



Exploring brain insulin resistance in adults with bipolar depression using extracellular vesicles of neuronal origin

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ABSTRACT

Accumulating evidence suggests that disrupted insulin signaling is involved in bipolar disorder (BD) pathogenesis. Herein, we aimed to directly explore the potential role of neuronal insulin signaling using an innovative technique based on biomarkers derived from plasma extracellular vesicles enriched for neuronal origin (NEVs). We leveraged plasma samples from a randomized, double-blind, placebo-controlled, 12-week clinical trial evaluating infliximab as a treatment of bipolar depression. We isolated NEVs using immunoprecipitation against neuronal marker L1CAM from samples collected at baseline and weeks 2, 6 and 12 (endpoint) and measured NEV biomarkers using immunoassays. We assessed neuronal insulin signaling at its first node (IRS-1) and along the canonical (Akt, GSK-3 β , p70S6K) and alternative (ERK1/2, JNK and p38-MAPK) pathways. A subset of participants ($n = 27$) also underwent whole-brain magnetic resonance imaging (MRI) at baseline and endpoint. Pre-treatment, NEV biomarkers of insulin signaling were independently associated with cognitive function and MRI measures (i.e. hippocampal and ventromedial prefrontal cortex [vmPFC] volumes). In fact, the association between IRS-1 phosphorylation at serine site 312 (pS312-IRS-1), an indicator of insulin resistance, and cognitive dysfunction was mediated by vmPFC volume. In the longitudinal analysis, patients treated with infliximab, a tumor necrosis factor-alpha antagonist with known insulin sensitizing properties, compared to those treated with placebo, had augmented phosphorylation of proteins from the alternative pathway. Infliximab responders had significant increases in phosphorylated JNK levels, relative to infliximab non-responders and placebo responders. In addition, treatment with infliximab resulted in increase in MRI measures of brain volume; treatment-related changes in the dorsolateral prefrontal cortex volume were mediated by changes in biomarkers from the insulin alternative pathway. In conclusion, our findings support the idea that brain insulin signaling is a target for further mechanistic and therapeutic investigations.

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1. Introduction

Converging evidence indicates that disrupted insulin signaling is relevant to bipolar disorder (BD) pathophysiology. A recent genome-wide association study has documented that the insulin signaling pathway is implicated in BD etiology (Stahl et al., 2019). In addition, metabolic disorders (i.e. type 2 diabetes mellitus [T2DM], obesity) are highly prevalent in BD (Vancampfort et al., 2015a); individuals with BD exhibit a 2-fold increased risk for T2DM (Vancampfort et al., 2015b). While there is an iatrogenic component (e.g. use of psychotropic agents with adverse metabolic effects) that may add to this excessive risk, higher levels of insulin resistance (IR) are observed in drug naïve and/or newly diagnosed cohorts (Coello et al., 2019; Guha et al., 2014). The occurrence of IR in BD is also associated with a more complex illness presentation, as evidenced by higher rates of non-recovery, chronicity and suboptimal treatment response (Calkin et al., 2015; Mansur et al., 2016; Ruzickova et al., 2003). Results from neuroimaging studies indicate that individuals with BD and IR/T2DM, have smaller hippocampal/cortical volumes, as well as neurochemical abnormalities, when compared to adults with BD without IR/T2DM (Hajek et al., 2014, 2015).

As a result, the use of insulin sensitizers in the treatment of BD (e.g. insulin, pioglitazone and liraglutide) has been investigated (Aftab et al., 2019; Mansur et al., 2017; McIntyre et al., 2012). Results have been mixed. A clinical trial showed that intranasal insulin was associated with an improvement in neurocognitive function in euthymic patients with BD (McIntyre et al., 2012), whereas another study documented a lack of antidepressant efficacy of pioglitazone in bipolar depression (Aftab et al., 2019). A significant limitation of this line of research is the fact that these studies were based on measures of peripheral insulin sensitivity as surrogates for brain sensitivity. Hitherto, it has been assumed that peripheral measures of IR (e.g. homeostatic model assessment of insulin resistance [HOMA-IR], Matsuda index) reflect brain insulin sensitivity, which has not been well characterized (Kullmann et al., 2016; Sartorius et al., 2015). Overall, brain insulin sensitivity has not yet been extensively tested as a clinically relevant mechanism or target for interventions in psychiatry or, until recently, in neurology.

