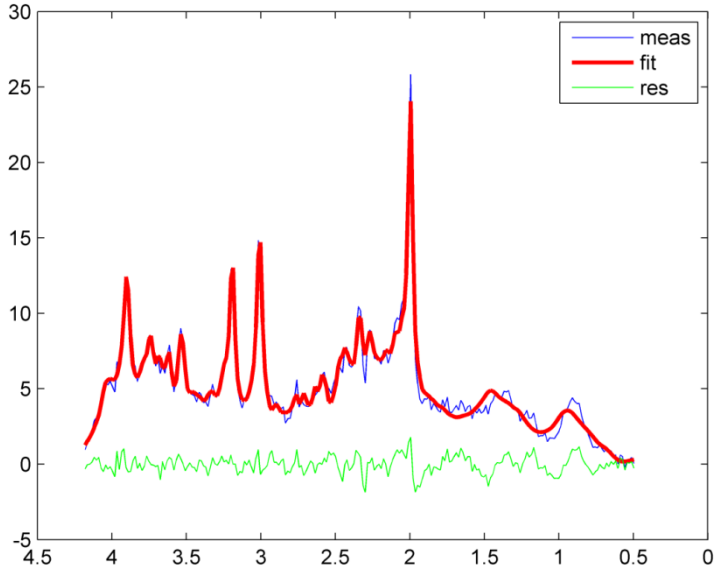
Supplement Figure 1: An example of a two-dimensional JPRESS spectrum of the ACC in a BD subject (top), its fitted spectrum (middle), and the residual of the fit (bottom). Note that the color scale is amplified x20 in the residual.



Supplement Figure 2: One-dimensional projection of the acquired JPRESS spectrum displayed in Figure 1. Measured spectrum in blue, fitted in red and residual in green.



3.3. Capítulo III – Association between *CACNA1C* gene rs100737 polymorphism and glutamatergic neurometabolites in bipolar disorder

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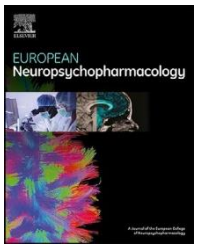

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Association between *CACNA1C* gene rs100737 polymorphism and glutamatergic neurometabolites in bipolar disorder

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Glutamine;
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^{1}H-MRS

Abstract
Abnormalities in Ca^{2+} homeostasis in Bipolar Disorders (BD) have been associated with impairments in glutamatergic receptors and voltage-gated calcium channels. Increased anterior cingulate cortex (ACC) glutamatergic neurometabolites have been consistently disclosed in BD by proton magnetic resonance spectroscopy (^{1}H-MRS). A single nucleotide polymorphism (SNP) in the *CACNA1C* gene (rs1006737), which encodes the alpha 1-C subunit of the L-type calcium channel, has been associated with BD and is reported to modulate intra-cellular Ca^{2+}. Thus, this study aimed to explore the association of the *CACNA1C* genotype with ACC glutamatergic metabolites measured by ^{1}H-MRS in both BD and HC subjects. A total of 194 subjects (121 euthymic BD type I patients and 73 healthy controls (HC) were genotyped for *CACNA1C* rs1006737, underwent a 3-Tesla ^{1}H-MRS imaging examination and ACC glutamatergic metabolite were assessed. We found overall increased glutamatergic metabolites in AA carriers in BD. Specifically, higher Glx/Cr was observed in subjects with the AA genotype compared to both AG and GG in the overall sample (BD + HC). Also, female individuals in the BD group with AA genotype were

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found to have higher Glx/Cr compared to those with other genotypes. *CACNA1C* AA carriers in use of anticonvulsant medication had higher estimated Glutamine (Glx-Glu) than the other genotypes. Thus, this study suggests an association between calcium channel genetics and increased glutamatergic metabolites in BD, possibly playing a synergic role in intracellular Ca^{2+} overload and excitotoxicity.

1. Introduction

Although the neurobiology of Bipolar Disorders (BD) is still poorly understood, glutamatergic abnormalities have emerged over recent years as one of the main neurobiological findings associated with the disorder. Increased glutamatergic activity in BD has been demonstrated by *post-mortem* studies in prefrontal cortex (Hashimoto et al., 2007), evidenced by down-regulation of glutamatergic receptor expression in several cortical regions (Mc Cullumsmith et al., 2007), as well as by *in vivo* studies confirming elevated glutamate (Glu) in cerebral spinal fluid (CSF), serum and plasma of BD patients (Sanacora et al., 2008). Similarly, brain proton magnetic resonance spectroscopy (^{1}H-MRS) studies have documented increased Glx [Glu + glutamine (Gln)] in several cortical regions in BD such as the frontal cortex (Gigante et al., 2012; Chitty et al., 2013), including the anterior cingulate cortex (ACC) (Soeiro-de-Souza et al., 2018; Scotti-Muzzi et al., 2021b). Increased glutamatergic activity is believed to promote supra-activation of glutamatergic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, increasing calcium post-synaptic influx, resulting in Ca^{2+} homeostasis dysfunction and ultimately in neuronal excitotoxicity (Mehta et al., 2012). However, the genetic background and molecular pathways associated with such increased glutamatergic metabolites and impaired calcium homeostasis in BD is still poorly understood. In the last few decades, several genome-wide association studies (GWAS) have consistently associated single nucleotide polymorphisms (SNPs) within the *CACNA1C* gene (e.g., rs1006737) with BD (Ferreira et al., 2008; Sklar et al., 2008, 2011; Stah et al., 2019) as confirmed by candidate gene association studies (Khalid et al., 2018; Mosheva et al., 2020), genetic risk score analysis (Croarkin et al., 2018) as well as meta-analyses (Liu et al., 2011; Nurnberger et al., 2014) and a review on the association of *CACNA1C* rs1006737 and BD (Ou et al., 2015). *CACNA1C* encodes the alpha 1c (CAV1.2) subunit of the voltage-gated, L-type calcium channel (LTCC) and thus regulates Ca^{2+} influx and subsequent cell depolarization (Sinnegger-Brauns et al., 2009).

Besides being considered a risk factor for BD, the AA genotype of the SNP rs1006737 has been reported as a mediator of structural and functional variations in both healthy subjects and BD patients (Jogia et al., 2011; Perrier et al., 2011). For instance, the rs1006737 risk allele has been associated with increased volumes and activation (Jogia et al., 2011; Tesli et al., 2013; Sumner et al., 2015) of the amygdala during cognitive tasks in BD subjects, along with reduced pre-frontal cortex activation (Sumner et al., 2015). Indeed, lower cortical thickness in parietal and frontal cortices have been observed in BD subjects carrying the *CACNA1C* risk allele (Smedler et al., 2019), including the ACC (Soeiro-de-Souza et al., 2017; Smedler et al., 2019), whose thinning has been correlated with subject age (Soeiro-de-Souza et al., 2017). Thus, *CACNA1C* gene is associated with structural and functional changes in frontal and subcortical brain regions that mediate emotion processing and cognition (Bigos et al., 2010; Wang et al., 2011; Soeiro-de-Souza et al., 2012; Dima et al., 2013; Radua et al., 2013; Sumner et al., 2015; Smedler et al., 2019; Shonibare et al., 2021), such as the ACC (Soeiro-de-Souza et al., 2017), which, however, appeared to be influenced by the female sex (Dao et al., 2009; Witt et al., 2014; Takeuchi et al., 2018).

Glutamate excitotoxicity may be attenuated or reverted by neurotrophins (eg., Brain-Derived Neurotrophic Factor- BDNF) via the extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) pathway (Almeida et al., 2005). Also, cognitive and synaptic abnormalities caused by hemizygosity of the psychiatric risk gene *CACNA1C* in mice may be rescued by the effect of neurotrophins via these same signaling pathways (Tigaret et al., 2021). Strikingly, variations in *CACNA1C* has also been reported to modulate serum BDNF in BD subjects (Smedler et al., 2021), corroborating the intricate relationships among the genetic background (eg., *BDNF, CACNA1C*), glutamatergic system and intracellular calcium abnormalities in BD.

Impairments in voltage-gated calcium channels (VGCC) in BD (Uemura et al., 2016) have been corroborated by a robust meta-analysis involving 642 BD patients and 404 healthy control subjects reporting increased intracellular Ca^{2+} in both platelets and lymphocytes of manic, depressed and medication-free BD patients (Harrison et al., 2019). Although it is unclear whether this enhanced intracellular Ca^{2+} derives from VGCC dysfunction (Uemura et al., 2016; Harrison et al., 2019) or enhanced glutamatergic metabolites and activity (Soeiro-de-Souza et al., 2018; Scotti-Muzzi et al., 2021b), the interplay between these pathways has been largely neglected in BD. In mice, *CACNA1C* is highly expressed in glutamatergic neurons and its deletion promotes manifestation of features similar to those observed in BD, such as cognitive decline, impaired synaptic plasticity, reduced sociability, and hyperactivity (Kabir et al., 2017; Dedic et al., 2018). Thus, cortical dysfunctions associated with the *CACNA1C* risk allele (Erk et al., 2014; Paulus et., 2014) may likely be attributed to enhanced Ca^{2+} influx, associated with either a disrupted *CACNA1C*-calmodulin-MAPK/ERK signaling pathway or impairments in glutamatergic receptor activity.

To date, however, no studies have examined the influence of the established risk gene *CACNA1C* on cortical glutamatergic levels in BD. Therefore, the aim of the present

Table 1 Demographic and clinical data for BD-I and HC subjects.

	BD-I (121 subjects)	HC (73 subjects)	p value
Age (SD)	31.88 (0.84)	28.58 (0.97)	**0.014**
Sex (M/F)	39/83	37/36	**0.015***
Education Years	13.14 (0.28)	13.30 (0.36)	0.74
Illness Duration (SD)	6.96 (6.0)		-
GM (SD)	0.60 (0.004)	0.62 (0.005)	**0.006**
WM	0.176 (0.002)	0.175 (0.004)	0.80
CSF	0.21 (0.05)	0.20 (0.05)	**0.025**
CACNA1C rs 1006737 (AA/AG/GG)$^{+}$	(9/43/69)	(5/29/39)	0.78*
Lithium users	90	-	-
Lithium monotherapy	39	-	-
Anticonvulsant users	37	-	-
Antipsychotic users	46	-	-
Lithium +Anticonvulsants	21	-	-
Lithium +antipsychotics	30	-	-

* *t*-test.**$\chi 2$ test, significance $p<0.05$

$^{+}$ Hardy-Weinberg Equilibrium: $\chi 2 = 0.00$, $p = 0.2$

study was to investigate the association of the *CACNA1C* genotype with ACC glutamatergic metabolites measured by ^{1}H-MRS in both BD and HC subjects. We hypothesized the AA genotype might be associated with higher glutamatergic metabolites in BD, theoretically contributing to increased Ca^{2+} levels and neuronal excitotoxicity.

2. Material and methods

2.1 Sampling

The study sample was derived from a database produced in a recent study by our group reporting increased ACC Glx/Cr and Glu/Cr in a large population of BD subjects compared with healthy controls, in addition to modulation of these metabolites by anticonvulsant medications (Soeiro-de-Souza et al., 2018; Scotti-Muzzi et al., 2021a). The present sample comprised 121 euthymic BD type I patients and 73 healthy control (HC) subjects (aged 18-45 years) enrolled in the BIP-USP MRS STUDY and evaluated from 2008 to 2016. These subjects were evaluated over a 4-year period by three research programs focused on BD at the University of São Paulo. Diagnoses were established by trained psychiatrists based on the Structured Clinical Interview (SCID-I/P) for DSM-IV TR (First et al., 1996; American Psychiatric Association 2000). The patients assessed had been on stable medication regimens, as described in **Table 1**, for at least 2 months prior to the ^{1}H-MRS scanning session. Individuals with neurological or medical disorders, head trauma, current/past (3 months) substance abuse (including illegal substances), as well as individuals treated with electroconvulsive therapy in the past six months or reporting heavy episodic drinking over the past 3 months were not enrolled on the study. The Young Mania Rating Scale (YMRS) (Young et al., 1978) and the Hamilton Depression Rat- ing Scale (HDRS) (Hamilton, 1967) were applied to assess residual sub-threshold depressive and manic symptoms. Patients were considered euthymic if they scored <7 on both the YMRS and the HDRS, had no change in pharmacological prescription in the last three months and met the DSM criteria for euthymia. The euthymic BD patients were in use of different combinations of lithium, anticonvulsants and antipsychotics (**Table 1**).

Healthy subjects also had no current or past history of psychiatric disorders according to Mini International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998). In addition, HCs had no family history, in first-degree relatives, of mood or psychotic disorders and had not been in use of any psychotropic medicines for at least three months before enrollment, according to a semi-structured in- terview. Subjects with a history of substance abuse within the 3 months leading up to enrollment were excluded from the study. The research ethics committee of the University of São Paulo approved the study. Written informed consent was obtained from all study participants.

2.2. Image acquisition

Brain MRI exams were performed on a 3.0 T magnetic resonance scanner (Intera Achieva, PHILIPS Healthcare, Best, the Netherlands) with an 8-channel head coil. Each brain exam included anatomical images acquired with a 3D-T1 Fast Field Echo (3D-T1 FFE) sequence; time of echo (TE)/time of repetition (TR)/time of inver- sion (TI) = 3.2/7/900 ms; flip angle (FA) = 8°; FOV= 240mm x 240 mm x 180 mm; matrix = 240 × 240), and magnetic resonance spectroscopy (MRS) acquisition. Single-voxel ^{1}H-MRS was performed using the PRESS sequence with number of scans (NS) of 160, TR of 1500 ms and TE of 80 ms. The choice of TE was based on results from a previous study on optimization of Glu detection (Schubert et al., 2004). MRS was preceded by an automatic pre-acquisition that included adjustment of the transmitter-receiver, optimization of the tilt angle for water suppression and homogenization of the field for the selected volume of interest (VOI). Voxel size was set at $2 \times 2 \times 2$ cm^3 for all patients and controls. The voxel was positioned in the ACC using anatomical guidelines as a reference. The voxel was placed on midsagittal T1-weighted images, anterior to the genu of the corpus callosum, with the ventral edge aligned with the dorsal corner of the genu, and centered on the midline of axial images as shown in **Fig. 1**. An

unsuppressed water spectrum of the same voxel was also acquired for eddy current correction and reference purposes.

2.3. ^{1}H-MRS quantification

Metabolites were quantified using LC Model (Provencher, 1993) and a basis set was simulated for TE=80ms including: Alanine, Aspartate, Creatine, Phosphocreatine, GABA, Glucose, Glutamine, Glutamate, Glycerophosphocholine, Phosphocholine, myo-Inositol, Lactate, N-acetylaspartate, N-Acetylaspartylglutamate, Scyllo- inositol, Taurine, Guanidinoacetate, macromolecules and lipid signals. To control spectral quality between the groups, frequency width at half maximum (FWHM) and signal-to-noise ratio (SNR) were also recorded for each spectrum. In order to ensure the accuracy of the measurements obtained, only metabolite results with Cramer-Rao Lower Bound (CRLB) values $<$ 20% were considered, in accordance with technical references (Kreis, 2004). Metabolite ratios were calculated relative to Cr concentration. Glu/Cr and Glx/Cr were considered for the statistical analysis, where the latter represents the sum of Glu and Gln. Estimated Gln was calculated as follows: Est. Gln = Glx/Cr-Glu/Cr. Since the normal metabolic concentration varies considerably between gray matter (GM) and white matter (WM) (Gasparovic et al., 2006), the fraction of GM in the voxel needed to be taken into account in the analysis. To this end, brain tissue in the three-dimensional T1-weighted brain images was extracted using the brain extraction tool (BET), and then segmented into GM, WM and cerebrospinal (CSF) using the FAST algorithm, both available from the open source FSL software (http://www.fmrib.ox.ac.uk/fsl). Finally, the MRS voxel mask was superimposed onto the segmented images using a Python script developed in-house. The GM brain tissue fraction (f_{GM}) was calculated for each voxel [f_{GM}= %GM/(%GM +%WM)], and f_{GM} was used as a covariate when comparing metabolites between groups

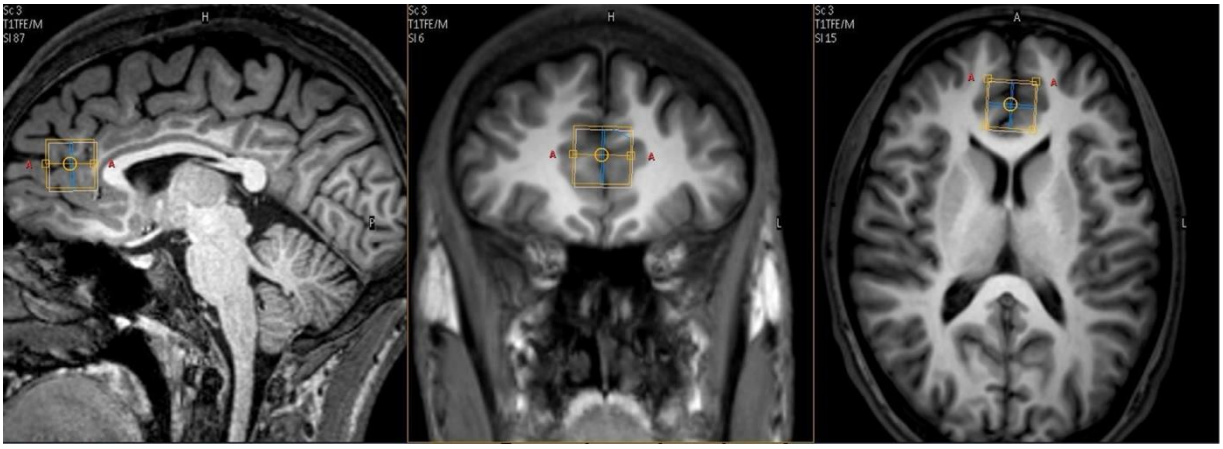
2.4 DNA extraction and genotyping

DNA was obtained from peripheral blood on the day of MRI exams, according to the *salting-out* protocol (Laitinen et al., 1994) and then genotyped for *CACNA1C* rs1006737 using real-time PCR allelic discrimination. PCR amplification for rs1006737 was performed in 5 μl reactions with 5 ng of template DNA, 1 $\times$ TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA), 1 $\times$ each primer and probe assay, and H_2O. Thermal cycling consisted of initial de- naturation for 10 min at 95°C, followed by 40 cycles of denatura- tion at 95°C for 15 s and annealing at 60°C for 1 min. Fluorescence detection was performed in the annealing step. Amplification and allelic discrimination were performed on a 7500 Real-Time System (Applied Biosystems, Foster City, CA). Quality control of Real-time PCR results was done by direct sequencing on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The geno- type distribution was in Hardy-Weinberg equilibrium ($\chi 2 = 0.00$, $p = 0.2$).