There has been increasing interest in the use of neuronal origin-enriched extracellular vesicles (NEVs) as a source of neuronal-specific biomarkers. Extracellular vesicles are particles naturally released by all cells, including neurons (Faure et al., 2006), which can cross the blood-brain barrier and be detected in peripheral blood. The isolation of NEVs by immunocapture, based on surface markers reflecting their cellular origin, allows for the assessment of intra-cellular signaling mediators typically undetectable in plasma and potentially reflecting abnormalities in neuronal metabolism (Fiandaca et al., 2015; Kapogiannis et al., 2015; Mustapic et al., 2017). This approach has been extensively used to investigate neuronal insulin signaling in mechanistic and interventional studies (Athauda et al., 2019; Bassil et al., 2017; Goetzl et al., 2019; Kapogiannis et al., 2015; Mustapic et al., 2019; Wijtenburg et al., 2019a). Given their origin and biogenesis neuronal NEVs effectively sample the cytoplasm and endosomal compartments of neurons offer a neuron-specific window into insulin metabolism compared to the soluble phase of plasma. Using a series of protein discovery arrays, we have shown that neuronal EVs have much higher levels of intracellular signaling mediators of the insulin pathway compared to serum, but also total NEVs and other subpopulations of NEVs derived from the same plasma sample, perhaps, as a result of the higher metabolic rate of neurons than most other cells (Mustapic et al., 2017). Based on these theoretical considerations and empirical observations, we and collaborators have used insulin signaling mediators in neuronal EVs as biomarkers for clinical Alzheimer's (Kapogiannis et al., 2015), preclinical Alzheimer's and age-associated cognitive decline (Eren et al., 2020; Kapogiannis et al., 2019b), schizophrenia (Kapogiannis et al., 2019a; Wijtenburg et al., 2019b), and also demonstrated their relationship with brain cortical volume (Mullins et al., 2017;

Walker et al., 2020), whereas, recently, another group demonstrated that they may be used as biomarkers for depression (Nasca et al., 2020). However, this method has not yet been applied in a population of individuals with BD.

We recently completed a randomized, double-blind, placebo-controlled, clinical trial evaluating infliximab as a treatment for bipolar depression (McIntyre et al., 2019). Infliximab is a TNF- α antagonist primarily used for the treatment of autoimmune diseases. It has been previously shown to affect insulin signaling (Bissell et al., 2016; Corrado et al., 2019; Mendez-Garcia et al., 2018). In the study primary outcome, we did not observe a significant effect of infliximab on depressive symptoms. In this *post-hoc*, exploratory analyses we aimed initially to describe the associations between NEV biomarkers of neuronal insulin signaling, and clinical and biological variables of interest (i.e., cognition, MRI measures). We then investigated the effects of treatment with infliximab, as well the relationship between changes in NEV biomarkers and change in trial outcomes. We focused herein on pS312-IRS-1 and in molecules from insulin's two main downstream pathways: the canonical pathway (i.e., PI3K/Akt), which affects glucose and lipids homeostasis; and the alternative pathway (i.e., Ras/MAP kinase), which is implicated in the regulation of apoptosis and cell proliferation/differentiation. For analysis of MRI measures, we used a region of interest (ROI) approach and investigated the anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (dlPFC), ventromedial prefrontal cortex (vmPFC) and hippocampus (Haukvik et al., 2018; Lu et al., 2019; Wang et al., 2019). Our primary hypothesis was that infliximab-treated participants, compared to placebo-treated individuals, would exhibit activation of insulin signaling in NEVs. Furthermore, we hypothesized that changes in insulin signaling biomarkers would be associated with antidepressant response.

2. Methods and materials

2.1. Study design

The present NEV biomarker study was based on samples from a clinical trial of infliximab for bipolar depression performed from October 1, 2015, and April 30, 2018 to assess the effects of adjunctive infliximab on depressive symptom severity for 12 weeks. The clinical study was a multisite, randomized, double-blind, placebo-controlled, parallel-group, fixed-dose trial. Participants were individuals with bipolar I/II depression meeting inflammatory criteria (McIntyre et al., 2019). The study was approved by the Institutional Ethics Board at the University Health Network, Toronto, ON, Canada, as well as Stanford University, Palo Alto, California. The study is registered with [ClinicalTrials.gov](https://clinicaltrials.gov) (Identifier: NCT02363738).

2.2. Participants

Eligible participants were male and female outpatients between the ages of 18 and 65 who met Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) criteria for a current major depressive episode as part of bipolar I/II disorder. All subjects provided written informed consent after receiving a complete description of the study. The diagnosis of BD was confirmed using the Mini-International Neuropsychiatric Interview (M.I.N.I.) 5.0.0 for the DSM-IV-TR and interview responses were compared to DSM-5 diagnostic criteria to confirm BD I/II diagnosis. A total score of 22 or higher on the Montgomery-Asberg Depression Rating Scale (MADRS) and a total score of less than 12 on the Young Mania Rating Scale (YMRS) for participants was required for inclusion in the study. No changes in their medication for 4 weeks prior to baseline as well as during the 12-week study was also required. There were no inclusion or exclusion criteria based on their past or current psychiatric medication history.

Participants were also required to meet 1 of the following biochemical or phenotypic inflammatory criteria at baseline: CRP of 5

mg/L or more; obesity (i.e., ethnicity-specific waist circumference or body mass index ≥ 30 and increased triglyceride levels, decreased high-density lipoprotein cholesterol level, or elevated blood pressure; type 1 or 2 diabetes; inflammatory bowel disorder; rheumatologic disorder; daily cigarette smoking; or migraine headaches. (McIntyre et al., 2019). Exclusion criteria included a concurrent psychiatric disorder that was a primary focus of clinical attention; a history of schizophrenia; active psychotic symptoms; substance abuse and/or dependence in the previous 6 months; electroconvulsive therapy in the previous 6 months; at risk of suicide or actively suicidal; clinically significant unstable medical illness determined by physical examination and laboratory testing; current or previous exposure to anti-TNF biologic agents; or previous immediate hypersensitivity response (e.g., anaphylaxis) to a plasma-derived or recombinant immunoglobulin product.