2.5 Statistical analysis

The sample was first tested for homogeneity. Normality was checked using the Kolmogorov-Smirnov test. Normally- distributed variables were studied using χ^2 tests for categorical variables and *t*-tests for continuous variables. Non-normally distributed variables (e.g., Glx/Cr and Glu/Cr) were compared using the univariate generalized linear model (GLM), where ACC glutamatergic metabolites (Glu/Cr and Glx/Cr) entered as dependent variables, the *CACNA1C* rs1006737 genotype (AA, AG or GG) as fixed variable, while sex, age and fGM were covariates for all analyses. Mood stabilizing medications (lithium and anticonvulsants) were considered as covariates in the exploratory analyses on the medication effect. Each group was analyzed using a separate model to investigate the association between *CACNA1C* and neurometabolites. The regression coefficients (coef) are expressed together with their 95% confidence intervals (95% CI). Partial eta squared (ηp^2) is provided as a measure of effect size. All statistical analyses were carried out using IBM SPSS version 20.

Fig. 1- ACC Location in a MRI scan



3. Results

3.1 Socio-demographic and genetic distribution data

The socio-demographic and clinical features are shown in **Table 1**. Significant between-group differences were ob- served for the following parameters: age, sex, gray matter (GM) and cerebral spinal fluid (CSF). These differences were controlled for in the statistical analyses by entering fGM (see Material and Methods), age and sex in the statistical model as co-variables. BD patients had higher mean age and CSF values than HCs, whereas controls had larger GM volume than patients. There was also a significant predominance of female individuals in the BD group as compared to males. No group differences in education (years), illness duration or white matter volume were detected. No difference in distribution of *CACNA1C* rs1006737 genotypes (AA/AG/GG) was found between groups. The samples did not deviate from the Hardy-Weinberg equilibrium (**Table 1**).

3.2. Influence of *CACNA1C* rs 1006737 on GM, WM and CSF

No influence of *CACNA1C* rs 1006737 on GM, WM and CSF was observed for the BD or HC groups (**Table S2**).

3.3. Influence of *CACNA1C* rs 1006737 on glutamatergic metabolites

The influence of the *CACNA1C* rs 1006737 genotype on Glx/Cr in the overall sample (BD-I + HC) is shown in **Table 2**. Overall, significantly higher Glx/Cr was detected in AA carriers compared to AG (B: 0.09; 95% CI: 0.005 to 0.17; $p = 0.03$; $\eta p^2=0.02$) and GG (B: 0.09; 95%CI: 0.005 to 0.17; $p = 0.03$; $\eta p^2=0.02$) genotype carriers, although no significant difference in this ratio was observed between AG and GG carriers (B: -0.09; 95%CI: -0.055 to 0.037; $p = 0.69$; $\eta p^2=0.02$). (**Fig. 2A**). Assessment of the effect of the *CACNA1C* rs1006737 genotype on Glu/Cr and estimated Gln in the overall sample showed the same tendency, with AA genotype carriers exhibiting higher levels, but this difference did not reach statistical significance (**Table 2**).

Assessment of the effect of the *CACNA1C* rs 1006737 genotype on glutamatergic metabolites in each group revealed an influence only in the BD group **(Table 3**). BD patients with the AA genotype ($n = 9$) had significantly higher Glx/Cr levels than GG carriers ($n = 69$) (**Table 3**; **Fig. 2B**; B:0.11; 95%CI: 0.005 to 0.22; $p = 0.04$; $\eta p^2=0.03$) and marginally non-significantly higher levels than AG carriers (**Table 3**; B: 0.11; 95%CI: -0.002 to 0.22; $p = 0.054$; $\eta p^2=0.03$), but there was no significant difference in levels between AG and GG genotype (**Table 3; Fig. 2B**; B: 0.001; 95%CI: -0.06 to 0.06; $p = 0.96$; $\eta p^2=0.03$).

Regarding Glu/Cr and estimated Gln, a similar tendency was observed, where carriers of the AA genotype had higher metabolites levels, but this difference did not reach statistical significance (**Table 3**). In the HC group, no significant differences in concentrations were found among genotypes, but AA carriers again had higher levels of all glutamatergic metabolites (**Table 3**).

When assessing the effect of the A allele (subjects with A vs. without A allelle) in both BD and HC groups, no significant differences in glutamatergic metabolites were observed (**Table S3**).

Table 2 Glutamatergic metabolites for the three *CACNA1C* (rs 1006737) genotypes in total sample (BD-I + HC).

	AA (n = 14)	AG (n = 70)	GG (n = 105)
Glx/Cr (SD)	1.14 (0.18)[a]	1.05 (0.13)[b]	1.05 (0.15)[b]
Glu/Cr (SD)	1.06 (0.03)[a]	0.99 (0.01)[a]	1.0 (0.01)[a]
Estimated Gln/Cr (SD)	0.09 (0.01)[a]	0.05 (0.008)[a]	0.05 (0.007)[a]

Legend: Means with different letters on same line differ significantly according to univariate generalized linear model (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

Fig. 2- Glx/Cr for the three *CACNA1C* (rs 1006737)genotypes in total sample (BD-I + HC) (A), BD-I group (B) and female BD-I group (C).
Medians with different letters on same line differ significantly according to univariate generalized linear model (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

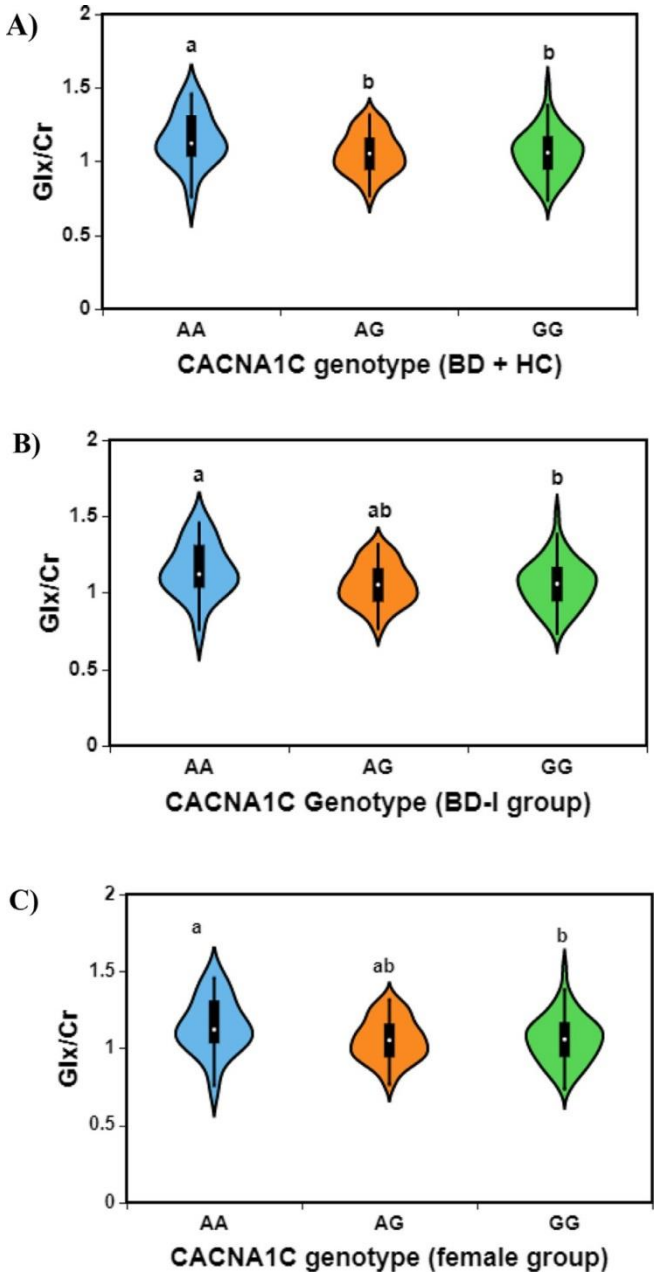


Table 3 Within-group comparison of glutamatergic metabolites by *CACNA1C* (rs 1006737) genotype.

	Healthy Control			Bipolar Disorder		
CACNA1C	AA	AG	GG	AA	AG	GG
(rs 1006737)	$N = 5$	$n = 29$	$n = 39$	$n = 9$	$n = 43$	$n = 69$
GLX/Cr (SD)	1. 09 [a]	1.0[a]	1.03[a]	1.16[a]	1.08[ab]	1.07[b]
	(0.13)	(0.11)	(0.16)	(0.20)	(0.14)	(0.15)
Glu/Cr (SD)	1.01[a]	0.97[a]	0.99[a]	1.07[a]	1.01 [a]	1.00[a]
	(0.09)	(0.10)	(0.13)	(0.19)	(0.11)	(0.11)
Estimated	0.08[a]	0.03[a]	0.04[a]	0.09[a]	0.07[a]	0.06[a]
Gln/Cr (SD)	(0.03)	(0.03)	(0.05)	(0.08)	(0.07)	(0.07)
Cr (SD)	4.5[a]	3.1[a]	3.8[a]	3.9[a]	3.7[a]	3.8[a]
	(0.68)	(1.1)	(1.1)	(1.2)	(0.8)	(1.0)

Legend: Means with different letters on same line differ significantly according to univariate generalized linear model (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

Table 4 Influence of sex and *CACNAC1C* (rs 1006737) genotype on metabolites in BD-I (A) and HC groups (B).

A) Bipolar Disorder

		Male		Female		
CACNAC 1C	AA	AG	GG	AA	AG	GG
(rs 1006737)	$N = 1$	$n = 20$	$n = 18$	$n = 8$	$n = 23$	$n = 51$
GLX/Cr (SD)	1.14[a]	1.07[a]	1.13[a]	1.17[a]	1.08[ab]	1.04[b]
		(0.15)	(0.14)	(0.22)	(0.13)	(0.15)
Glu/Cr (SD)	1.05[a]	0.98[a]	1.04[a]	1.08[a]	1.03[a]	0.99[a]
		(0.12)	(0.09)	(0.19)	(0.10)	(0.11)
Estimated Gln/Cr	0.09[a]	0.09[a]	0.08[a]	0.09[a]	0.05[a]	0.05[a]
(SD)		(0.08)	(0.08)	(0.09)	(0.06)	(0.07)
Cr (SD)	3.2[a]	3.6[a]	3.9[a]	4.1[a]	3.8[a]	3.7[a]
		(0.85)	(1.2)	(1.2)	(0.8)	(1.0)

B) Healthy Control

		Male		Female		
CACNA1C	AA	AG	GG	AA	AG	GG
(rs 1006737)	$N = 5$	$n = 9$	$n = 23$	$n = 0$	$n = 20$	$n = 16$
GLX/Cr (SD)	1.09[a]	1.04[a]	1.03[a]		0.99[a]	1.04[a]
	(0.13)	(0.13)	(0.18)		(0.11)	(0.15)
Glu/Cr (SD)	1.01[a]	1.01[a]	0.97[a]		0.96[a]	1.00[a]
	(0.09)	(0.12)	(0.13)		(0.10)	(0.12)
Estimated Gln/Cr	0.08[a]	0.03[a]	0.04[a]		0.03[a]	0.04[a]
(SD)	(0.03)	(0.04)	(0.06)		(0.03)	(0.04)

Legend: Means with different letters on same line differ significantly according to univariate generalized linear model (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

3.5 Influence of *CACNA1C* rs 1006737 on glutamatergic metabolites by sex

When stratifying the sample by sex, a sex-mediated ef- fect of *CACNA1C* rs 1006737 on ACC Glx/Cr and Glu/Cr was seen in the BD group (**Table 4**). Female BD patients carrying the AA genotype (*n* = 8) had higher Glx/Cr than those with the GG (**Table 4 A** ; **Fig. 2 C** ; B = 0.13; 95%CI 0.01 to 0.24; *p* = 0.03; ηp 2 = 0.06) but not with the AG (B = 0.09; 95%CI: -0.03 to 0.22; *p* = 0.13; ηp 2 = 0.06) genotype, while AG did not differ from GG for these metabolites in females (B = 0.03; 95%CI -0.04 to 0.11; *p* = 0.41; ηp 2 = 0.06).

Similarly, female BD patients with the AA genotype had marginally non-significantly higher Glu/Cr than females with the GG genotype (**Table 4 A** ; B = 0.09; 2.26; 95% CI: -0.002 to 0.18; *p* = 0.05; ηp 2 = 0.05). This finding was not observed between AA and AG (B = 0.05; 95% CI: -0.046 to 0.15; *p* = 0.28; ηp 2 = 0.05) or AG and GG (B = 0.03; 95% CI:-0.025 to 0.09; *p* = 0.24; ηp 2 = 0.05) carriers. Estimated Gln also did not significantly differ among genotypes in male or female BD groups (**Table 4 A**). There were no other differences among genotypes in the male BD group (**Table 4 A**) or in the HC groups for both sexes (**Table 4 B**), although the analy-ses were compromised by the few subjects carrying the AA genotype in these sub-groups.

3.5. Influence of *CACNA1C* rs 1006737 on glutamatergic metabolites by medication type

In order to assess the influence of the main medication types (lithium and anticonvulsants) on the results, we entered both these variables as covariates, together with sex, age and fGM. When controlling for anticonvulsants, sub- jects with AA genotype (*n* = 9) showed significantly higher Glx/Cr than GG (67 subjects; B = 0.11; 95% CI -0.002 to 0.21; *p* = 0.04; ηp 2 = 0.03) and marginally non significantly higher than AG (41 subjects; B = 0.11; 95% CI -0.002 to 0.22; *p* = 0.05; ηp 2 = 0.03). When controlling for lithium, the Glx/Cr was marginally non-significantly higher in AA geno- type (*n* = 9 subjects) in comparison to AG (41 subjects; B = 0.11; 95%CI -0.003 to 0.22; *p* = 0.057; ηp 2 = 0.03) and GG (67 subjects; B = 0.10; 95% CI -0.002 to 0.22; *p* = 0.054; ηp 2 = 0.03). We further assessed the influence of *CACNA1C* rs 1006737 on glutamatergic metabolites according to each mediation regimen separately (**Table S4**). BD AA carriers in use of anticonvulsant medication had significantly higher esti-mated Gln than AG genotype carriers (**Table S4A** ; B = 0.11; 95%CI: 0.004 to 0.22; *p* = 0.04; ηp 2 = 0.12), a finding not observed for the other pair-wise comparisons within anticonvulsant users (**Table S4A**). Within anticonvulsant non-users, those with the AA genotype had a marginally non-significantly higher Glx/Cr than carriers of the GG genotype (**Table S4A** ; B = 0.12; 95%CI -0.003 to 0.24; *p* = 0.056; ηp 2 = 0.05) and there were no significant differences for the pair-wise comparisons of the other genotypes within this group (**Table S4A**). Also, no significant differences in any of the glutamatergic metabolites were detected among the genotypes within lithium users and non-users (**Table S4B**).

4. Discussion

To the best of our knowledge, this is the first study to assess the influence of the *CACNA1C* gene on ACC glutamatergic neurometabolites in BD. The study results confirmed our hypothesis of an association between calcium channel genet-ics and glutamatergic metabolites exclusively in BD, since the latter were found to be increased in subjects carrying the *CACNA1C* rs1006737 AA genotype. This increase appears to be influenced by female sex in BD, and patients with the AA genotype in use of anticonvulsants had in-creased estimated Gln (Glx-Glu) than the other genotypes. Taken together, these results suggest a complex interaction among the disease condition, *CACNA1C* genotype, sex, mood-stabilizing medication and glutamatergic metabolites in BD.

The present study confirms increased ACC Glx/Cr in euthymic BD, as previously reported for part of this sample by Soeiro-de-Souza et al. (2018), and likewise by a recent meta-analysis (Scotti-Muzzi et al., 2021b). Increased gluta-matergic metabolites in BD has been interpreted to result from increased glutamatergic neurotransmission associated with supra-activation of glutamatergic receptors, increased calcium post-synaptic influx and ultimately excitotoxicity (Mehta et al., 2012). The results showed that the increased ACC Glx/Cr observed in BD is modulated by the AA genotype of *CACNA1C* rs1006737, where BD patients with this genotype exhibited higher Glx/Cr. Curiously, when stratifying the BD group by sex, this effect was observed in women only and, within this sub-group, the AA genotype was also associated with marginally significantly higher Glu/Cr. Thus, the mechanism by which the *CACNA1C* rs1006737 genotype AA confers risk for BD seems to be related to increased glutamatergic metabolites, particularly in women. This finding is in line with reports that *CACNA1C* rs1006737 A is a risk al-lele for psychiatric illness such as BD, in women only, modulating sex-driven differences in behavior, mood and cognition (Dao et al., 2009; Witt et al., 2014; Takeuchi et al., 2018). Moreover, other SNP of *CACNA1C* such as rs10774035, in strong linkage disequilibrium with the G/A rs1006737 polymorphism, was associated with impaired recovery from

schizophrenia-spectrum disorder in women but not in men (Heilbronner et al., 2015), and the risk variant rs1024582 has been associated with decreased fronto-limbic activity during working memory tasks only in female healthy individuals (Takeuchi et al., 2018). Therefore, we speculate that the AA genotype might confer less resilience (Michels et al., 2018) to women with BD type I during a BD mood episode (e.g., mania or depression) relative to men, predisposing females to worse outcomes associated with increased glutamatergic insult.

Overall, our data suggest an interplay between the *CACNA1C*-calmodulin-MAPK/ERK and NMDA-mediated glutamatergic signaling pathways. Although the Cav1.2 encoded by *CACNA1C* is thought to mediate LTP and synaptic plasticity, independently of the NMDA receptor activity by activating cAMP response element binding CREB-dependent transcription (Moosmang et al., 2005), there is some evidence that LTP induction may be initiated by both NMDAR dependent activity and voltage-gated Ca^{2+} channel mechanisms (Grover and Teyler, 1990; Morgan and Teyler, 1999). Therefore, subjects with AA genotype might show higher intracellular calcium (Uemura et al 2016) modulated not only by NMDA receptor activity, but also by the *CACNA1C*- calmodulin-MAPK/ERK pathway, likely explaining the abnormal intracellular calcium homeostasis implicated in the pathophysiology of BD (Warsh et al., 2004; Uemura et al., 2016; Harrison et al., 2019) as well as changes in BDNF content (Smedler et al., 2021).

Although the mechanism by which calcium channels and glutamatergic pathways interact to enhance intracellular Ca^{2+} is poorly understood, some calcium channel subunits share homology with glutamate receptor membrane proteins (Nicoll et al., 2006), and changes in calcium channels may serve as a signal for neurotransmitter release through protein interactions (Mochida et al., 1998). Thus, we hypothesize that glutamatergic receptor proteins complexes interact with calcium channels to sense, and respond to, changes in intracellular calcium concentration. This notion is corroborated by some reports showing that mice embryonic deletion of *CACNA1C* in forebrain glutamatergic neurons results in impaired synaptic plasticity, reduced social and cognitive performances, hyperactivity and anxiety- related behavior (Kabir et al., 2017; Dedic et al., 2018), features commonly observed in BD patients. Furthermore, ACC deletion of Cav1.2 Ca^{2+} channels induced impaired learning in mice (Jeon et al., 2010), and the *CACNA1C* haplo-insufficiency resulted in changes in mood-related behaviors in female (but not male) mice, suggesting a sex-specific role mediated by the *CACNA1C* gene (Dao et al., 2009). Also, the knockdown of *CACNA1C* in mouse hippocampal cells challenged by glutamatergic insult resulted in excessive mitochondrial reactive oxygen species formation and calcium influx (Michels et al., 2018). Therefore, the pre-frontal thinning mediated by the *CACNA1C* allele A (Soeiro- de-Souza et al., 2017) may derive from glutamatergic insult in BD, possibly in response to successive manic episodes (Abé et al., 2021).

Although mood stabilizers, particularly the anticonvulsants, have been demonstrated to influence the ACC glutamatergic metabolites content in euthymic BD subjects (Soeiro-de-Souza et al., 2018; Scotti-Muzzi et al., 2021a), AA carriers still showed increased Glx/Cr after controlling for anticonvulsants. On the other hand, AA carriers in use of this medication class exhibited higher estimated Gln levels. This finding is in line with the results reported by Soeiro- de-Souza et al. (2018) for part of the sample studied, which demonstrated lower Glu/Cr among patients using anticonvulsants. Indeed, anticonvulsant medication has been associated with decreased Glx (Strawn et al., 2012) and Glu (Soeiro-de-Souza et al., 2018; Scotti-Muzzi et al., 2021b) as well as increased Gln (Soeiro-de-Souza et al., 2015) and a recent meta-analysis on ACC metabolites in BD correlated increased Gln with euthymia (Scotti-Muzzi et al., 2021b). Therefore, anticonvulsants may promote a shift in Glu-Gln balance across neurons and astrocytes in euthymic BD patients with the AA genotype, since Gln has been considered a "non-excitatory" form of stored Glu (Walls et al., 2015; Cooper and Jeitner, 2016). This might be a possible mechanism underlying its moodstabilizing effect (Scotti-Muzzi et al., 2021b).

The present study has some strengths and limitations. Strengths include the large sample for a ^{1}H-MRS study in- volving, homogeneous BD type I patients in the same mood state, as well as its pioneering approach for a hypothesis-driven study assessing the influence of the *CACNA1C* risk polymorphism on glutamatergic neurometabolites in a cortical region associated with emotional and cognitive control such as the ACC. However, the sample may not be considered especially robust for a genetic study, particularly the sub-analyses stratified by sex and medication type, whose conclusions should be cautiously interpreted and need further confirmation by larger studies. The BD group sample was larger than the HC group (including the female sub- sample), possibly constituting a source of bias for the genotype/group and genotype/sex exploratory analyses. Thus, the increased glutamatergic metabolites associated with the AA genotype, observed in the female BD group only, warrants further confirmation by larger studies.

In conclusion, our results support the hypothesis that voltage-gated calcium channels and increased glutamatergic pathways synergically contribute to neuronal excitotoxicity in BD, particularly among women. This study also opens new avenues for

pharmacogenetic studies involving the *CACNA1C* gene, since euthymic BD patients with the AA genotype using anticonvulsants appear to have higher estimated Gln and may potentially be better responders to Ca^{2+} channel blockers such as lamotrigine. However, further studies are required to better elucidate this shared mechanism of calcium homeostasis regulation in BD, investigating *CACNA1C* gene-sex-medication-neurometabolite in- teractions in larger samples.

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Declaration of Competing Interest

The authors have no conflict of interest to declare.

CRediT authorship contribution statement

Estêvão Scotti-Muzzi: Conceptualization, Formal analy- sis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Thais Chile:** Investiga- tion, Methodology. **Homero Vallada:** Investigation, Method- ology. **Maria Concepción Garcia Otaduy:** Formal analysis, Funding acquisition, Investigation, Methodology, Software. **Márcio Gerhardt Soeiro-de-Souza:** Conceptualization, For- mal analysis, Funding acquisition, Investigation, Methodol- ogy, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing.

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Supplementary materials

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Supplementary Material

Table S1 - Glutamatergic metabolite comparison between BD-I and HC

Glutamatergic metabolites	BD-I (121 subjects)	HC (73 subjects)
Glx/Cr (SD)	1.08 (0.15)[a]	1.03 (0.14)[b]
Glu/Cr (SD)	1.01 (0.11)[a]	0.98 (0.12)[b]
Estimated Gln/Cr (SD)	0.06 (0.07)[a]	0.04 (0.05)[b]

Legend: Means with different letters in the same line are significantly different according to univariate *generalized linear model* (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

Table S2- Influence of *CACNA1C* rs 1006737 on GM, WM and CSF in Bipolar Disorder and Healthy Controls.

Variables	**Healthy Control**			**Bipolar Disorder**		
Genotype	**AA N=5**	**AG n=29**	**GG n=39**	**AA n=9**	**AG n=43**	**GG n=69**
GM (SD)	0.60 [a] (0.05)	0.62 [a] (0.04)	0.62 [a] (0.05)	0.60 [a] (0.04)	0.60 [a] (0.05)	0.60 [a] (0.04)
WM (SD)	0.17 [a] (0.06)	0.17 [a] (0.03)	0.17 [a] (0.03)	0.19 [a] (0.03)	0.17 [a] (0.02)	0.17 [a] (0.03)
CSF (SD)	0.22 [a] (0.03)	0.19 [a] (0.04)	0.19 [a] (0.05)	0.20 [a] (0.06)	0.21 [a] (0.05)	0.21 [a] (0.05)

Legends: Means with different letters in the same line are significantly different according to univariate *generalized linear model* (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

Table S3- Influence of *CACNA1C* rs 1006737 (A allele) on glutamatergic metabolites in Healthy Controls and Bipolar Disorder.

	Healthy Control		**p**	**Bipolar Disorder**		**p**
Cacna1c (rs 1006737)	**With A (AG + AG) n=35**	**Without A (GG) n=38**		**With A (AA + AG) n=51**	**Without A (GG) n=68**	
GLX/Cr (SD)	1.02 (0.12)	1.03 (0.16)	0.8	1.11 (0.16)	1.07 (0.15)	0.40
Glu/Cr (SD)	0.98 (0.10)	0.99 (0.13)	0.8	1.02 (0.12)	1.00 (0.11)	0.49

Legends: Means with different letters in the same line are significantly different according to univariate *generalized linear model* (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

Table S4- Influence of *CACNA$_1$C* rs 1006737 on glutamatergic metabolites of BD-I anticonvulsant users (A) and lithium users (B).

A)

	Anticonvulsant users			Anticonvulsant non-users		
CACNA1C (rs 1006737)	**AA N=2**	**AG n=12**	**GG n=23**	**AA n=7**	**AG n=29**	**GG n=44**
Glx/Cr (SD)	1.16[a] (0.03)	1.01[a] (0.13)	1.07[a] (1.07)	1.16[a] (0.23)	1.10 [a] (0.13)	1.06[a] (0.13)
Glu/Cr (SD)	1.01[a] (0.05)	0.94[a] (0.10)	0.99[a] (0.13)	1.10[a] (0.20)	1.03[a] (0.10)	1.01[a] (0.10)
Estimated Gln/Cr (SD)	0.15[a] (0.08)	0.06[b] (0.05)	0.07[ab] (0.08)	0.07[a] (0.08)	0.07[a] (0.08)	0.05[a] (0.06)

B)

	Lithium users			Lithium non-users		
CACNA1C (rs 1006737)	**AA N=8**	**AG n=34**	**GG n=45**	**AA n=1**	**AG n=7**	**GG n=21**
Glx/Cr (SD)	1.16 [a] (0.22)	1.09 [a] (0.16)	1.06 [a] (0.13)	1.19 [a]	0.99 (0.15) [a]	1.08 (0.13) [a]
Glu/Cr (SD)	1.09 [a] (0.18)	1.02 [a] (0.10)	0.99 [a] (0.11)	0.97 [a]	0.96 (0.14) [a]	1.02 (0.10) [a]
Estimated Gln/Cr (SD)	0.07[a] (0.07)	0.07 [a] (0.07)	0.06 [a] (0.07)	0.21[a]	0.03 (0.05) [a]	0.05 (0.06) [a]

Legend: Means with different letters in the same line are significantly different according to univariate *generalized linear model* (GLM) at 5% confidence level ($p \leq 0.05$). The variables age, sex and fGM entered as co-variables in the GLM.

3.4. Capítulo IV – *BDNF* rs6265 differentially influences neurometabolites in the anterior cingulate of healthy and bipolar disorder subjects

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ORIGINAL RESEARCH

BDNF rs6265 differentially influences neurometabolites in the anterior cingulate of healthy and bipolar disorder subjects

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Abstract
Brain-derived neurotrophic factor (BDNF) is the most abundant brain neurotrophin and plays a critical role in neuronal growth, survival and plasticity, implicated in the pathophysiology of Bipolar Disorders (BD). The single-nucleotide polymorphism in the *BDNF* gene (*BDNF* rs6265) has been associated with decreased hippocampal BDNF secretion and volume in *met* carriers in different populations, although the *val* allele has been reported to be more frequent in BD patients. The anterior cingulate cortex (ACC) is a key center integrating cognitive and affective neuronal connections, where consistent alterations in brain metabolites such as Glx (Glutamate + Glutamine) and N-acetylaspartate (NAA) have been consistently reported in BD. However, little is known about the influence of *BDNF* rs6265 on neurochemical profile in the ACC of Healthy Controls (HC) and BD subjects. The aim of this study was to assess the influence of *BDNF* rs6265 on ACC neurometabolites (Glx, NAA and total creatine- Cr) in 124 euthymic BD type I patients and 76 HC, who were genotyped for *BDNF* rs6265 and underwent a 3-Tesla proton magnetic resonance imaging and spectroscopy scan (^{1} H-MRS) using a PRESS ACC single-voxel (8cm^{3}) sequence. *BDNF* rs6265 polymorphism showed a significant two-way interaction (diagnosis × genotype) in relation to NAA/Cr and total Cr. While *met* carriers presented increased NAA/Cr in HC, BD-I subjects with the *val* allele revealed higher total Cr, denoting an enhanced ACC metabolism likely associated with increased glutamatergic metabolites observed in BD-I *val* carriers. However, these results were replicated only in men. Therefore, our results support evidences that the *BDNF* rs6265 polymorphism exerts a complex pleiotropic effect on ACC metabolites influenced by the diagnosis and sex.

Keywords Creatine · Glx · ^{1}H-MRS · N-acetylaspartate · Pleiotropic effect · Val66Met polymorphism

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Introduction

Bipolar Disorder (BD) is a severe chronic illness associated with structural and functional dysfunctions in fronto-limbic circuitries (Strakowski et al., 2012; Maletic and Raison., 2014), particularly in the anterior cingulate cortex (ACC), a key center integrating cognitive and affective neuronal connections. In BD, there are consistent reports of ACC thinning (Hibar et al., 2017), alterations in white matter tracts (Vederine et al., 2011; Nortje et al., 2013), as well as increased levels of glutamatergic and choline metabolites (Scotti-Muzzi et al., 2021) within the ACC. Therefore, alterations in the morphology, connectivity and neurochemistry of the ACC appear to be key features for BD neurobiology.

Brain-derived neurotrophic factor (BDNF) is the most abundant brain neurotrophin and plays a critical role in neuronal health (Hofer et al., 1990), modulating synaptic transmission (Kowiaoski et al., 2017). While the pro-BDNF induces neuronal apoptosis, the mature BDNF plays a neurotrophic role by binding to tropomyosin-related kinase receptor B (TrkB), promoting a downstream signal cascade that leads to neuronal differentiation, survival and plasticity (Boulle et al., 2012; Mitchelmore & Gede, 2014). Consequently, BDNF has been extensively studied in both healthy controls (HC) and in psychiatric disorders such as BD, in relation to cognition (Kowiaoski et al., 2017; Mandolini et al., 2019) as well as brain morphology and function (Egan et al., 2003; Harrisberger et al., 2014, 2015; Kennedy et al., 2021). Although the neurotrophic properties of BDNF have been associated with brain metabolism (Markham et al., 2014) and alterations in neurometabolites such as Glx (Glutamate + Glutamine) and N-acetylaspartate (NAA) have been reported in BD (Croarkin et al., 2015; Ehrlich et al., 2015; Soeiro-de-Souza et al., 2018 a,b; Scotti-Muzzi et al., 2021), very few studies have addressed whether BDNF influences these neurometabolites in BD.

Proton magnetic resonance spectroscopy (1 H-MRS) is a non-invasive technique useful for assessing several metabolites involved in brain cellular and mitochondrial metabolism, such as N-acetylaspartate (NAA), Creatine (Cr) and Choline (Cho), Glutamate (Glu) and Glx (Glu + Gln) (Buonocore & Maddock, 2015). NAA is an amino acid synthesized exclusively in neuronal mitochondria whose decline has been commonly interpreted as mitochondrial dysfunction associated with reduced neuronal energy metabolism (Stork & Renshaw, 2005). Creatine (Cr) is commonly referred to as a combination of creatine and phosphocreatine which plays a critical role in energy metabolism, providing ATP for neuronal growth and survival via the Cr/PCr/CK system (Rackayova et al., 2017). Glx refers to the sum of glutamate (Glu) and glutamine (Gln), being a proxy of brain glutamatergic metabolites (Buonocore & Maddock, 2015).The mitochondrial dysfunction hypothesis for BD has long been proposed based on evidence of reduced expression of mitochondrial electron transport chain proteins, increased mtDNA deletion and mutations (Clay et al., 2011), increased intracellular Ca2+ (Harrison et al., 2021), and increased oxidative stress markers (Brown et al., 2014; Soeiro-de-Souza et al., 2013). Also, compelling evidence from ^{1}H-MRS data supports mitochondrial and cellular dysfunctions in BD (Stork & Renshaw, 2005), such as: decreased intracellular pH, elevated lactate (Clay et al., 2011), decreased creatine phosphate (Clay et al., 2011; Stork & Renshaw, 2005), decreased NAA levels (Croarkin et al., 2015; Ehrlich et al., 2015), as well as elevated Choline (Cho), Glx and Glu (Scotti-Muzzi et al., 2021; Soeiro-de-Souza et al., 2018 a,b), particularly in the ACC.

BDNF is encoded by the *BDNF* gene, located on chromosome 11p13 (Pruunsild et al., 2007), whose common single-nucleotide polymorphism (SNP) (*BDNF* rs6265 or Val66Met) causes a valine (*val*) to methionine (*met*) change at position 66 of the pro-BDNF protein. This polymorphism has been associated with decreased transport of BNDF mRNA to dendrites and reduced packaging and, in HC, with lower hippocampal BDNF secretion in *met* carriers (Egan et al., 2003; Chen et., 2004; Baj et al., 2013). BDNF is highly expressed in brain regions associated with cognitive and affective control, such as the hippocampus and the anterior cingulate cortex (ACC) (Kowiaoski et al., 2017; Strakowski et al., 2012; Camuso et al., 2022). Although the *met* allele of *BDNF* rs6265 has been associated with poorer cognitive performance (Kowiaoski et al., 2017; Mandolini et al., 2019), as well as with impaired brain morphology and function (Egan et al., 2003; Lang et al., 2009; Harrisberger et al., 2014; 2015; Kennedy et al., 2021), particularly in the hippocampus of healthy subjects and individuals with mood disorders, the *val* allele has been consistently associated with BD (Neves-Pereira et al., 2002; Sklar et al., 2002; Li et al., 2016; Paul et al., 2021).

To date, six studies have addressed the association of *BDNF* rs6265 and brain metabolites using ^{1}H-MRS, and only two have focused on psychiatric disorders. Whereas Frey et al., (2007) reported lower pre-frontal Cr levels in *met* carriers relative to *val* homozygotes in BD, Gruber et al., (2012) observed lower NAA/Cr levels in the hippocampus of *met* allele carriers in a mixed sample of schizophrenic, BD and healthy subjects. So far, there are no studies in psychiatric disorders involving the ACC neurometabolites and *BDNF* rs6265. Regarding studies involving healthy subjects, two such investigations have reported that *BDNF* rs6265 *met* carriers had increased NAA in the ACC (Gallinat et al., 2010; Martens et al., 2021), but decreased NAA levels in the hippocampus (Egan et al., 2003; Stern et al., 2008) compared to non-carriers, suggesting the *met* allele modulates NAA differentially in distinct brain regions. Also in controls, increased Cr in the dorsolateral pre-frontal cortex has been associated with the *met* allele (Frey et al., 2007), while another study reported decreased Cr levels in the hippocampus (Gallinat et al., 2010).

Our research team has recently demonstrated increased NAA levels in a group of euthymic BD patients relative to HCs, particularly in lithium-treated patients (Soeiro-de- Souza et al., 2018b). Considering the BDNF *met* allele has been

associated with increased NAA and Cr in healthy controls (Frey et al., 2007; Gallinat et al., 2010; Martens et al., 2021), the aim of this study was to investigate whether the *BDNF* rs6265 *met* allele distinctly modulates ACC metabolites associated with brain metabolism in euthymic BD and HC subjects. Also, we have recently demonstrated that BD subjects in this same sample carrying the AA genotype of the *CACNA1C (rs1006737*) gene had increased glutamatergic metabolites within the ACC (Scotti-Muzzi et al., 2022) and variations in *CACNA1C* have been reported to modulate serum BDNF in healthy and BD subjects (Smedler et al., 2021). Thus, this study sought also to investigate whether *BDNF* rs6265 influences the glutamatergic metabolies (Glx and Glu), as well as its influence on NAA levels in euthymic lithium-treated patients.