2.3. Procedures

Participants were enrolled at the Mood Disorders Psychopharmacology Unit (MDPU) in Toronto and Stanford University in Palo Alto. Only individuals enrolled at the MDPU site had blood samples taken and composed the sample analyzed herein. All participants meeting eligibility criteria at screening were randomized to either receive intravenous infliximab (5 mg/kg) or placebo (saline solution matched to infliximab in color and consistency) administered adjunctively to their existing guideline-congruent pharmacologic regimen for BD.

The infliximab dose and infusion schedule selected for this study was based on a previously published clinical trial with infliximab for a mood disorders population (Raison et al., 2013). Participants received the infusion over a period of 120 min by a rheumatology infusion registered nurse. All participants completed the baseline infusion within one month of completing the screening assessment. Infliximab and placebo were prepared and dispensed in a concealed 250 mL infusion bag matched in color and consistency by hospital pharmacists who did not have contact with any of the participants. The randomization schedule was computer generated in blocks of 6 by a research team member who had no contact with the participants.

Participants, outcome assessors, principal investigators, and infusion nurses were all blinded to treatment randomization. Depressive symptoms severity was measured using the MADRS at every study visit. Cognitive assessment was conducted at weeks 0, 2, and 12 and consisted of the Digit Symbol Substitution Test (DSST), a measure of executive function and processing speed. Analysis of fasting plasma glucose and insulin were completed using standard methods. Basal state IR was calculated from fasting plasma glucose and fasting insulin using the HOMA2 calculator (<http://www.dtu.ox.ac.uk>) (Levy et al., 1998). The methods for the quantification of plasma TNF- α were previously described (Mansur et al., 2020).

2.4. Neuroimaging

A subsample ($n = 27$) completed magnetic resonance imaging (MRI) scans at baseline and endpoint. There were no baseline differences in age ($p = 0.638$), sex ($p = 0.496$) and MADRS total score ($p = 0.720$) between subjects that did or did not participate in MRI scans. High resolution 3D T1-weighted images were acquired from 14 participants in the placebo cohort and 13 participants from the infliximab group. Images were obtained on a General Electric Signa HDxt 1.5-T scanner at Toronto General Hospital in Toronto, Canada. The scanning parameters for the 3D T1-weighted fast spoiled gradient echo sequence were as follows: slice thickness = 1 mm, repetition time = 10.74 ms, echo time = 4.20 msec, inversion time = 450 msec, matrix size = 256 mm \times 256 mm, field of view = 220 mm, flip angle = 15°, voxel size = 0.86 \times 0.85 \times 1 mm³, scan duration = 15 min 03 s. There was a total of 146 slices produced in the axial plane.

Reconstruction of patient cortical tissue was preformed using the Freesurfer (v6.0) imaging analysis suite, which is freely available online

(<https://surfer.nmr.mgh.harvard.edu/>). The technical details of the procedures are described in prior publications (Dale et al., 1999; Fischl et al., 1999). Given that patients were scanned at two separate time points, images were automatically processed using the longitudinal stream of Freesurfer in order to extract reliable thickness estimates (Reuter et al., 2012). First, images were cross-sectionally processed for each timepoint using the default pipeline. Briefly, the preprocessing involves correcting for motion and magnetic inhomogeneities, removing non-brain tissue, registering the images to Talairach space and segmenting the white and gray matter tissues. The boundary between white matter and gray matter (i.e. the white surface) is first calculated and then inflated to determine the boundary between gray matter and cerebral spinal fluid (i.e. the pial surface). Once completed, all time points for a single patient are used to create an unbiased within-subject template for each patient, which is then run again through the Freesurfer pipeline described above. In the final step, the processed template is used to resample the individual time points, which further reduces variability across time. A priori regions using the Desikan-Killiany atlas were exported to SPSS for further analysis. These regions included: ACC (Freesurfer label: bilateral caudal and rostral ACC), dlPFC (Freesurfer label: bilateral superior frontal, rostral middle frontal and caudal middle frontal), vmPFC (Freesurfer label: bilateral medial orbitofrontal and lateral orbitofrontal) and hippocampus (Freesurfer label: bilateral hippocampus).

2.5. NEV isolation and protein quantification

The team at the National Institute on Aging analyzed all available plasma samples from the clinical study and conducted EV isolation and biomarker measurements blindly. Plasma samples were defrosted on ice and defibrinated by 30 min incubation with Thrombin (System Biosciences, Inc., Mountainview, CA) at room temperature adding protease and phosphatase inhibitors. A pellet containing total EVs was acquired after precipitation with Exoquick™ (System Biosciences, Inc., Mountainview, CA) for 1 h and centrifugation at 1500 \times g for 20 min at 4 °C. Total EVs were re-suspended in 0.5 mL of Ultra-pure distilled water with the manufacturer-recommended concentration of protease and phosphatase inhibitors. To immunocapture L1CAM + NEVs the suspension was incubated for 1 h at 4 °C with 4 μ g of mouse anti-human CD171 (L1CAM) biotinylated antibody (clone 5G3) (Thermo Scientific, Inc., Waltham, MA), followed by incubation with 25 μ L of Pierce™ Streptavidin Plus UltraLink™ Resin (Thermo Scientific, Inc., Waltham, MA) for 30 min at 4 °C. After centrifugation at 800 \times g for 10 min at 4 °C and removal of supernatant, NEVs were eluted with 200 μ L of 0.1 M glycine. Then, beads were sedimented by centrifugation at 4500 \times g for 5 min at 4 °C and the supernatants containing NEVs were transferred to clean tubes. pH was neutralized with 1 M tris-HCl, and samples underwent 2 freeze thaw cycles with M-PER™ protein extraction reagent (Thermo Scientific, Inc., Waltham, MA) supplemented with protease and phosphatase inhibitors. The final suspensions containing NEV proteins were stored at -80 °C. Samples were thawed and vortexed twice prior to protein measurements.