Materials and methods

Sampling

The present sample comprised 124 euthymic BD type I patients and 76 healthy control (HC) subjects (aged 18–45 years), enrolled from the study reported by Soeiro-de-Souza et al., (2018 a,b). These subjects were evaluated over a 4-year period by three research programs focused on BD at the University of São Paulo. Diagnoses were established by trained psychiatrists based on the Structured Clinical Interview (SCID-I/P) for DSM-IV TR (APA, 2000; First et al., 1996). The patients assessed had been on stable medica- tion regimens for at least 2 months prior to the ^{1}H-MRS scanning session. Individuals with neurological or medical disorders, head trauma, current/past (3 months) substance abuse (including illegal substances), as well as individu- als treated with electroconvulsive therapy in the past six months or reporting heavy episodic drinking over the past 3 months were not enrolled on the study. The Young Mania Rating Scale (YMRS) (Young et al., 1978) and the Ham- ilton Depression Rating Scale (HDRS) (Hamilton, 1967) were applied to assess residual sub-threshold depressive and manic symptoms. Patients were considered euthymic if they scored < 7 on both the YMRS and the HDRS, had no change in pharmacological prescription in the last three months and met the DSM criteria for euthymia. The euthymic BD patients were in use of different combinations of lithium, anticonvulsants and antipsychotics.

Healthy subjects also had no current or past history of psychiatric disorders according to the Mini International Neuropsychiatric Interview (MINI) (Sheehan, 1998). In addition, HCs had no family history, in first-degree rela- tives, of mood or psychotic disorders and had not been in use of any psychotropic medicines for at least three months before enrollment, as reported in a semi-structured interview. Subjects with a history of substance abuse within the 3 months leading up to enrollment were excluded from the study. The Research Ethics Committee of the University of São Paulo approved the study. Written informed consent was obtained from all study participants.

Image acquisition

Brain MRI exams were performed on a 3.0T magnetic reso- nance scanner (Intera Achieva, PHILIPS Healthcare, Best, the Netherlands) with an 8-channel head coil. Each brain exam included anatomical images acquired with a 3D-T1 Fast Field Echo (3D-T1 FFE) sequence; time of echo (TE)/ time of repetition (TR)/time of inversion (TI) = 3.2/7/900 ms; flip angle (FA) = 8°; FOV = 240 mm x 240 mm x 180 mm; matrix = 240 × 240), and magnetic resonance spectroscopy (MRS) acquisition. Single-voxel ^{1}H-MRS was performed using the PRESS sequence with number of scans (NS) of 160, TR of 1500 ms and TE of 80 ms. MRS was preceded by an automatic pre-acquisition that included adjustment of the transmitter-receiver, optimization of the tilt angle for water suppression and homogenization of the field for the selected volume of interest (VOI). Voxel size was set at 2 × 2 × 2 cm^3 for all patients and controls. The voxel was positioned in the ACC using anatomical guidelines as a reference and placed on midsagittal T1-weighted images, anterior to the genu of the corpus callosum, with the ventral edge aligned with the dorsal corner of the genu, and centered on the midline of axial images as shown in Fig. 1. An unsuppressed water spectrum of the same voxel was also acquired for eddy cur- rent correction and reference purposes.

^{1}H-MRS quantification

Metabolites were quantified using LCModel (Provencher, 2003) and a basis set was simulated for TE = 80ms includ- ing: Alanine, Aspartate, Creatine, Phosphocreatine, GABA, Glucose, Glutamine, Glutamate, Glycerophosphocholine, Phosphocholine, myo-Inositol, Lactate, N-acetylaspar- tate, N-Acetylaspartylglutamate, Scyllo-inositol, Taurine, Guanidinoacetate, macromolecules and lipid signals. To control spectral quality between the groups, frequency width at half maximum (FWHM) and signal-to-noise ratio (SNR) were also recorded for each spectrum. In order to ensure the

accuracy of the measurements obtained, only metabolite results with Cramer-Rao Lower Bound (CRLB) values < 20% were considered, in accordance with technical references (Kreis, 2004). Metabolite ratios were calculated relative to Cr concentration. NAA/Cr, Cho/Cr, Glx/Cr, Glu/ Cr and Cr were the metabolites of interest for the statistical analysis. Since the normal metabolic concentration varies considerably between gray matter (GM) and white matter (WM) (Gasparovic et al., 2006), the fraction of GM in the voxel needed to be taken into account in the analysis. To this end, brain tissue in the three-dimensional T1-weighted brain images was extracted using the brain extraction tool (BET), and then segmented into GM, WM and cerebrospinal fluid (CSF) using the FAST algorithm, both available from the open source FSL software (http://www.fmrib.ox.ac.uk/ fsl). Finally, the MRS voxel mask was superimposed onto the segmented images using a Python script developed in-house. The GM brain tissue fraction (f_{GM}) was calculated for each voxel [f_{GM}= %GM/ (%GM +%WM)], and f_{GM} was used as a covariate when comparing metabolites between groups.

Tissue segmentation into CSF, WM and GM was also used to estimate metabolite concentrations in mM by using water as a reference, as described by Gasparovic et al., (2006). Metabolite concentrations were estimated by tak- ing into account the different water content in WM, GM and CSF (36.11 M, 43.33 M and 53.89 M, respectively) as well as the different relaxation properties of water in CSF (T1 = 4.16 s, T2 = 0.5 s), GM (T1 = 1.82 s, T2 = 0.10 s) and WM (T1 = 1.08 s, T2 = 0.07 s) (Gasparovic et al., 2006).
For T1, metabolites of NAA, Glu and Glx were assumed as 1.47s, 1.27s and 1.20s in GM, and as 1.35s, 1.17s and 0.96s in WM, respectively (Stanisz et al., 2005). For T2, NAA values of 0.247s in GM and 0.295s in WM were used, respectively. For Glu and Glx (T2), a value of 0.2s was assumed (Mlynárik et al., 2001).

DNA extraction and genotyping

DNA was obtained from peripheral blood on the day of MRI exams, according to the salting-out protocol (Laitinen et al., 1994) and then genotyped for *BDNF* rs6265 using real-time PCR allelic discrimination. PCR amplification for rs1006737 was performed in 5 µl reactions with 5 ng of template DNA, 1× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA), 1× each primer and probe assay, and H_2O. Thermal cycling consisted of initial denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Fluorescence detection was performed in the anneal- ing step. Amplification and allelic discrimination were per- formed on a 7500 Real-Time System (Applied Biosystems, Foster City, CA). Quality control of Real-time PCR results was done by direct sequencing on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The genotype distribution was in Hardy-Weinberg equilib- rium (x2 = 0.00, p = 0.2).

Statistical analysis

The sample was first tested for homogeneity. Normality was checked using the Shapiro-Wilk test. Normally distributed variables were studied using x^2 tests for categorical variables and *t*-tests for continuous variables. Non-normally distributed variables (e.g. Cr) were compared using the univariate generalized linear model (GLM). We fitted a two (diagnosis × genotype) and three-way interaction analysis (diagnosis × genotype × sex) using a generalized linear model (GLM), considering a gaussian distribution and iden- tity link function via a maximum-likelihood estimation. Age and fGM entered as continuous variables to reduce residual confounding and the analyses were performed in Stata 16 (StataCorp, College Station, TX, USA). Each group was also analyzed using a separate model to investigate the asso- ciation between *BDNF* rs6265 and neurometabolites. The regression coefficients (coef) are expressed together with their 95% confidence intervals (95% CI). Partial eta squared (ηp^2) is provided as a measure of effect size.

Results

Sociodemographic and genetic distribution data

Clinical and sociodemographic data are shown in Table 1. Significant between- group differences were observed for age, sex, gray matter (GM) and cerebral spinal fluid (CSF). Whilst BD patients had higher mean age and CSF values than HCs, the control group had larger GM volume than the BD group. Also, a predominance of females over males was noted in the BD group. There were no between-group differences for the other parameters.

The *BDNF* allele frequency was in the Hardy-Weinberg equilibrium. Within the BD-I group, 36.3% had the *met* allele and 63.7% were non-carriers, whereas in the HC group, 38.2% carried the *met* allele versus 61.8% non-carri- ers. There was no difference between HC and BD-I in rela- tion to allele frequency (Table 1).

Regarding the medication regimen (Table 1), 74.6% of the BD-I sample was using lithium (32.2% on lithium monotherapy), 29.8% anticonvulsants, and 37.1% were on second-generation antipsychotics.

Influence of *BDNF* rs6265 on ACC metabolites

The 2-way interaction model (Fig. 2 A) revealed a significant interaction between genotype with NAA/Cr in the overall sample since subjects with the *met* allele (n = 74) presented significantly higher NAA/Cr than those without this allele (n = 126). When assessing the differences between genotypes in each group separately (BD-I and HC), we found that HC subjects carrying the *met* allele (n = 29) presented increased ACC NAA/Cr relative to non-carriers (n = 47) (B: 0.05; 95% CI: 0.01 to 0.09; p = 0.01; $\eta p^2 = 0.07$), a finding not observed within the BD-I group (Fig. 2 B). However, there were no differences between groups (BD-I vs. HC) in relation to NAA/Cr in the overall sample and neither when stratified by each genotype (Fig. 2 A).

Fig. 1 ACC ^{1}H-MRS voxel placement

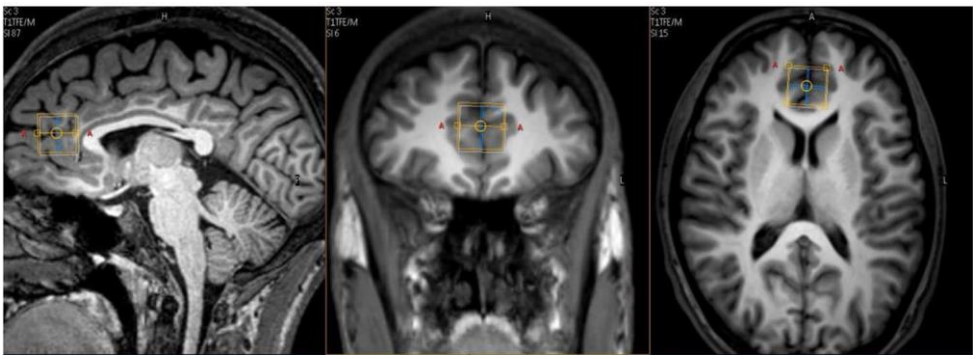

Table 1 Demographic and clini- cal data for BD-I and HC subjects

Variables	BD-I (124 subjects)	HC (76 subjects)	p value
Age (sd)	31.88 (0.84)	28.58 (0.97)	**0.014**
Sex (M/F)	40/84	39/37	**0.015***
Education years (sd)	13.14 (0.28)	13.30 (0.36)	0.74
Illness duration (sd)	6.96 (6.0)		-
Grey Matter (sd)	0.60 (0.004)	0.62 (0.005)	**0.006**
White matter (sd)	0.176 (0.002)	0.175 (0.004)	0.80
Cerebral spinal fluid (sd)	0.21 (0.05)	0.20 (0.05)	**0.025**
BDNF genotype (with met/ without met)	(45/79)	(29/47)	0.79**
Medication regimen:			
-Lithium monotherapy	40		
-Lithum + Valproate	21		
-Lithium + Atypical Antipsychotics	30		

*t test, ** x2 test, significância p < 0.05; + Equilíbrio de Hardy-Weinberg: x2 = 0.65, p = 0.2

The 2-way interaction (diagnosis × *BDNF* rs6265 genotype) analysis also revealed a significant interac tion between genotype and total Cr in the overall sample (Fig. 3 A). Subjects with the *met* allele (n = 74) presented significantly lower total Cr than those without this allele (n = 126) (Fig. 3 A). Such a finding was replicated only within the BD-I group (Fig. 3 B), where the presence of the *met* allele was associated with lower levels of total creatine in relation to non-carriers in BD-I group (B: -0.39; 95% CI: -0.74 to -0.04; p = 0.03; $\eta p^2 = 0.02$). However, there was no difference between groups (BD-I vs. and HC) in relation to total Cr regardless the genotype (Fig. 3 A).

On the other hand, we found a significant group effect (BD-I vs. HC) only for Glx/Cr since increased levels of this metabolite was found in BD-I group (n = 124) relative to HC (n = 76), a finding replicated only in those male subjects with the *val* allele (Fig. 4 A). Also, marginally non-significantly increased Glu/Cr in BD-I in relation to HC was observed, particularly among *val* carriers (**Figure** S1). However, we did not detect a significant interaction between the *BDNF* rs6265 genotype and Glx/Cr in the overall sample (Fig. 4 A).

Influence of sex

The three-way interaction model (diagnosis × *BDNF* rs6265 genotype × sex) revealed that male subjects in the overall sample showed marginally non-significant higher levels of both NAA/Cr (**Figure** S2) and total Cr (**Figure** S3) in relation to females. Within each group, we observed that healthy male control carriers of the *met* allele had higher NAA/Cr than non-carriers (B: 0.07; 95% CI: 0.003 to 0.14; $p = 0.04$; $\eta p^2 = 0.11$; Table 2 A), and male BD patients carrying the BDNF *met* allele had lower levels of total Cr than male non-carriers (B: -0.53; 95% CI: − 1.0 to -0.06; $p = 0.04$; $\eta p^2 = 0.12$; Table 2 B). In relation to Glx/Cr, the group effect (BD-I vs. HC) observed among *val* carriers (Fig. 4 A) was replicated only in men (Fig. 4 B).

Influence of medication

Table S1 **A and B** reveal no mutual influence of the *BDNF* rs6265 polymorphism and lithium on NAA levels in the BD group, but show slightly lower total Cr in *met* carriers than non-carriers in patients on lithium monotherapy (**Table** S1**B**).

Discussion

This is the first study assessing the influence of *BDNF* rs6265 on ACC neurometabolites in 200 subjects (124 BD-I and 76 HC), representing the largest investigation of its kind. We found that *BDNF* rs6265 polymorphism differentially modulates metabolites in BD and HC groups, since it influenced NAA/Cr in HC and total Cr in BD patients. While healthy *met* allele carriers of the *BDNF* rs6265 polymorphism showed increased NAA/Cr within the ACC, BD-I *val*/*val* homozygotes had higher total Cr levels relative to *met* carriers. Although no differences between BD-I and HC were detected for NAA/Cr and total Cr, BD-I subjects showed increased Glx/Cr in relation to HC, a finding replicated only among *val* carriers. However, sex appeared to modulate the influence of *BDNF* rs6265 genotype on ACC metabolites since these results were replicated only within the male sub-group.

The increased NAA/Cr levels observed among *met* carriers in the HC group is consistent with the few previous ^{1}H-MRS studies assessing the effect of *BDNF* rs6265 polymorphism on brain neurometabolites. While Gallinat et al., (2010) observed significantly increased ACC levels of NAA in *met* carriers compared to non-carriers among 82 healthy volunteers, Martens et al., (2021) reported increased NAA within the ACC of *met* carriers relative to *val*/*val* homozygotes in two different healthy cohorts comprising 30 and 98 subjects, respectively. These authors have postulated that these higher NAA/Cr levels in the ACC of healthy individuals carrying the *met* allele might protect them against psychiatric disorders because decreased brain NAA has been interpreted as an indicator of neuronal dysfunction (Stork & Renshaw, 2005). However, such a putative protective role of the *met* allele against BD-I regarding NAA was not entirely confirmed by our data since we failed to find an interaction between group and NAA/Cr.

On the other hand, we observed that total Cr was significantly increased in *val*/*val* homozygotes in relation to *met* carriers in the overall sample, a finding that was replicated only within the BD patients but not in HCs. This result is in agreement with those reported by Frey et al., (2007) who found higher prefrontal Cr levels in BD with val/val than *met* carriers. Similarly, Gallinat et al., (2010) observed higher hippocampal Cr among healthy subjects without the *met* allele. Total Cr denotes creatine content together with its phosphorylation product, phosphocreatine, which are in near-equilibrium exchange, being interconverted by the enzyme creatine kinase in both mitochondria and cytosol. The creatine/phosphocreatine system plays a key role in brain energetics since it buffers and transports high-energy phosphate bonds across the brain (Maddock and Buonocore, 2015). Thus, the increased total Cr observed in those BD-I with the *val* allele may suggest an enhanced ACC metabolic activity. The literature reports that BDNF plays a central role in energy balance by controlling the mitochondrial function, thermogenesis, tissue differentiation and modulating glucose metabolism (Markham et al., 2014; Di Rosa et al., 2021). In addition, aside from its role as the main excitatory neurotransmitter in central nervous system (CNS), glutamate plays a key role in brain energetic metabolism since it can itself be used as an energy substrate in the tricarboxylic acid (TCA) cycle, resulting in ATP generation (Yu et al., 1982; Walls et al., 2015; Karaca et al., 2015). Therefore, the increased energetic metabolism observed among BD-I *val* carriers, which is the allele more consistently associated with BD (Neves-Pereira et al., 2002; Sklar et al., 2002; Li et al., 2016; Paul et al., 2021), may be attributed to the higher glutamatergic mebolites observed in BD-I *val* carriers. Indeed, increased ACC glutamatergic metabolites have been considered a hallmark in BD in different mood states (Soeiro-de-Souza et al., 2018a; Scotti-Muzzi et al., 2021). In addition, we have recently demonstrated that BD subjects in this same sample carrying the AA genotype of the *CACNA1C (rs1006737*) gene had increased glutamatergic metabolites within the ACC (Scotti-Muzzi et al., 2022), supporting the hypothesis that BD-I subjects with both the *CACNA1C (rs1006737*) AA and *BDNF* rs6265 *val* genotypes might show increased excitotoxicity within the ACC and worse outcome.

The apparent distinct effect of the *met* allele in BD and HC in relation to ACC metabolites corroborates its known pleiotropic effect (Tsai, 2018), which depends on several factors such as brain region, cortical maturation stage (Camuso et al., 2022), ethnicity, age and sex (Hosang et al., 2014; Tsai, 2018). Aside from the diagnosis status (BD-I vs. HC), sex appeared to modulate the influence of *BDNF* rs6265 polymorphism on NAA/Cr and total Cr. The three-way (diagnosis × *BDNF* rs6265 genotype × sex) interac-

tion model showed a marginally non-significant main effect for sex on NAA/Cr and total Cr in the overall sample and

the influence of *BDNF* rs6265 genotype on both NAA/Cr and total Cr was replicated only within the male subgroup. Similarly, Martens et al., (2021) reported higher NAA con- tent in the ACC of *met* carriers in their healthy male sample. In addition, the increased Glx/Cr in BD-I group relative to HC observed among *val* carriers was observed only in men. The literature corroborates such modulation of the *BDNF* rs6265 polymorphism by sex. For instance, Wei & Berman (2019) showed that healthy women carrying the *BDNF met* variant exhibited atypical activation of the hippocampus in the presence of estradiol and, in schizophrenia, female *met* carriers had an earlier age of psychosis onset (Decoster et al., 2011). Also, the *met* allele has been reported to confer susceptibility to Alzheimer's disease in women, but not in men (Fukumoto et al., 2010), and predict worse cognitive function during normal cognitive aging (Laing et al., 2012). The pleiotropic effect of *BDNF* polymorphism appears also to be modulated by the brain region (Camuso et al., 2022) since apparent opposite effects of *met* allele have been reported in the hippocampus, where it has been associated with decreased neuronal BDNF secretion (Baj et al., 2013; Chen et al., 2004; Egan et al., 2003), lower hippocampal NAA (Egan et al., 2003; Stern et al., 2008; Gruber et al., 2012), as well as smaller hippocampal volume (Har- risberger et al., 2014; Pereira et al., 2017) in both HC subjects and individuals with psychiatric disorders (e.g. BD). On the other hand, several studies have consistently associated the *val* allele with BD (Neves-Pereira et al., 2002; Sklar et al., 2002; Paul et al., 2021), a finding confirmed by a meta-analysis showing the *val* allele was significantly associated with BD in Europeans, but not in Asians (Li et al., 2016). Thus, the pleiotropic effect of *BDNF* in different brain regions (e.g. ACC and hippocampus) may likely contribute for the fronto-subcortical disintegration (Strakowski et al., 2012; Maletic and Raison., 2014), ultimately conferring susceptibility or protection for BD.