The isolation of EVs by immunoprecipitation has a low yield, perhaps due to competition for binding with non-specific epitopes in the complex plasma matrix. This difficulty can be addressed with concentrating the EVs and removing the greater part of soluble material prior to immunoprecipitation. Our protocol takes this into consideration by combining two EV isolation steps. First, total EVs in plasma are concentrated using ExoQuick® (System Biosciences), a proprietary polymer solution that allows the sedimentation of EVs from low volumes of plasma and other biofluids. This step provides a high-yield/low-purity EV preparation that is then subjected to immunoprecipitation targeting the neuronal-specific marker L1CAM. This two-step immunoprecipitation protocol results in the isolation of nanoparticles from human plasma showing a size range and morphology compatible with EVs as showed by nanoparticle tracking analysis and electron microscopy (Figs. 2 and 3 in Mustapic

et al., 2017; Supplementary figs. 1 and 2 in Kapogiannis, Mustapic, 2019b; Supplementary fig. 1 in Nogueras-Ortiz et al., 2020), and a 3-fold enrichment for the neuronal-specific markers L1CAM, enolase-2 and neurofilament light compared to non-specific EVs immunoprecipitated targeting the general EV marker CD81 and EV-depleted plasma (Fig. 4 in Mustapic et al., 2017; Supplementary fig 4 in Kapogiannis, Mustapic, 2019b; Supplementary fig. 1 in Nogueras-Ortiz et al., 2020).

We quantified phosphorylated IRS-1 (S312) (cat no. K150HLD), ERK1/2 (T202/Y204; T185/Y187), JNK (T183/Y185), p38 (T180/Y182) (MAP kinase phosphoprotein panel, cat no. K15101D), Akt (S473), GSK-3 β (S9), and p70S6K (T421/S424) (Akt signaling panel, cat no. K15115D), using MESO SCALE DISCOVERY[®] (MSD) electrochemiluminescence plate assays. Plates were read using a MESO QuickPlex SQ120 imager and the Workbench Software 4.0 (Meso Scale Discovery, Rockville, MD). We also quantified Alix (or else human programmed cell death 6-interacting protein (PDCD6IP) (cat no. CSB-EL017673HU) (Cusabio Biotech Co., LTD, Houston, TX), an established EV marker enriched in exosomes (Kowal et al., 2016), to assess differential NEV yield. The detection of both transmembrane and intra-vesicular EV markers, as well as the absence of negative EV markers and markers of peripheral contamination, are required to confirm the sequential purification of EVs from neat plasma using ExoQuick[®] sedimentation of total EVs followed by immunoprecipitation of neuronal EVs. EV purification of immunoprecipitated EVs has been previously confirmed by western blots showing enrichment for transmembrane and intra-vesicular EV markers (i.e. CD9, CD81 and alix), low levels of apolipoprotein A1 and absence of the Golgi-specific negative EV marker GM-130, in comparison with EV-depleted plasma and total EVs (Supplementary figure 1c in Nogueras-Ortiz, Mahairaki, 2020); Fig. 2 in Mustapic et al., 2017; Supplementary fig. 3 in Kapogiannis, Mustapic, 2019b).

Alix plates were read using the Synergy[™] H1 microplate reader set to 450 nm and the Gen5[™] microplate data collection software (BioTek Instruments, Winooski, VT). The optimum dilution for each assay was determined using serial dilutions of test samples. For Alix, lysed NEVs were diluted 1:4 with the supplied sample diluent. No other assay required sample dilution. For Alix, the concentration was determined using a standard curve separately for each plate using standards provided by the manufacturer and the four-parameter logistic regression curve-fit. For MSD phospho-protein assays, a standard curve could not be constructed and thus we analyzed the electrochemiluminescence signal.

All assays were conducted in duplicate and the calculated quality control measurements are compiled in Supplementary Table 1. Duplicate NEV isolates from a healthy participant were also included as internal control (IC) on every plate to assess between-plate variability. The IC was used to determine a correction factor (IC signal for a given plate divided by the average of IC signals in all plates), which was used to normalize raw signals from each plate.

The limit of detection (LOD), defined as mean of the blank plus 2.5 the standard deviation (SD) of the blank, was calculated from electrochemiluminescence signal for MSD phospho-assays and colorimetric signal for the Alix ELISA. The lowest limit of quantification (LLOQ) (defined as the concentration of the standard with i) signal above the LOD, ii) CV among duplicates < 20%, and iii) recovery > 80% and <120%), was calculated for each plate for Alix assays, and the mean LLOQ was used as the global LLOQ.