Although lithium did not greatly influence the effect of *BDNF* rs6265 on NAA in the BD group, *met* carriers showed slightly (non-significant) decreased levels of total Cr only in BD subjects on lithium monotherapy, but not in those treated with lithium plus valproate. Given the previous study by our group in this same euthymic BD sample demonstrated that increased NAA levels in the ACC were influenced by lithium treatment (Soeiro-de-Souza et al., 2018b), the higher metabolic status observed in *val* carriers might also be influenced by lithium treatment, or this group might be better responders to lithium. In fact, lithium treatment has been shown to increase BDNF expression in cortical and hippocampal neurons (De-Paula et al., 2016) and the *BDNF* rs6265 polymorphism has been associated with response to lithium (Pagani et al., 2019; Paul et al., 2021).

The present study has some strengths and limitations. The study strengths include its large sample (200 subjects) for a ^{1}H-MRS study involving a homogeneous BD type I sample in the same mood state, assessing a cortical region associated with emotional and cognitive control, such as the ACC. To the best of our knowledge, this is the third (but largest) study evaluating the influence of the

BDNF rs6265 on cortical neurometabolites in BD, and the first on the ACC. However, the sample may not be considered especially robust for a genetic study, particularly in relation to the interaction analyses since, due to the multiple testing, *p* values ranging from 0.05 to 0.01 may be possibly attributed to type I error (false-positive). Therefore, our conclusions should be interpreted cautiously, requiring further confirmation by larger studies. Also, since differences were found in NAA/Cr but not in absolute levels of NAA between genotypes, the influence of Cr levels on the result cannot be ruled out. However, Cr levels do not appear to have impacted any of the other metabolites assessed (e.g. Glu/Cr, Glx/Cr), a finding which reduces the likelihood that the increased NAA/Cr are totally confounded by differences in creatine-normalized dat a (Buonocore & Maddock, 2015).

Conclusion

This study supports evidences that the *BDNF* rs6265 polymorphism exerts a pleiotropic effect influenced by the diagnosis and sex. While in HC, the *met* allele was associated with increased NAA/Cr and possibly better cortical fitness, in stable BD-I subjects, the *val* allele was associated with increased total Cr, denoting an enhanced ACC metabolism likely associated with increased glutamatergic metabolites observed in BD-I *val* carriers. These findings appeared to be influenced by sex since they were replicated only in men. Further studies should investigate the influence of *BDNF* rs6265 on ACC metabolites in healthy individuals at risk for BD (e.g. first degree relatives) in order to confirm the putative pleiotropic effect role of *BDNF* rs6265 polymorphism mediating changes in ACC metabolites.

Figure 2- A: Forest plot ahowing the two-way interaction (diagnosis × *BDNF* rs6265 genotype) via a generalized linear model (GLM) in relation to NAA/Cr; and **B**: Comparison of NAA/Cr between *BDNF* rs6265 *met* allele carriers and non-carriers in BD-I and HC groups. In the Forest plot (**A**), circles denote interaction effects and diamonds the main effects. The widths of the horizontal lines and diamonds denote the 95% CI. In the box plot (**B**), medians with different letters on same line differ significantly according to GLM at 5% confidence level ≤ 0.05)

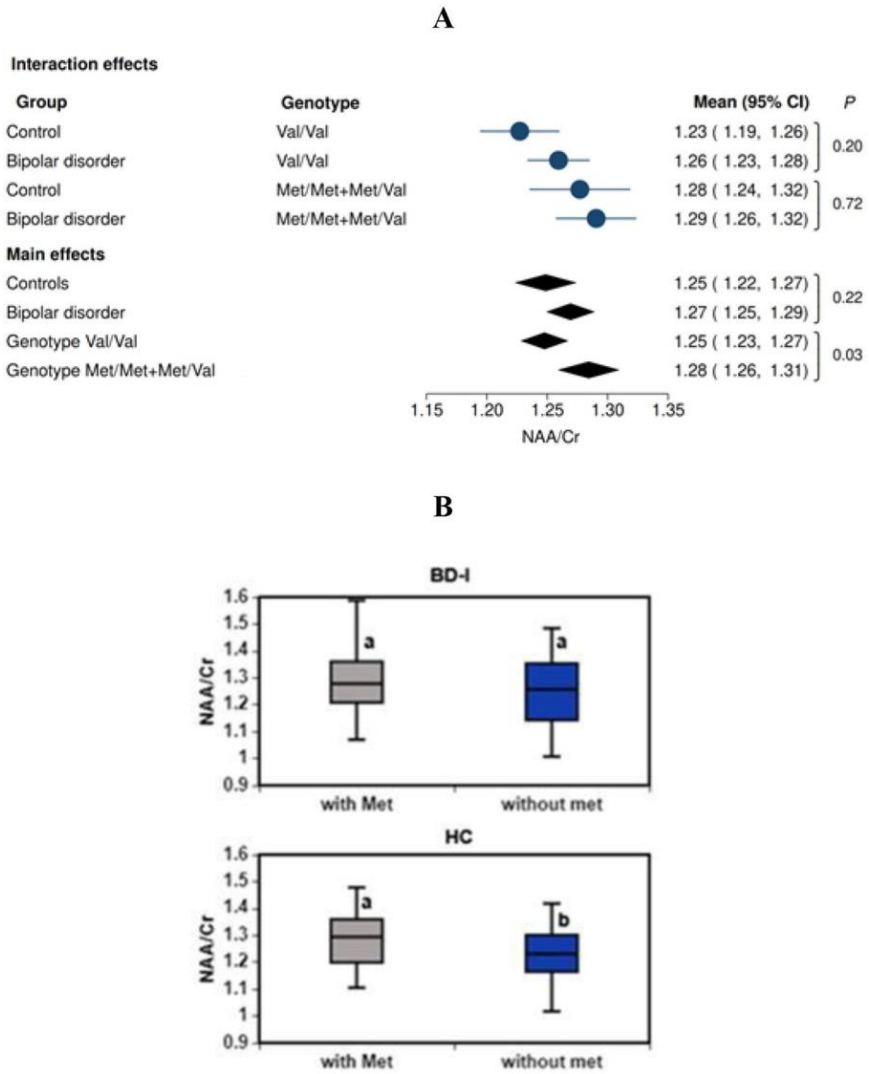

Figure 3- A: Forest plot showing the two-way interaction (diagnosis × *BDNF* rs6265 genotype) via a generalized linear model (GLM) in relation to the total Cr; and **B**: Comparison of total Cr between *BDNF* rs6265 *met* allele carriers and non-carriers in BD-I and HC groups. In the Forest plot (A), circles denote interaction effects and diamonds the main effects. The widths of the horizontal lines and diamonds denote the 95% CI. In the box plot (B), medians with different letters on same line differ significantly according to (GLM) at 5% confidence level ($p \leq 0.05$)

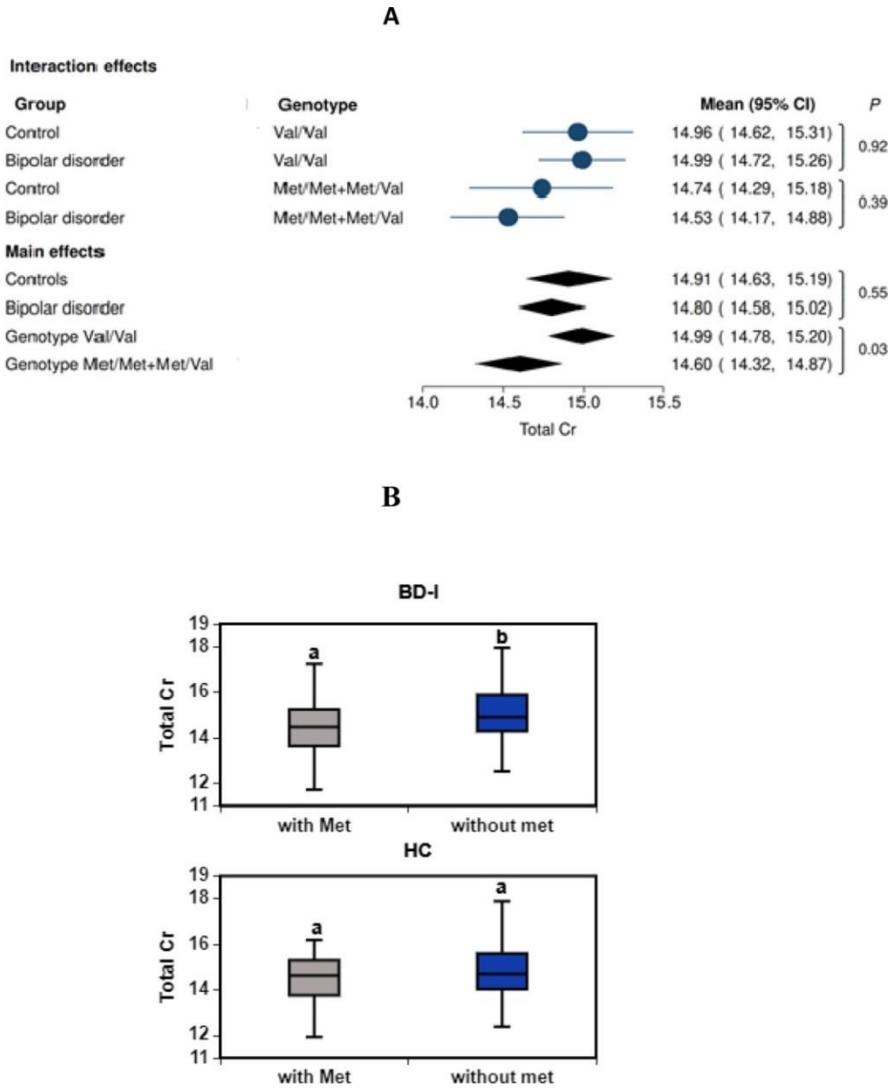

Figure 4- A: Forest plot showing the two-way interaction (diagnosis × genotype) and, **B**: three-way interaction (diagnosis × genotype × sex) via a generalized linear model (GLM) in relation to Glx/Cr. The widths of the horizontal lines and diamonds denote the 95% CI

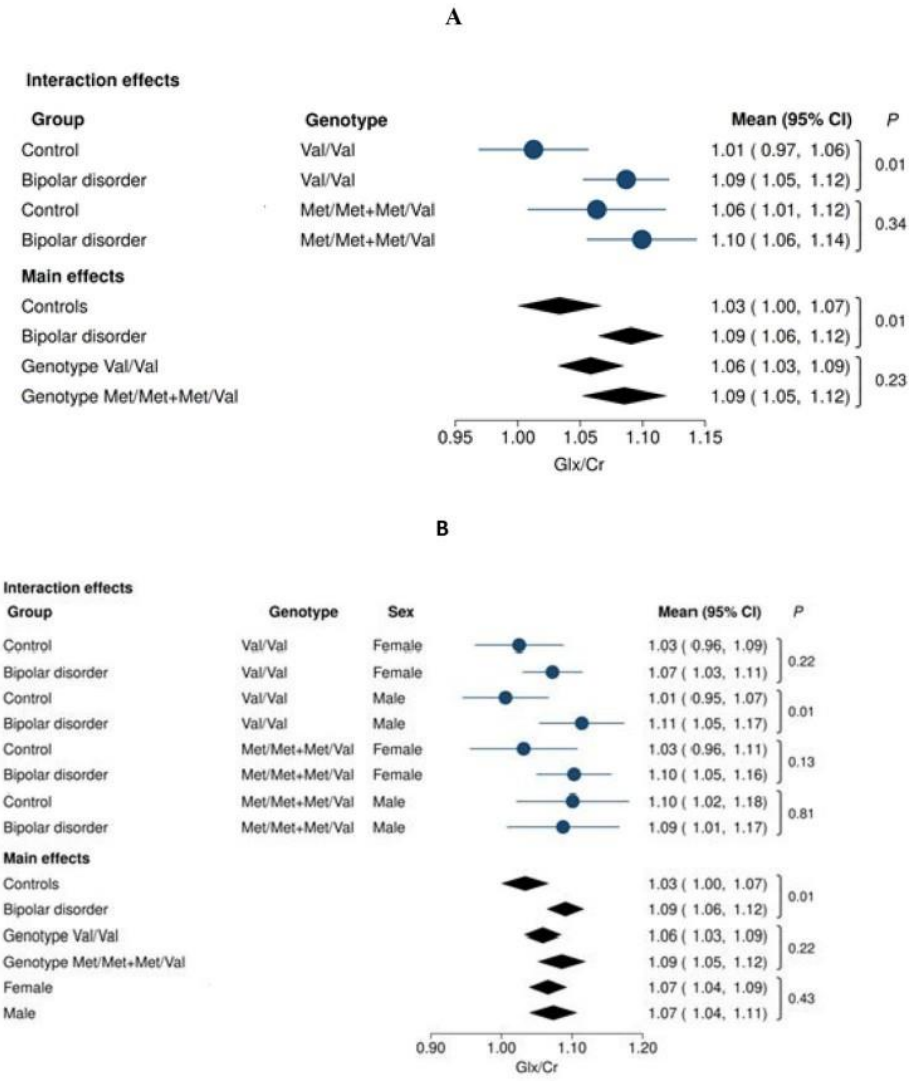

Table 2 Comparison of NAA/Cr (A) and total Cr between *BDNF* rs6265 *met* allele carriers and non-carriers in male and female groups within HCs. Medians with different letters on same line differ significantly according to univariate *generalized linear model* (GLM) at 5% confidence level ($p \leq 0.05$), using the Sidak correction for multiple comparisons

	A					
	Healthy Control					
Sex	**Male**			**Female**		
BDNF	**With met**	**Without met**	**p-value**	**With met**	**Without met**	**p-value**
(rs 6265)	**(AG +AA), n=14**	**(GG); n=25**		**(AG +AA); n= 15**	**(GG); n=22**	
NAA/Cr (SD)	1.32 (0.09)	1.25 (0.09)	**0.04**	1.25 (0.10)	1.21 (0.06)	0.24
Total Cr (SD)	14.45 (1.47)	14.98 (1.62)	0.32	14.67 (1.09)	14.84 (1.16)	0.83
	B					
	Bipolar Disorder					
Sex	**Male**			**Female**		
BDNF	**With met**	**Without met**	**p-value**	**With met**	**Without met**	**p-value**
(rs 6265)	**(AG +AA), n=15**	**(GG); n=25**		**(AG +AA); n= 32**	**(GG); n=52**	
NAA/Cr (SD)	1.31 (0.12)	1.27 (0.14)	0.52	1.27 (0.13)	1.24 (0.11)	0.23
Total Cr (SD)	14.54 (0.99)	15.28 (1.29)	**0.04**	14.55 (1.37)	14.95 (1.21)	0.23

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Author contributions Conception and study design (ES-M, MCGO, MGS-S), data collection or acquisition (E-SM, TC, HV, MCGO, MGS-S), statistical analysis (ES-M and MGS-S), interpretation of re- sults (ES-M and MGS-S), drafting the manuscript or revising it criti- cally for important intellectual content (ES-M, MCGO, MGS-S), and approval of final version to be published and agreement to be account- able for the integrity and accuracy of all aspects of the study (E-SM, TC, HV, MCGO, MGS-S).

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Data availability The data supporting the findings of this study are available from the corresponding author on reasonable request.

Declarations

Ethical approval The Research Ethics Committee of the University of São Paulo approved the study.

Consent to participate Written informed consent was obtained from all study participants.

Consent to publish All those listed as authors are qualified for author- ship and all who are qualified to be authors are listed as authors on the byline.

Competing interests None of the authors report biomedical financial interests or potential conflicts of interest.

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Supplementary Figures

Figure S1-Forest plot showing the two-way interaction (diagnosis × *BDNF* rs6265 genotype) via a generalized linear model (GLM) in relation to GLX/Cr. Circles denote interaction effects and diamonds the main effects. The widths of the horizontal lines and *diamonds denote* the 95% CI.

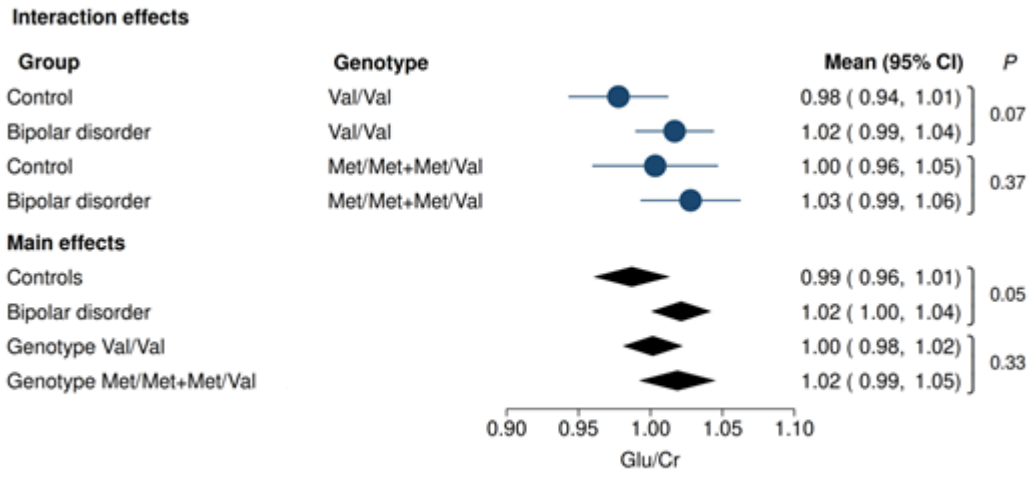


Figure S2- Forest plot showing the three-way interaction (diagnosis × genotype ×sex) via a generalized linear model (GLM) in relation to NAA/Cr. Circles denote interaction effects and diamonds the main effects. The widths of the horizontal lines and *diamonds denote* the 95% CI.

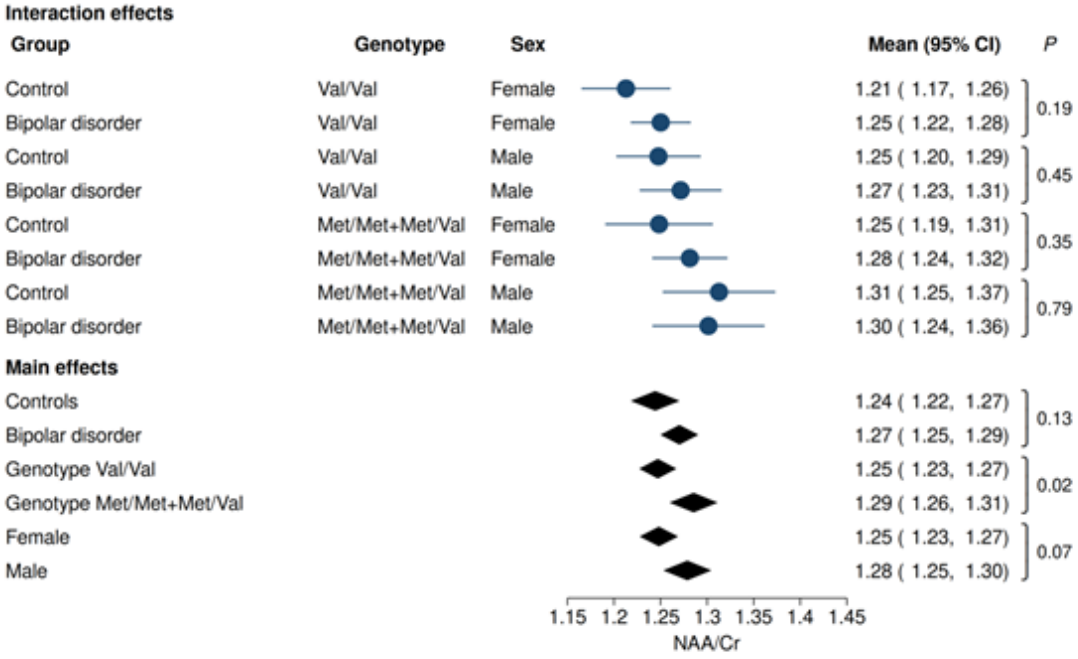
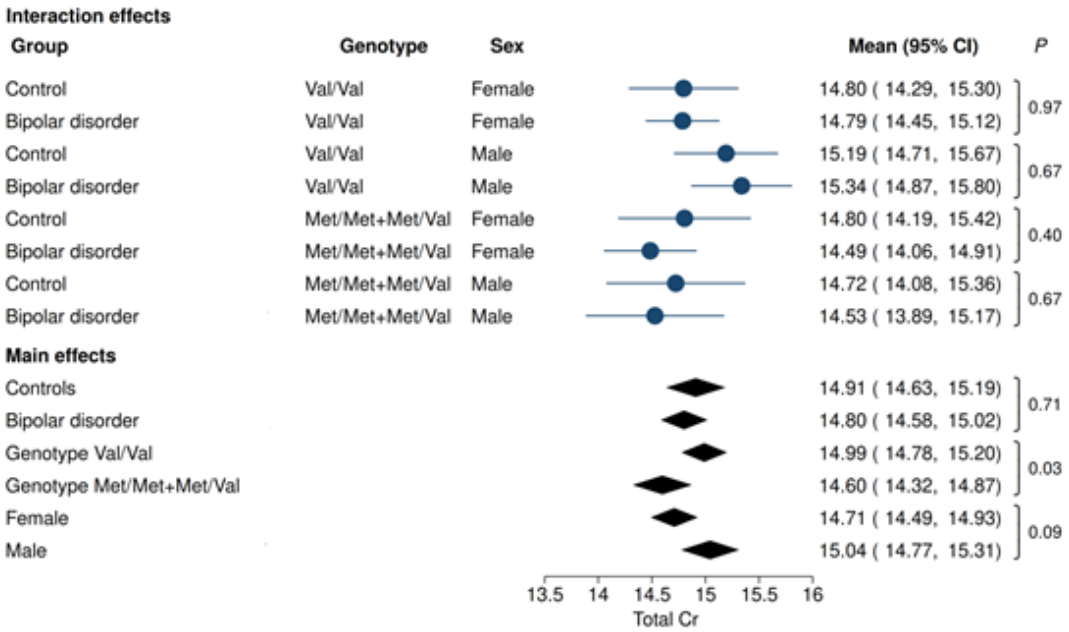


Figure S3- Forest plot showing the three-way interaction (diagnosis × genotype × sex) via a generalized linear model (GLM) in relation to total Cr. Circles denote interaction effects and diamonds the main effects. The widths of the horizontal lines and *diamonds denote* the 95% CI.



Supplementary Tables

Table S1- Mutual influence of *BDNF* (rs 6265) and mood stabilizing medication on NAA/Cr and total Cr in BD-I subjects. Statistical analysis was performed using the univariate *generalized linear model* (GLM) at 5% confidence level ($p \leq 0.05$) and multiple comparisons were adjusted by the Sidack correction.

A

	With met			**Without met**		
***BDNF* (rs 6265)**	**Li users (n=38)**	**Li non-users (n=9)**	**p-value**	**Li users (n=56)**	**Li- non users (n=21)**	**p-value**
NAA/Cr (SD)	1.29 (0.13)	1.25 (0.08)	0.10	1.24 (0.12)	1.25 (0.13)	0.97
Total Cr (SD)	14.4 (0.19)	14.9 (0.40)	0.32	15.07 (0.17)	15.09 (0.27)	0.94

B

	Lithium monotherapy			**Lithium + Valproate**		
***BDNF* (rs 6265)**	**w/met (n=15)**	**Without met (25)**	**p-value**	**With met (n=7)**	**Without met (n=14)**	**p-value**
NAA/Cr (SD)	1.32 (0.03)	1.27 (0.02)	0.27	1.27 (0.05)	1.24 (0.03)	0.74
Total Cr (SD)	14.49 (1.05)	15.17 (1.32)	**0.05**	14.41 (0.44)	14.49 (0.30)	0.89

4. DISCUSSÃO GERAL

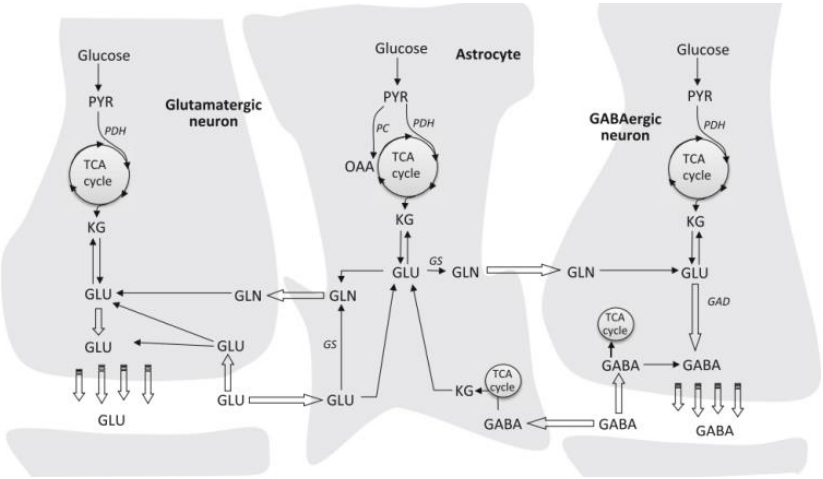
4.1. Polimorfismos genéticos funcionais e o sistema glutamatérgico

No trabalho de meta-análise (**Capítulo I**) sobre as alterações de neurometabólitos no CCA de pacientes com TAB, encontramos um aumento de metabólitos glutamatérgicos (Glx: glutamato + glutamina) no TAB em relação a controles saudáveis (Scotti-Muzzi et al., 2021a). Este é um dos achados neurobiológicos mais consistentes no TAB já que corrobora estudos de meta-análises prévios focados em região frontal (Gigante et al., 2012; Chitty et al., 2013) bem como meta-análises posteriores mostrando aumento de Glx (Ino et al., 2022) e Gln (Chabert et al., 2022) no cíngulo anteior de pacientes bipolares em relação à contoles. Além disso, nossos dados de meta-análise demonstraram que, no estado de eutimia, o aumento de Glx foi associado à glutamina e não ao glutamato (Scotti-Muzzi et al., 2021a), dado este confirmado pelo trabalho de Chabert et al. (2022).

Entretanto, devido à heterogeneidade do TAB e o escasso número de estudos realizados em diferentes estados de humor, ainda não é possível concluir quais dos componentes do Glx (glutamato, glutamina ou ambos) encontra(m)-se elevado(s) no TAB e se há uma variação de Glx em diferentes fases de humor. Portanto, as alterações no sistema glutamatérgico em cíngulo anterior no TAB encontram-se ainda longe de um perfeito entendimento, sobretudo nos estados de depressão, (hipo) mania e mistos.

De fato, ainda não compreendemos os mecanismos subjacentes à elevação de neurometabólitos glutamatérgicos no CCA no TAB. O glutamato é um aminoácido sintetizado nos neurônios glutamatérgicos a partir do alfa-cetoglutarato, um intermediário do ciclo dos ácidos tricarboxílicos, e exerce seu papel de neurotransmissor excitatório ao ligar-se a receptores glutamatérgicos pós-sinápticos. O glutamato sináptico é então re-captado por neurônios e, sobretudo, por astrócitos via transportadores de membranas específicos, evitando-se, assim, seu acúmulo e potencial excito-toxicidade (Walls et al., 2015), como mostrado na **Figura 1**:

Figura 1 – Esquema mostrando o metabolismo do glutamate, glutamina e GABA entre neurônios glutamatérgicos, GABAergicos e astrócitos.



Fonte: Walls et al. (2015).

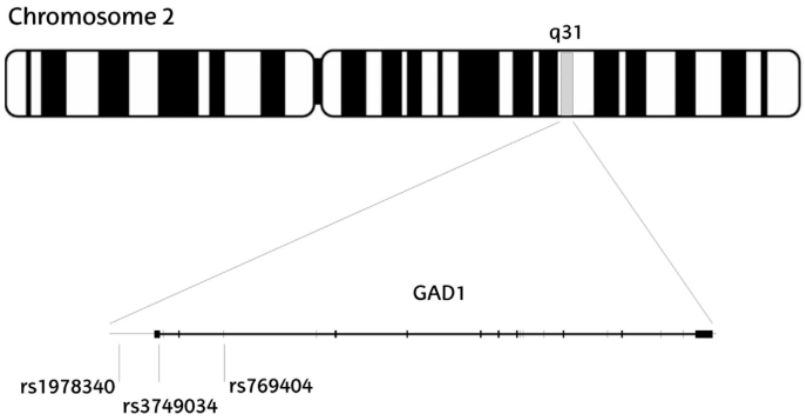
O glutamato sintetizado segue duas rotas principais entre neurônios glutamatérgicos, GABAérgicos e astrócitos. A primeira consiste na sua entrada nos astrócitos, onde é convertido em glutamina pela enzima glutamina sintetase, a qual é depois transportada de volta para os neurônios glutamatérgicos e re-convertida em glutamato (**Figura 1**; Walls et al. 2015). A segunda via é caracterizada pela entrada da glutamina nos neurônios GABAergicos, onde é convertida a glutamato (**Figura 1**) e, somado aquele oriundo do ciclo do ácido tricarboxílico (TCA), serve de substrato para a síntese de GABA catalizada pela enzima glutamato descarboxilase (GAD) (Tian et al., 1999). A enzima GAD apresenta duas isoformas, a GAD65 localizada em terminais sinápticos e a GAD 67, encontrada no corpo celular do neurônio, a qual é responsável pela síntese da maior parte do GABA (Martin; Rimvall, 1993; Tian et al., 1999).

No estudo reportado no **Capítulo II** (Scotti-Muzzi et al., 2021b), explorou-se a hipótese de que o aumento de glutamato no CCA de pacientes com TAB-I estaria associado aos polimorfismos (SNPs) no gene *GAD*1 (rs3749034, rs1978340, rs769390, rs11542313). Avaliou-se a associação destes SNPs com o índice Glu/GABA no CCA de 88 indivíduos (50 portadores de TAB-I em eutimia e 38 controles saudáveis). Os resultados mostraram que pacientes com TAB-I portadores do alelo A do polimorfismo *GAD1* rs1978340 apresentaram níveis mais elevados da relação Glu/GABA comparado com os inidivíduos com TAB-I não portadores desse alelo, o qual foi influenciado mais pelos níveis absolutos de Glu do que GABA. Estes resultados não foram verificados na amostra controle e em relação aos demais SNPs analisados (rs11542313, rs3749034 e rs769390), os quais não influenciaram os níveis da relação Glu/GABA em ambos os

grupos analisados (Scotti-Muzzi et al., 2021b). Entretanto, os níveis da relação Glu/GABA mosrou-se reduzida na amostra total de TAB-I em relação aos controles saudáveis, sobretudo naqueles em uso de anticonvulsivante e antipsicóticos atípicos, sugerindo ser este um efeito induzido pelos estabilizadores de humor no estado de eutimia.

Embora o alelo G da variante *GAD* rs3749034, relacionado a uma expressão deficitária do gene *GAD*1 e, portanto, da enzima GAD67, seja mais frequentemente associado ao TAB, os SNPs rs1978340 e rs3749034 encontram-se em desequilíbrio de ligação (Arrúe et al., 2019), como observado na **Figura 2**. De fato, um estudo prévio de associação genética demonstrou que o alelo A do polimorfismo *GAD* rs1978340 estava associado ao TAB (Lundorf et al., 2005). Assim, conclui-se que o mecanismo pelo qual o alelo A do polimorfismo rs1978340 encontra-se associado ao TAB relaciona-se possivelmente a uma atividade deficitária da enzima GAD67, resultando em níveis elevados de Glu/GABA como verificado pelo nosso estudo (Scotti-Muzzi et al., 2021b). Por outro lado, aqueles portadores do genótipo GG apresentam níveis reduzidos desse índice e portando parecem apresentar neuroproteção em relação a excito-toxicidade glutamatérgica.

Figura 2 – Localização cromossômica do gene *Gad* 1 e seus polimorfismos funcionais.



Fonte: Arrúe et al. (2019).

4.2. Polimorfismos funcionais, canais de cálcio e neurometabólitos glutamatérgicos

Postula-se que a elevação dos níveis de neurometabólitos glutamatérgicos no CCA de pacientes com TAB (Scotti-Muzzi et al., 2021a) levaria a uma hiperativação

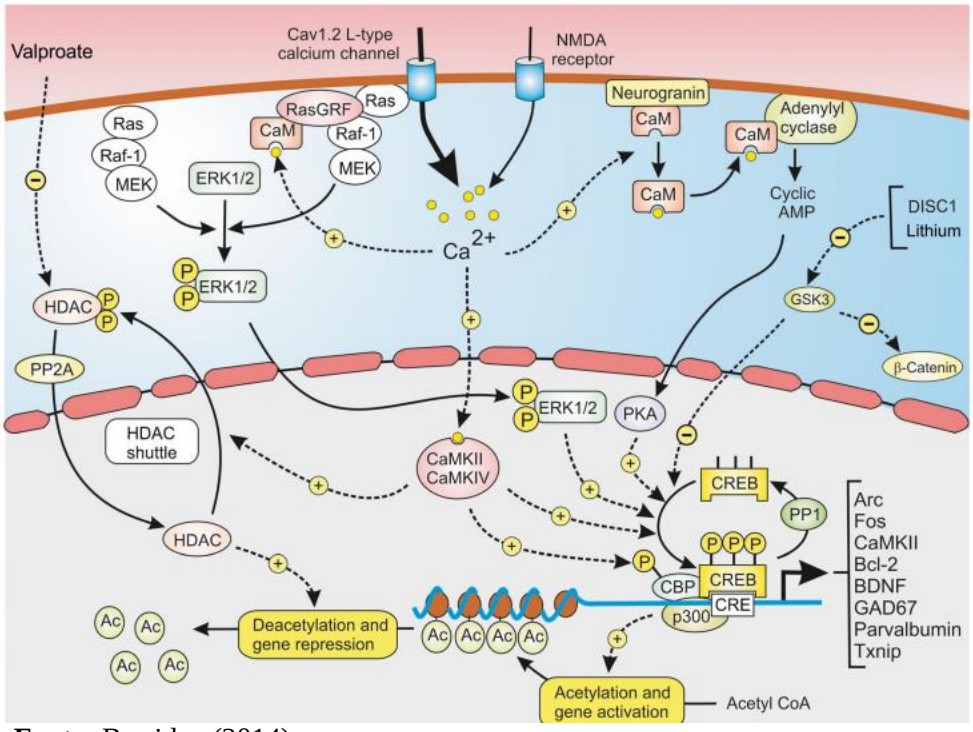
dos receptores glutamatérgicos pós-sinápticos dos tipos N-metil-D-aspartato (NMDA) e α-amino-3-hydroxi-5-metil-4- ácido isoxazolepropionico (AMPA), resultando em um aumento no influxo de cálcio e a um estado de excitabilidade neuronal (Mehta et al., 2013). De fato, a elevação do Ca^{+2} intracelular em linfócitos e plaquetas é um dos achados neurobiológico mais robustos encontrados em pacientes com TAB como demostrado por meta-análise envolvendo 642 pacientes e 404 controles (Harrison et al., 2019). Entretanto, além da hiperatividade glutamatérgica, o aumento do Ca^{+2} intracelular celular no TAB também tem sido atribuído às alterações em canais de cálcio dependentes de voltagem (CCDV) (Uemura; Green; Warsh, 2016).