Samples that had mean CV \geq 20% were excluded from the analysis independent of their levels. Additional censoring criteria consisted of LLOQs for assays that had standard curve. For Alix, all samples were above the LLOQ and within the linear range of the standard curve. For MSD phospho-assays, given that no standard curve was available, we excluded samples below the LOD.

2.6. Characterization of NEVs by immunoblotting and nanoparticle tracking analysis

Sequential EV purification was confirmed by assessing positive and negative EV protein markers in EV-depleted plasma, total EV isolates and immunoprecipitated NEVs using immunoblotting. The protein concentration was determined using the Bradford protein assay (Biorad, Hercules, CA). Ten μ g of total protein per sample was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% bis-tris gels in MOPS SDS running buffer (NuPAGE[®] Novex[®] SDS-PAGE system; Thermo Fisher Scientific) and transferred to polyvinylidene fluoride membranes (iBlot[®] 2 gel transfer system; Thermo Fisher Scientific). Membranes were blocked using the Odyssey[®] blocking buffer in TBS (Licor Biosciences, Lincoln, NE) for 1 h at RT and incubated overnight at 4 °C with fluorescently labelled antibodies (IRDye[®] 680RD antibody infrared dye; Licor Biosciences) targeting the cis-golgi marker GM-130 (0.2 μ g/mL; RRID: AB_880266, cat. no. 52649, Abcam, Cambridge, MA) used as a negative EV marker, and the membrane and intra-vesicular positive EV markers CD81 (1:500; cat. no. EXOAB-CD81A-1, System Biosciences) and alix (0.4 μ g/mL; RRID: AB_11023702, cat. no. NBP1-90201, Novus Biologicals, Littleton, CO), respectively. Enrichment of NEVs by L1CAM/CD171 immunoprecipitation was confirmed using the neuron-specific anti- β -III-tubulin antibody (RRID: AB_444319, cat. no. 18207, Abcam, Cambridge, MA). Antibody excess was washed five times with tris buffer saline supplemented with 0.05% tween-20 detergent for 5 min and blots were scanned using the Odyssey[®] CLx imaging system (Licor Biosciences). Ten μ L of intact EVs were used for determination of particle concentration and diameter using nanoparticle tracking analysis (NTA) (Nanosight NS500; Malvern, Amesbury, UK).

2.7. Statistical analysis

To evaluate between-group differences in demographic baseline characteristics, non-parametric (i.e. Mann-Whitney U) and Chi-square tests were used. A two-tailed alpha value of 0.05 was used to denote statistical significance. The Kolmogorov–Smirnov test was used to determine normality. Biomarker values were natural log transformed to minimize skewness. Principal components analysis (PCA) was used as a data reduction strategy. We used varimax rotation with Kaiser Normalization and determined factors by Eigen values > 1; only individual variable contributions of >0.5 qualified for loading a component. The resulting Bartlett factor scores were subsequently entered into the models.

An intent-to-treat analysis (i.e., all participants who were randomized) was used to analyze changes over time of factors and individual biomarkers levels. Due to the non-normal distribution of biomarkers and clinical outcomes, generalized estimating equation (GEE) models were used. For analysis with factors and MRI measures as outcomes, linear distributions were used. For individual biomarkers as outcomes, best fit was found with gamma distribution with log link specification and an independent covariance structure. For analyses with MADRS and DSST as the outcome, negative binomial models with log link specification and autoregressive covariance structure (AR-1) were selected. The independent variables were treatment group (i.e. infliximab vs. placebo), time (as a categorical variable), and group \times time interaction. For non-linear models, the estimated β coefficients were transformed into rate ratio (RR) estimates. The concentration of Alix, a commonly used canonical EV marker, was included as covariate in all analyses that involve biomarkers to normalize for differential EV yield between samples. Similarly, all analysis with MRI measures included adjustment for intracranial volume (ICV). As there were differences in MRI measures between treatment groups at baseline (e.g. hippocampus: $p < 0.001$), baseline values were included in the longitudinal models. To assess mediation, we used the PROCESS macro (Hayes, 2013) in SPSS v24.0 (IBM Corp., 2013). Bootstrapping with 5000 resamples was performed

to determine bias-corrected (asymmetric) 95% confidence intervals for indirect effects (Preacher and Hayes, 2008). No interim analysis was conducted.

3. Results

3.1. Demographics and clinical characteristics

A total of 55 participants were randomized and were included in the analysis herein. Baseline sociodemographic and clinical characteristics of the intent-to-treat population are described in [Supplementary Table 2](#). There were no statistically significant demographic or clinical differences between groups. Twenty-four of 27 infliximab-randomized participants (88.8%) and 23 of 28 placebo-randomized participants (85.7%) received all three infusions. A total of 43 participants (78.2%) completed all 12 weeks; differences in study completion rates between treatment groups in this subsample were not statistically significant ($p = 0.469$). There was no statistically significant (all p s > 0.1), differences in biomarkers at baseline between subjects in the infliximab and placebo groups.