O gene *CACNA1C*, codificador da subunidade alfa 1c dos CCDV do tipo L (Sinnegger-Brauns et al., 2009), apresenta polimorfismos funcionais (ex: rs1006737) que têm sido consistentemente associados ao TAB por estudos genéticos de associação ampla (GWAS) (Ferreira et al., 2008; Sklar et al., 2008; Stahl et al., 2019), estudos de associação com genes candidatos (Khalid et al., 2018; Mosheva et al., 2020), análises de risco genético (Croarkin et al., 2018) bem como por revisões (Ou et al., 2015; Gordovez; McMahon, 2020; Harrison et al., 2022) e meta-análises (Liu et al., 2011; Nurnberger et al., 2014). Entretanto, os mecanismos neurais desse polimorfismo a as manifestações fenotípicas no TAB ainda não são bem compreendidos (Harrison et al., 2022; Jiang et al., 2023). Assim, no estudo apresentado no **Capítulo III** avaliou-se, de forma pioneira, a associação entre o polimorfismo funcional rs100737 no gene *CACNA1C* e neurometabólitos glutamatérgicos no cíngulo anterior em uma amostra de 194 indivíduos composta de 121 portadores de TAB-I eutímicos e 73 controles saudáveis (Scotti-Muzzi et al., 2022). No grupo de pacientes com TAB-I, observamos um aumento dos neurometabólitos glutamatérgicos (Glx/Cr, Glu/Cr e Gln estimada) em relação aos controles saudáveis, corroborando, em parte, os resultados revelados pela meta-análise (Scotti-Muzzi et al., 2021a) no **Capítulo I**. A variante genética rs100737 no gene *CACNA1C* influenciou os níveis de Glx/Cr no CCA na medida em que os níveis elevados desse metabólito foram observados naqueles portadores do genótipo AA tanto na amostra total (TAB-I + HC) quanto nos pacientes com TAB-I, mas não nos controles saudáveis. A mesma tendência foi observada em relação ao Glu/Cr e Gln estimada, porém sem alcançar significância estatística. Ademais, o fator sexo pareceu influenciar nessa modulação já que verificaram-se níveis de Glx/Cr aumentados em carreadores do genótipo AA do sexo feminino mas não no masculino do grupo TAB-I.

Estes resultados revelaram que há uma relação entre o sistema glutamatérgico e alterações nos canais de cálcio no CCA em pacientes com TAB-I, possivelmente contribuindo para um possível aumento do influxo intracelular de cálcio observado neste transtorno (Uemura; Green; Warsh, 2016; Harrison et al., 2019). Apesar dos mecanismos envolvendo a entrada de cálcio intracelular mediadas por receptores glutamatérgicos NMDA e canais de cálcio tipo L serem independentes (Berridge, 2014; **Figura 3**), nossos resultados sugerem uma "conversa molecular" entre esses dois sistemas no TAB-I, mas não em controles saudáveis (Scotti-Muzzi et al., 2022). Estes sistemas provavelmente interagem entre si uma vez que algumas subunidades de canais de cálcio guardam homologia com receptores glutamatérgcicos (Nicoll; Tomita; Bredt, 2006).

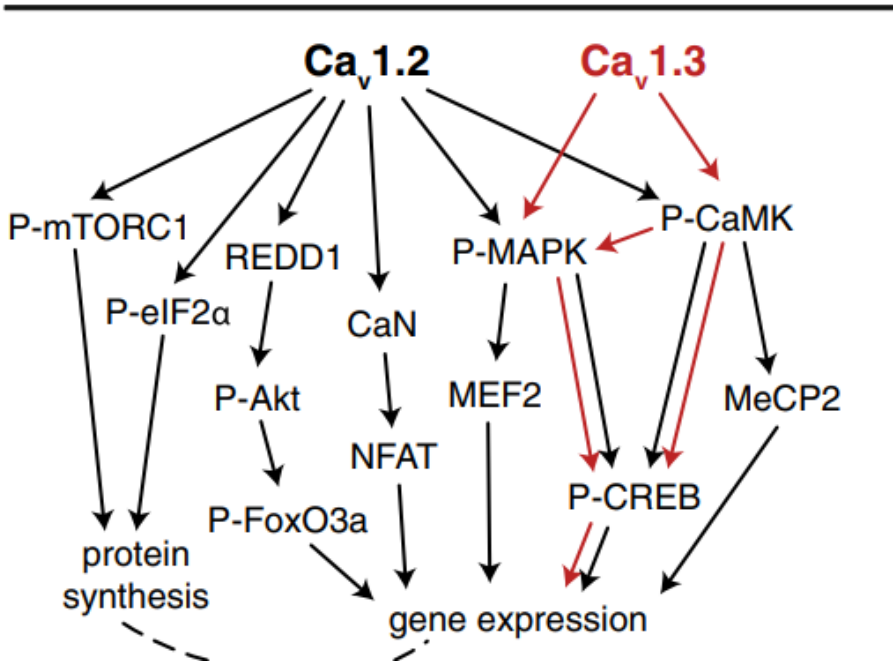
Como verificado nas **Figuras 3 e 4**, as vias comum de sinalização intracelular ativadas pelo Ca^{2+} são principalmente as vias de transcrição gênicas mediadas pela ativação da calmodulina (caM) e subsequentemente por proteínas quinases dependentes de Ca^{2+}/CaM bem como pela atividade fosforilativa de proteínas quinase ativadas por mitógeno (MAPK) e quinases regulada por sinal extracelular (ERK) (Berridge, 2014), as quais, juntamente com o próprio Ca^{2+}, estão envolvidas na transcrição de fatores de transcrição como o CREB ("cyclic AMP-responsive element-binding protein*"*) (Berger; Bartsch, 2014; Berridge, 2014; Kabir et al., 2017). Estudos com modelos animais defectivos para o gene *CACNA1*C confirmam a inativação da via MAPK/ERK (Moosman et al., 2005; Tigaret et al., 2021), a qual, juntamente com outras vias neurotróficas (ex: mTOR, AKT/FoxO), parecem ser as mais relacionadas nos eventos "downstream" desencadeados pela ativação dos canais de cálcio (**Figura 4**; Kabir et al., 2017). O CREB, por sua vez, é um fator de transcrição de vários genes relevantes para o TAB-I como a *GAD*-1 e o do fator neurotrófico *BDNF*, responsáveis pela codificação, respectivamente, das proteínas GAD67 e BDNF (**Figuras 3 e 5**), principalmente em neurônios glutamatérgicos (Lee et al., 2021). Além disso, o processo de fusão das vesículas contendo BDNF e membrana celular e consequente secreção neuronal do BDNF (**Figura 6**) também dependem do Ca^{2+} e de sua sinalização intracelular **(**Kwinter et al., 2009; Sharma et al., 2022). Portanto, as alterações na homeostase do Ca^{2+} intracelular têm implicações para as vias intracelulares dependentes do cálcio, síntese e secreção de BDNF (**Figura 6**, Sharma et al., 2022).

Figura 3 – Vias de sinalização intracelular mediadas pelo cálcio**.**



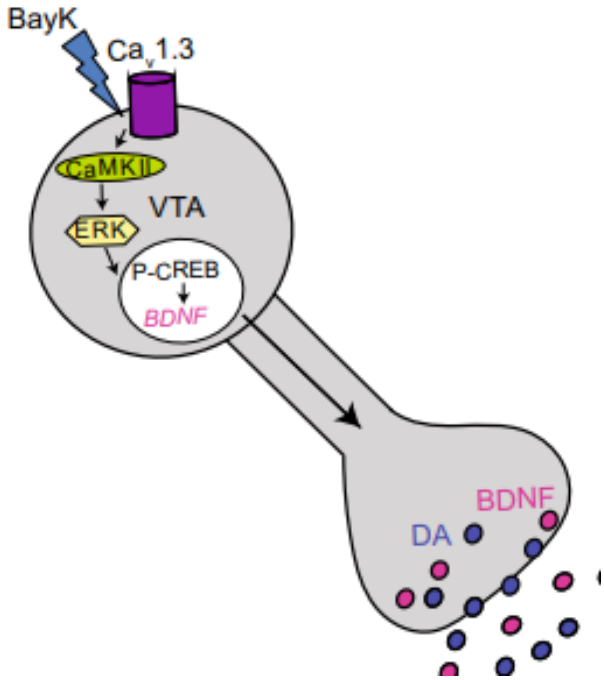
Fonte: Berridge (2014).

Figura 4 – Vias de sinalização intracelular mediadas pelo cálcio a partir de canais de cálcio do tipo L.



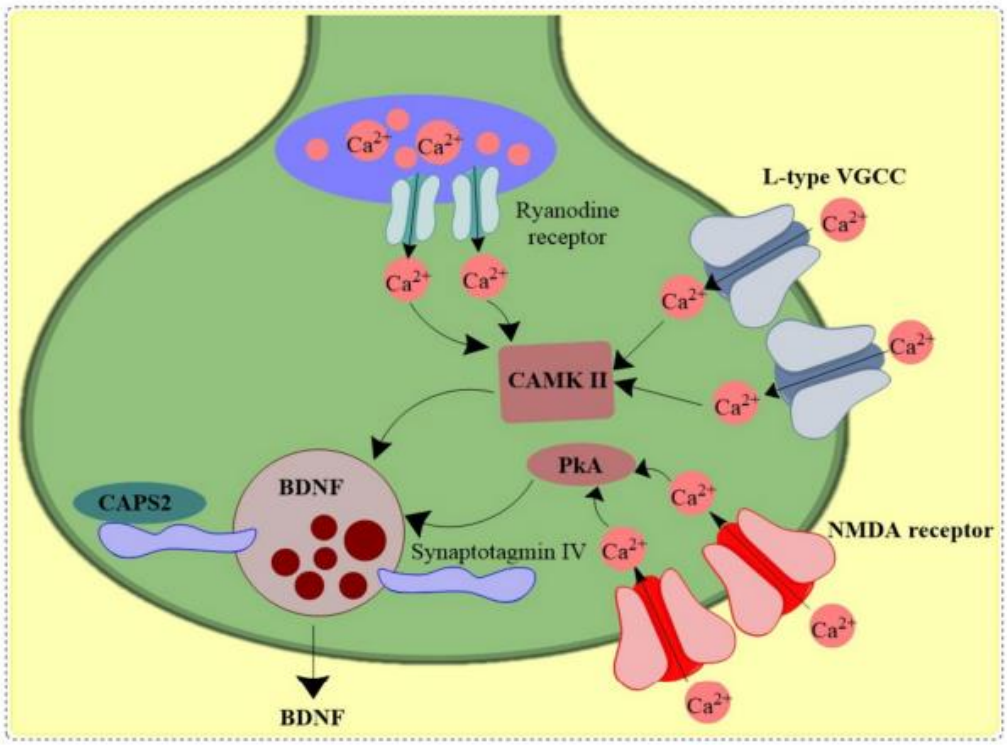
Fonte: Kabir et al. (2017).

Figura 5 – Via de sinalização intracelular neurotrófica mediada pelo cálcio a partir de canais de cálcio do tipo L responsável pela síntese de BDNF.



Fonte: Kabir et al. (2017).

Figura 6 – Influência dos receptores glutamátergicos NMDA e dos canais de cálcio do tipo L para o influxo de cálcio e empacotamento e secreção vesicular de BDNF.



Fonte: Sharma et al. (2022).

O fator sexo também pareceu influenciar a modulação do polimorfismo *CACNA1C* rs100737 nos neurometabólitos glutamatérgicos uma vez que o aumento de Glx/Cr nos carreadores de genótipo AA foi observado apenas em mulheres. Este resultado ratifica a literatura que reporta que o alelo A do *CACNA1C* rs1006737 como fator de risco para o TAB apenas no sexo feminino (Dao et al., 2009; Witt et al., 2014). Em coortes de indivíduos saudáveis, Takeuchi et al. (2018) observaram que o alelo de risco do *CACNA1C* rs1024582 estava assoado a uma atividade fronto-límbica reduzida sob tarefas cognitivas apenas em indivíduos saudáveis do sexo feminino e Bastos et al. (2023) recentemente também verificaram redução dos níveis séricos de BDNF em mulheres portadoras do haplótico AA dos polimorfismos rs1006737–rs4765913 do *CACNA1C* em uma amostra de 641 adultos jovens.

Sumarizando, podemos postular que no TAB, polimorfismos no gene *CACNA1*C resultariam em alterações na estrutura do canal de Ca^{2+} dependente de voltagem (Kabir et al., 2017), que, associado a um aumento na atividade glutamatérgica no CCA (Scotti-Muzzi et al., 2022), levaria à elevação dos níveis intracelulares de Ca^{2+} (Uemura; Green; Warsh, 2016; Harrison et al., 2019). Tal elevação traduziria a um estado de excitabilidade neuronal que levaria à desregulação de vias de sinalização intracelular de cálcio e neurotróficas como BDNF (Berridge, 2014; Harrison et al., 2022) e, em última análise, à morte neuronal (Mehta et al., 2013). Este modelo explicaria a redução cortical observada no CCA em pacientes bipolares (Hibar et al., 2017), o qual mostrou-se influenciada pelo alelo de risco do gene *CACNA1*C (Soeiro-de-Souza et al., 2017; Smedler et al., 2019).

4.3. Polimorfismos funcionais, fatores neurotróficos e metabolismo cerebral

Do ponto de vista dos neurometabólitos, tal perda neuronal reportada no CCA no TAB (Hibar et al., 2017) pode se traduzir em um aumento da ciclagem de fosfolipídeos de membrana como a colina resultante de sua degradação, levando a um aumento do pico de colina (Cho) no espectro de ^{1}H-MRS (Stork; Renshaw, 2005; Buonocore; Maddock, 2015). O aumento de Cho foi precisamente um dos resultados mais consistente revelado pela meta-análise sobre neurometabólitos no cíngulo anterior (Scotti-Muzzi et al., 2021a) apresentada no **Capítulo I**. Os resultados da meta-análise revelaram um aumento de colina no cíngulo em diversos estados de humor (eutímia e

depressão) bem como nos pacientes não medicados, sugerindo ser este um marcador sensível, porém não específico para o TAB (Scotti-Muzzi et al., 2021a).

Entretanto, os mecanismos subjacentes a essa perda neuronal e aumento de colina no CCA não são bem compreendidos. A secreção defectiva de BDNF resultante de alterações na sinalização de cálcio na via MEK/MAPK-CREB (**Figura 5**) é uma hipótese atrativa para explicar tais alterações morfológicas e metabólicas. O BDNF é a neutrotrofina mais abundante no cérebro e desempenha papel chave na neurogênese e plasticidade sináptica (Hofer et al., 1990; Kowiaoski et al., 2017). O polimorfismo funcional no gene *BDNF* (*BDNF* rs6265 or Val66Met) causa uma substituição de uma valina (*val*) por metionina (*met*) no códon 66 do pró-BDNF e tem sido associado a um transporte deficiente de RNAm, empacotamento e secreçãodo BDNF no hipocampo de portadores saudáveis do alelo *met* (Egan et al., 2003; Chen et al., 2004; Baj et al., 2011). No entanto, o alelo *val* do *BDNF* rs6265 tem sido mais associado ao TAB (Neves-Pereira et al., 2002; Sklar et al., 2002; Li; Chang; Xiao, 2016; Paul et al., 2021), embora a literatura careça de informações sobre a influência do referido polimorfismo nos neurometabólitos do cíngulo anterior de pacientes afetados pelo TAB.

O **capítulo IV** apresenta o artigo intitulado "*BDNF* rs6265 differentially influences neurometabolites in the anterior cingulate of healthy and bipolar disorder subjects" (Scotti-Muzzi et al., 2023) cujo objetivo foi avaliar a influência do polimorfismo *BDNF* rs6265 nos neurometabólitos do CCA em 124 indivíduos com TAB-I eutímicos e 76 controles saudáveis. Observou-se uma interação entre os níveis de NAA/Cr e o genótipo *met* do *BDNF* rs6265 na amostra completa (BD-I + C) e nos controles, mas não no grupo TAB-I. Nos controles, indivíduos portdores do alelo *met*, apresentaram níveis elevados de NAA/Cr em relação aos não carreadores desse alelo, sugerindo uma melhor saúde cortical já que níveis reduzidos de NAA são comumente interpretados como indicador de disfunção neuronal (Stork; Renshaw, 2005).

Por outro lado, no grupo de pacientes, observamos que aqueles portadores do alelo *val* apresentavam níveis elevados de creatina total, achado esse não verificado nos controles. Este resultado sugere que os carreadores do alelo *val* no grupo de bipolares apresentam uma atividade metabólica elevada já que o sistema creatina/fosfocreatina desempenha um papel chave no metabolismo energético cerebral, tamponando e transportando fosfatos de alta energia (Buonocore; Maddock, 2015).

Uma vez que verificamos que os pacientes com TAB-I apresentavam níveis elevados de Glx/Cr em relação aos controles e que tal achado foi observado apenas nos

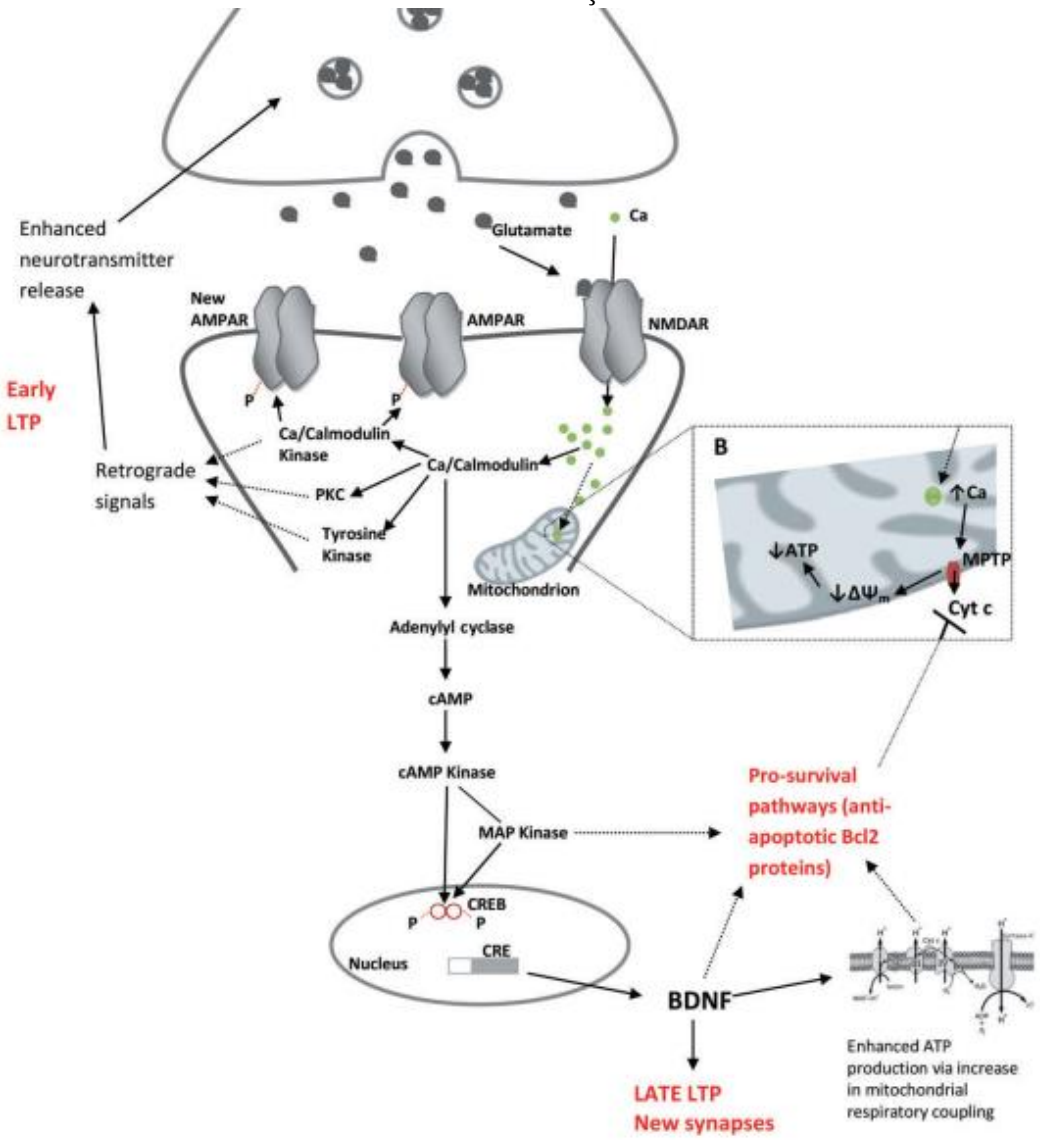
carreadores do alelo *val* (Scotti-Muzzi et al., 2023), atribuímos o metabolismo energético elevado neste grupo a um aumento de glutamato. Este metabólito, além de ser o principal neurotransmissor excitatório do SNC, pode servir de substrato no metabolismo energético do ciclo tricarboxílico, gerando ATP como observado na **Figura 1** (Walls et al., 2015; Karaca et al., 2015). A literatura confirma que o BDNF exerce papel importante no metabolismo energético controlando a função mitocondrial, termogênese e metabolismo da glicose (Markham et al., 2014; Di Rosa et al., 2021).

Além disso, o BDNF exerce seu papel neuroprotetor contra os danos excito-tóxicos causados pela atividade glutamatérgica elevada via cascatas de sinalização intracelular trkB-MEK/MAPK bem como pela manutenção da eficiência respiratória mitocondrial (**Figuras 3 e 7**; Markham et al., 2014). Uma vez que as mitocôndrias são as principais responsáveis pelo sequestro do Ca^{2+} intracelular (Wang; Thayer, 2002; Wu et al., 2004), o qual tem papel relevante na eficiência respiratória com geração de ATP (**Figura 7;** Markham et al., 2014), a desregulação do tamponamento de cálcio pode estar associada ao alelo de risco *val* para o TAB (Neves-Pereira et al., 2002; Sklar et al., 2002; Li; Chang; Xiao, 2016; Paul et al., 2021). De fato, verificamos que os pacientes carreadores desse alelo apresentavam níveis elevados de Glx/Cr em relação aos não-carreadores (Scotti-Muzzi et al., 2023).

Assim, nossos resultados apontam para uma inter-relação entre o metabolismo energético modulado pelo polimorfismo *BDNF* rs6265 e o sistema glutamatérgico, corroborando os resultados apresentados no capítulo III (Scotti-Muzzi et al., 2022) bem como aqueles revelados por Smedler et al. (2021) que reportaram que o gene *CACNA1C* é capaz de modular os níveis séricos de BDNF em indivíduos saudáveis e com TAB. Entretanto, evidências recentes mostraram que tal modulação do gene *CACNA1C* nos níveis séricos de BDNF é dependente do sexo (Bastos et al., 2023).

De fato, o fator sexo contribuiu para a modulação do polimorfismo *BDNF* rs6265 nos níveis e NAA/Cr, creatina total e Glx/Cr uma vez que os resultados reportados para estes metabólitos foram verificados apenas no sexo masculino. Os dados encontrados por Bastos et al. (2023) mostrando níveis elevados de BDNF sérico no sexo masculino em relação ao femino nos portadores do haplótipo AA dos polimorfismos rs1006737-rs4765913 do gene *CACNA1C* concordam com os nossos resultados o (Scotti- Muzzi et al., 2023).

Figura 7 – Influência do metabolismo mitocondrial e sinalização de cálcio na síntese de BDNF.

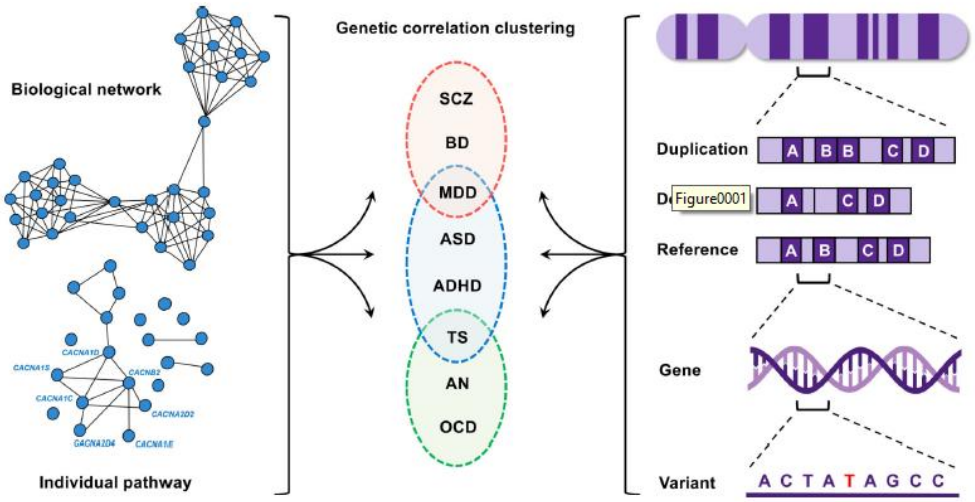


Fonte: Markham et al. (2014).

Estes dados corroboram o conhecido efeito pleiotrópico exercido pelo polimorfismo *BDNF* rs6265 (Tsai, 2018; Di Rosa et al., 2021), o qual é influenciado por fatores como o diagnóstico, etinicidade, idade e sexo (Hosang et al., 2014; Tsai, 2018). De fato, a pleiotropia parece ser um fenômeno comum na genética dos transtornos psiquiátricos, o que explica o fato de um mesmo polimorfismo (ex: *CACNA1*C e *BDNF*) estarem associados de forma distinta a diferentes transtornos como o TAB, esquizofrenia e Transtornos do espectro autista bem como a manifestações fenotípicas distintas nos indivíduos saudáveis (Lee; Feng; Smoller, 2021). A pleiotropia pode ter origem em diferentes níveis genômicos, desde SNPs, locis, genes até interação entre múltiplos genes e vias biológicas, resultando em fenótipos distintos (**Figura 8**). No caso

dos polimorfismos *BDNF* rs6265 e *CACNA1C* rs1006737, nossos dados sugerem que estes exercem efeito pleiotrópico nos neurometabólitos do CCA modulados pelos fatores diagnóstico (TAB-I vs. Indivíduos saudáveis) e o sexo.

Figura 8 – Pleiotropia em diferentes níveis genômicos**.**



Fonte: Lee, Feng e Smoller (2021).

4.4. Polimorfismos funcionais, estabilizadores de humor e neurometabólitos

Este estudo foi conduzido em pacientes bipolares em estado de eutimia medicados com estabilizadores de humor. Portanto, é imperativo considerar o efeito dessas medicações em relação aos polimorfismos genéticos analisados e os neurometabólitos. No trabalho de meta-análise (**Capítulo I**), foi observado que o estado de eutimia estava associado a uma elevação dos níveis de glutamina (Gln) no CCA (Scotti-Muzzi et al., 2021a), corroborando evidências prévias que os estabilizadores de humor parecem aumentar o índice Gln/Glu ou reduzir o Glu/Gln nos pacientes com TAB (Öngür et al., 2008; Soeiro-de-Souza et al., 2015; Kubo et al., 2017), principalmente em uso de anticonvulsivantes (Soeiro-de-Souza et al., 2018a).

Considerando que a ciclagem de glutamato (Glu) e glutamina (Gln) entre neurônios glutamatérgicos e astrócitos (**Figura 1**) parece ser uma aquisição evolutiva para tamponar a excito-toxicidade glutamatérgica já que a Gln é a forma "não excitatória" do glutamato (Walls et al., 2015; Cooper; Jeitner, 2016), nossos resultados apontam que um dos mecanismos de ação dos estabilizadores de humor seria a

modulação do ciclo Glu-Gln no estado de eutimia. Este fenômeno parece ocorrer principalmente na classe dos anticonvulsivantes, os quais são capazes tanto de reduzir o Glu (Friedman et al., 2004; Strawn et al., 2012) como aumentar a Gln (Soeiro-de-Souza et al., 2015).

Este achado foi confirmado pelo trabalho apresentado no **Capítulo II** (Scotti-Muzzi et al., 2021b) no qual verificamos que os pacientes bipolares apresentaram níveis reduzidos do índice Glu/GABA em relação aos controles, o qual foi mais influenciado pela redução nos níveis de Glu que aumento de GABA. Quando analisado de acordo com a medicação utilizada (lítio, anticonvulsivantes e antipsicóticos atípicos), observou-se que pacientes em uso de anticonvulsivantes e antipisicóticos atípicos (monoterapia ou em combinação) apresentaram níveis reduzidos de Glu/GABA em relação aos controles saudáveis. Além disso, o polimorfismo *GAD*1 rs1978340 parece atuar em sinergia com os estabilizadores de humor na redução dos níveis de Glu/GABA. Pacientes com genótipo GG de *GAD*1 rs1978340 em uso de anticonvulsivantes, lítio e antipisicóticos apresentaram índices reduzidos de Glu/GABA em relação aos controles saudáveis com mesmo genótipo (Scotti-Muzzi et al., 2021b).

Estes resultados apontam para uma possível relação epigenética entre os estabilizadores de humor e este polimorfismo, corroborando evidências prévias que o ácido valpróico pode exercer um efeito epigenético aumentando a expressão do RNA mensageiro da GAD67 em pacientes com TAB (Pisanu et al., 2018). Apesar da nossa sub-amostra de pacientes em uso de cada tipo de estabilizador de humor ser demasiado pequena, este resultado desvela uma hipótese sugestiva a respeito da farmacogenética dos estabilizadores de humor: seriam os carreadores do genótipo GG do polimorfismo *GAD*1 rs1978340 mais reponsivos aos estabilizadores de humor devido a uma atividade glutamatérgica reduzida no cíngulo anterior?

Por outro lado, os resultados do **Capítulo III** mostraram que os estabilizadores não afetaram a influência do polimorfismo *CACNA1C* rs100737 nos níveis de Glx, já que este metabólito permaneceu elevado nos portadores do genótipo AA independentemente do uso de anticonvulsivantes e lítio (Scotti-Muzzi et al., 2022). Entretanto, os carreadores do alelo AA do *CACNA1C* rs100737 em uso de anticonvulsivantes apresentaram níveis elevados de glutamina estimada em relação àqueles com genótipo AG ou GG. Estes resultados reforçam as evidências que os anticonvulsivantes favorecem a conversão do Glu para Gln, sobretudo a lamotrigina, um anticonvulsivante amplamente utilizado no tratamento do TAB. A literatura confirma

que, além dos canais de sódio dependentes de voltagem, os canais de cálcio também exercem papel chave na mediação tanto do efeito anticonvulsivante como neuroprotetor da lamotrigina (Dibué-Adjei et al., 2017) e que esta medicação é capaz de reduzir os níveis de glutamato e aumentar os de GABA (Cunningham; Jones, 2000).

Ademais, os canais de cálcio dependentes de voltagem são alvo de bloqueadores de canais de cálcio usados no tratamento de hipertensão arterial sistêmica (Braunwald, 1982) bem como por gabapentinóides (gabapentina e pregabalina) usados no tratamento de epilepsia e dor crônica (Hong et al., 2022). Portanto, o desenvolvimento de novos bloqueadores de canais de cálcio ou moduladores das vias de sinalização intracelular do cálcio são promissores estabilizadores de humor, guardando possível relação farmacogenômica com o gene *CACNA1C*.

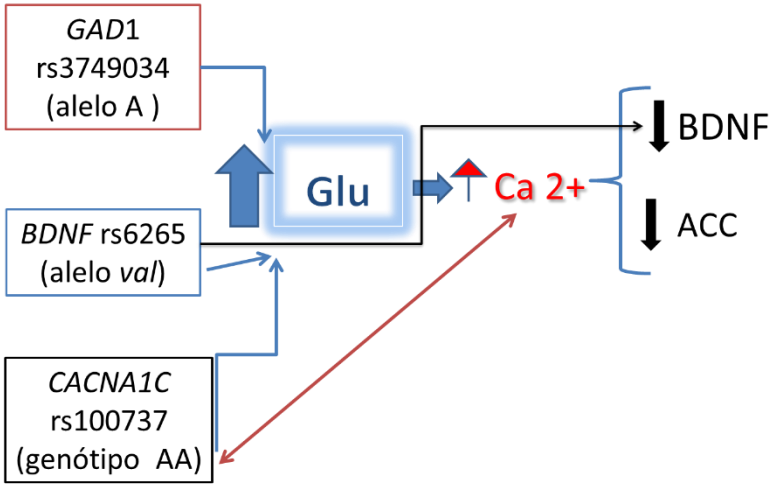
Embora nossa amostra seja considerada robusta para estudos de ^{1}H-MRS, ela é demasiada pequena para um estudo genético. Portanto, os resultados obtidos, principalmente as sub-análises relativas ao sexo e tipo de medicação devem ser replicadas em estudos com amostras maiores.

5. CONSIDERAÇÕES FINAIS

Nossos resultados confirmaram que a elevação de Glx (Glu + Gln) no CCA é um dos achados mais consistentes no TAB e revelaram possíveis mecanismos subjacentes a este fenômeno a partir do estudo da genética funcional. O aumento de neurometabólitos glutamatérgicos no cíngulo anterior no TAB mostrou-se influenciada pelos alelos A do polimorfismo *GAD* rs374903, genótipo AA do polimorfismo *CACNA1C* rs100737 e alelo *val* do *BDNF* rs6265. Portanto, este estudo aponta para a hipótese que a excito-toxicidade glutamatérgica no TAB resultaria de uma atividade deficitária da enzima GAD 67, a qual, juntamente com alterações nos canais de cálcio dependentes de voltagem (*CACNA1*C), levaria ao aumento do cálcio intracelular reportada no TAB.

Tal elevação estaria implicada na desregulação de vias de sinalização intracelular neurotróficas como BDNF e, em última análise, atrofia cortical do cíngulo anterior. Essa redução cortical reportada pela literatura foi corroborada pelos dados da meta-análise que demontraram que a elevação de Cho, atribuída a um aumento na ciclagem de fosfolipídeos de membrana resultante de morte celular, é um marcador para TAB. Desta forma, a principal hipótese derivada desse estudo é que a elevação de glutamato no TAB encontra-se associada a uma arquitetura poligênica relacionada com atividade da enzima GAD67, canais de cálcio e fatores neurotróficos como o BDNF. (**Figura 9**) Entretanto, os genes *BDNF* e *CACNA1C* mostraram um efeito pleiotrópico influenciados pelo diagnóstico (TAB *vs* C) e sexo.

Figura 9 – Esquema mostrando a influência dos polimorfismos testados nos níveis de glutamato no CCA e possíveus efeitos "dowstream".

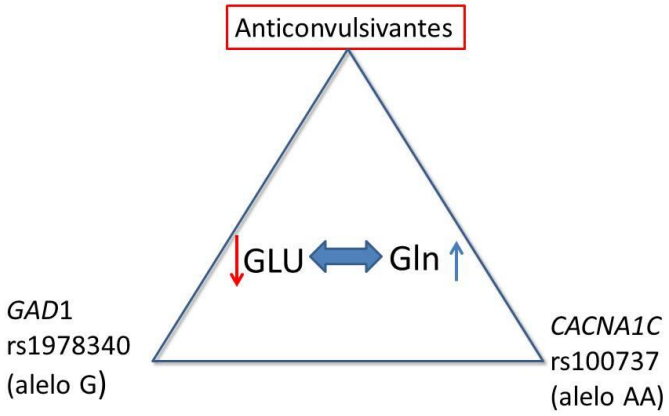


Fonte: autoria própria.

Além disso, a modulação dos neurometabólitos glutamatérgicos pelos SNPs parece depender das medicações estabilizadoras do humor e do estado de humor atual (**Figura 10**). Nosso estudo fornece evidências que no estado de eutímia ocorre uma elevação de Gln concomitante a uma redução de Glu, particularmente em resposta aos anticonvulsivantes.

Assim, postula-se que um dos mecanismos subjacentes à ação terapêutica dos anticonvulsivantes seja a conversão de Glu em Gln, resultando em estado de menor excito-toxicidade e neuroproteção. Tal resposta do Glu em relação a esta classe de estabilizadores de humor mostrou-se potencializada pelo *GAD*1 rs3749034 enquanto a elevação de Gln pareceu influenciada pelo *CACNA1C* rs100737, sugerindo uma possível relação farmacogenética entre esses polimorfismos e a resposta aos estabilizadores de humor (Figura 10).

Figura 10 – Influência dos anticonvulsivantes e polimorfismos genéticos funcionais (GAD1 rs3749034, CACNA1C rs100737) na conversão de Glu em Gln no CCA no estado de eutimia.



Fonte: autoria própria.

Ademais, nos controles saudáveis, o alelo *met* do *BDNF* rs6265 pareceu também conferir neuroproteção associado a níveis elevados de NAA/Cr. Desta forma, este estudo sugere que as variações genéticas funcionais avaliadas são candidatas para inclusão futura em testes farmacogenéticos para avaliação da resposta aos estabilizadores de humor bem como prognóstico no TAB.

Dessa forma, este trabalho é pioneiro na tentativa de melhor compreender a complexa relação entre alterações genéticas funcionais implicadas no TAB,

neurometabólitos em uma região cortical chave para o controle afetivo e cognitivo e o efeito dos estabilizadores de humor em uma população homogênica em eutimia.

Recomenda-se que trabalhos futuros devam avaliar tal relação em amostras maiores e em diferentes estados de humor e/ou estados afetivos (ex: distimia, depressão unipolar, depressão bipolar, depressão com sintomas psicóticos, mania com e sem sintomas psicóticos, hipomania, eutimia etc.), bem como em indivíduos medicados e não-medicados.

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