3.2. Characterization of plasma-derived EVs

According to established criteria (They et al., 2018), we characterized our EV preparations by determining the size and concentration of isolated EVs by Nanoparticle Tracking Analysis (NTA) and immunoblotting of positive and negative EV markers ([Supplementary Figure 1](#)). EV purification was confirmed by western blots showing enrichment for transmembrane and intravesicular EV markers (i.e., CD81 and alix), and absence of the Golgi-specific negative EV marker GM-130, in total EVs and NEVs compared to EV-depleted plasma ([Supplementary Fig. 1a-c](#)). A significant increase of β -III-tubulin levels in NEVs compared to total EVs and EV-depleted plasma confirmed the enrichment of NEVs by L1CAM/CD171 immunoprecipitation ([Supplementary Fig. 1d and e](#)). The size distribution of nanoparticles immunocaptured by L1CAM was consistent with a mixed EV population likely predominated by exosomes (50–100 nm) and microvesicles (100–200 nm) ([Supplementary Figure 1f](#)).

3.3. Baseline associations between NEV biomarkers, ROI volumes and clinical variables

PCA revealed two components. Factor 1 comprised of higher loadings for pAkt, pGSK-3 β and pp70S6K, and was termed “canonical pathway” (CP) factor. Factor 2 comprised of higher loadings for IRS-1 pS312, pERK1/2, pJNK and pp38-MAPK, and was termed “alternative pathway” (AP) factor ([Supplementary Table 3](#)).

Neither CP nor AP factor scores were significantly associated with any of the demographic variables at baseline. Antidiabetic, antipsychotic, antidepressant, lithium, and mood stabilizer use were not significantly associated with CP scores ($p = 0.304, 0.677, 0.076, 0.796, 0.974$, respectively) or AP scores ($p = 0.702, 0.556, 0.068, 0.312, 0.730$, respectively). Smoking and BMI were also not associated with CP ($p = 0.155, 0.651$, respectively) or AP ($p = 0.369, 0.994$, respectively) scores.

Baseline CP factor score, after adjustment for relevant confounders, was inversely associated with vmPFC volume ([Table 1](#)). The association between CP factor score and vmPFC volume remained significant following further adjustment for serum TNF- α ($\beta = -997.75, p = 0.013$) and HOMA-IR ($\beta = -909.05, p = 0.025$). All individual NEV biomarkers that comprised the CP factor were associated with vmPFC volumes (pAkt: $\beta = -1302.97, p < 0.001$; pGSK3 β : $\beta = -1205.62, p = 0.001$; p70S6K: $\beta = -1132.99, p = 0.002$).

The AP factor was associated with DSST total scores and plasma TNF- α , as well as vmPFC and hippocampal volumes. The association between the AP factor and DSST total scores and vmPFC volumes remained significant following further adjustment for serum TNF- α ($\beta = -0.051, p =$

Table 1

Association between neuronal origin-enriched extracellular vesicles (NEV) biomarkers factors, clinical and magnetic resonance imaging (MRI) measures, at baseline (single model for each outcome).

	CP		AP	
	β	p-value	β	p-value
MADRS ^a	0.194	0.870	-0.808	0.363
DSST ^b	0.029	0.440	-0.051	0.039
HOMA-IR ^a	0.241	0.196	0.172	0.195
TNF- α ^a	0.005	0.931	-0.109	0.016
dIPFC ^c	-2218.81	0.098	-836.06	0.330
vmPFC ^c	-929.74	0.019	-611.00	0.020
ACC ^c	53.83	0.841	337.94	0.058
Hippocampus ^c	-97.75	0.587	-220.04	0.045

CP = insulin canonical pathway factor (pAkt, pGSK3B, p70S6K).

AP = insulin alternative pathway factor (pS312-IRS-1, pERK1/2, pJNK, pp38-MAPK).

MADRS: Montgomery-Asberg Depression Rating Scale; DSST: Digit Symbol Substitution Test; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; TNF- α : tumor necrosis factor-alpha; dIPFC: dorsolateral prefrontal cortex; vmPFC: ventromedial prefrontal cortex; ACC: anterior cingulate cortex.

^a adjusted for age, sex and Alix concentration.

^b adjusted for age, sex, education and Alix concentration.

^c adjusted for age, sex, ICV and Alix concentration.

0.025, $\beta = -548.67, p = 0.046$, respectively) and HOMA-IR ($\beta = -0.050, p = 0.029$; $\beta = -808.96, p = 0.004$, respectively). The association between the AP factor and hippocampal volumes remained significant following further adjustment for HOMA-IR ($\beta = -139.77, p = 0.049$), but not serum TNF- α ($\beta = -184.92, p = 0.095$).

Of the individual NEV biomarkers, only pS312-IRS-1 was associated with DSST scores ($\beta = -0.053, p = 0.034$) and hippocampal volume ($\beta = -198.67, p = 0.014$). For TNF- α , we observed significant effects of pS312-IRS-1 ($\beta = -0.088, p = 0.010$) and pERK1/2 ($\beta = -0.144, p = 0.039$), whereas pS312-IRS-1 ($\beta = -569.83, p = 0.008$), pERK1/2 ($\beta = -1403.31, p < 0.001$) and pJNK ($\beta = -1133.80, p = 0.004$) significantly were associated with vmPFC volume.

Given that, at baseline, vmPFC volumes was significantly associated with DSST total scores ($r = 0.523, p = 0.005$), we then tested if vmPFC volumes mediated the association between NEV pS312-IRS-1 and DSST total scores (there was no association between hippocampal volumes and DSST total scores, $r = 0.268, p = 0.177$). Mediation analysis indicated that the relationship between NEV pS312-IRS-1 and DSST total score was fully mediated by vmPFC volume ([Fig. 1](#)). The 95% CI of the indirect effect of NEV pS312-IRS-1 on DSST total score through vmPFC volumes did not cross zero, indicating significant mediation. There was no exposure-mediator interaction ($p = 0.441$). The overall model, adjusted for relevant covariates, was significant ($F_{2,22} = 11.373, R^2 = 0.439, p < 0.001$).

3.4. Effects of treatment with infliximab on NEV biomarkers

After adjustment for age, sex and Alix concentration, we observed a significant treatment by time interaction for the AP factor ($\chi^2 = 8.437, df = 3, p = 0.038$), but not the CP factor ($\chi^2 = 1.712, df = 3, p = 0.634$). There was a significant increase in AP factor scores in infliximab-treated patients, relative to placebo, at week 6 ($\beta = 0.230, df = 1, p = 0.025$), but not at week 2 ($\beta = 0.078, df = 1, p = 0.306$) or week 12 ($\beta = 0.063, df = 1, p = 0.614$). There was no moderation by serum TNF- α (treatment by time by serum TNF- α interaction: $\chi^2 = 5.074, df = 3, p = 0.166$), or by HOMA-IR (treatment by time by HOMA-IR interaction: $\chi^2 = 2.127, df = 1, p = 0.145$), indicating that changes in CP factor scores were independent of changes in these parameters.

As for individual biomarkers that comprised the IAP factor, there were significant treatment by time interactions for pERK1/2 ($\chi^2 = 10.103, df = 3, p = 0.018$), pJNK ($\chi^2 = 9.018, df = 3, p = 0.029$) and pp38-MAPK ($\chi^2 = 8.253, df = 3, p = 0.041$). There were no significant

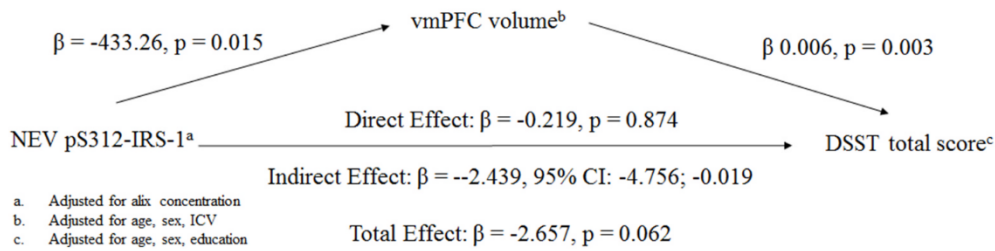


Fig. 1. Unstandardized regression coefficients from a bootstrap-mediation analysis indicating that bilateral ventromedial prefrontal cortex (vmPFC) volumes fully mediated the effects of neuronal origin-enriched extracellular vesicles (NEV) pS312-IRS-1 levels on Digit Symbol Substitution Test (DSST) total scores, at baseline.

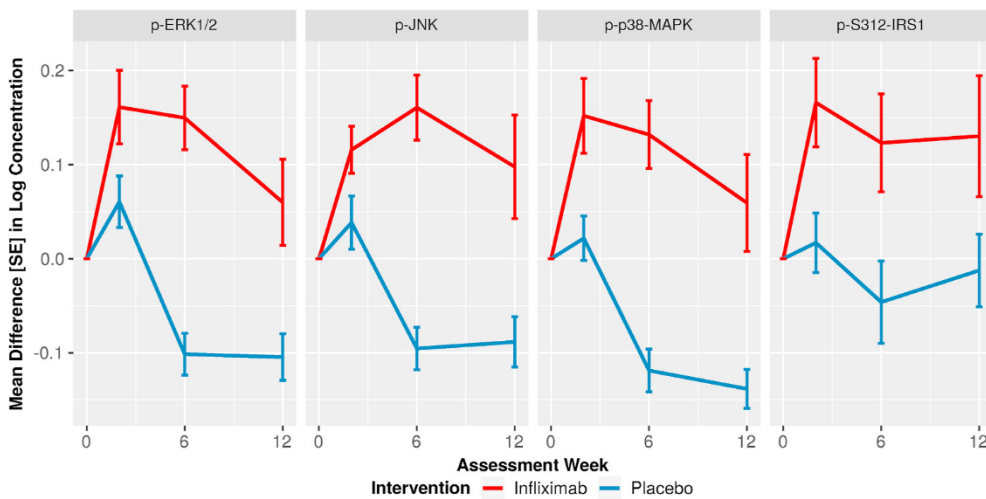


Fig. 2. Least squares mean changes in neuronal origin-enriched extracellular vesicles (NEV) biomarker levels comprising the alternative insulin signaling pathway, from baseline to week 12 in infliximab- and placebo-treated individuals with bipolar disorder. Error bars indicate standard errors. p-ERK1/2: phosphorylated extracellular signal-regulated protein kinases 1 and 2; p-JNK: phosphorylated c-Jun N-terminal kinase; p-p38-MAPK: phosphorylated p38 mitogen-activated protein kinase; pS312-IRS1: Serine-phosphorylated insulin receptor substrate-1; SE: standard error.

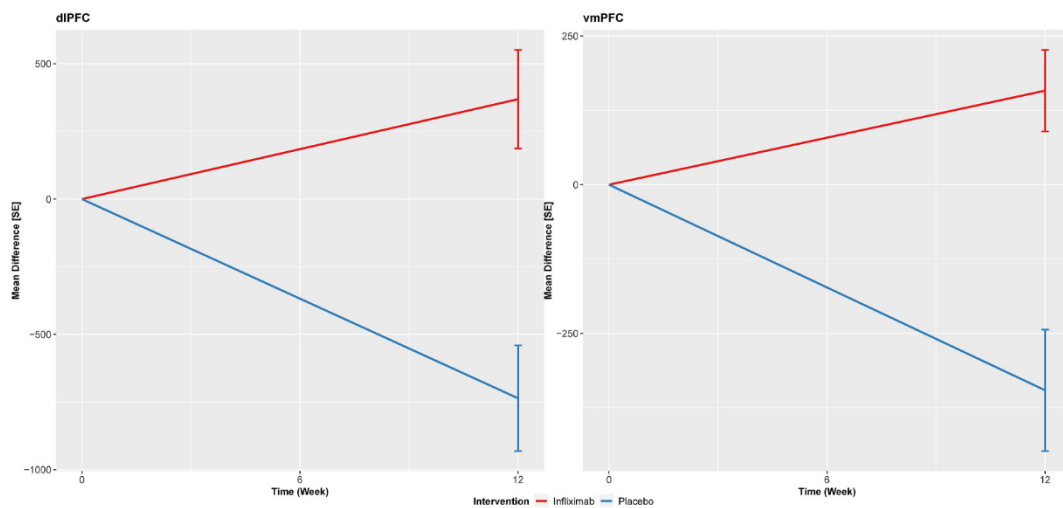


Fig. 3. Least squares mean changes in volume of regions of interest, from baseline to week 12 in infliximab- or placebo-treated individuals.

treatment by time interaction for pS312-IRS-1 ($\chi^2 = 2.855, df = 3, p = 0.414$).

There was a significant increase in pERK1/2 levels in infliximab-treated patients, relative to placebo-treated patients, at week 6 (RR = 1.047, $df = 1, p = 0.002$) and week 12 (RR = 1.043, $df = 1, p = 0.011$), but not week 2 (RR = 1.022, $df = 1, p = 0.140$). We observed similar patterns for pJNK (week 2: RR = 1.009, $df = 1, p = 0.423$; week 6: RR = 1.041, $df = 1, p = 0.005$; week 12: RR = 1.048, $df = 1, p = 0.012$), and pp38-MAPK (week 2: RR = 1.028, $df = 1, p = 0.068$; week 6: RR = 1.055, $df = 1, p = 0.005$; week 12: RR = 1.051, $df = 1, p = 0.015$) (Fig. 2).

3.5. NEV biomarkers and response to treatment

Treatment effects on depressive symptoms severity and cognitive function have been previously reported (Mansur et al., 2020; McIntyre et al., 2019). There was no moderating effect of changes in the CP ($\chi^2 = 5.313, df = 3, p = 0.150$) or AP ($\chi^2 = 3.725, df = 3, p = 0.293$) factors on depressive symptoms severity. As for the individual biomarkers that were shown to change over time, there were no significant time by treatment by biomarker interactions (all $ps > 0.1$). Nonetheless, at endpoint, infliximab responders had significant increases in pJNK levels, relative to infliximab non-responders ($p = 0.009$), placebo responders

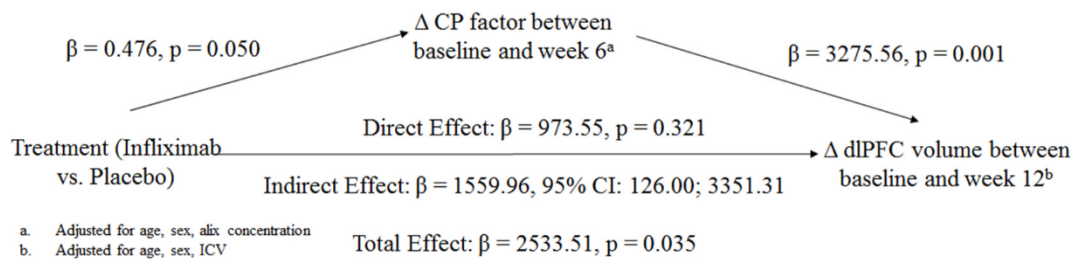


Fig. 4. Unstandardized regression coefficients from a bootstrap-mediation analysis indicating that changes in the insulin canonical pathway factor (CP) at week 6 fully mediated the effects of infliximab on dorsolateral prefrontal cortex (dlPFC) volumes at week 12.

($p = 0.029$) and placebo non-responders ($p = 0.003$) (Supplementary Fig. 1). For pERK1/2, significantly larger increases were observed in infliximab responders, relative to placebo non-responders ($p = 0.031$), but not infliximab non-responders ($p = 0.073$) or placebo responders ($p = 0.122$).

Moreover, after further adjustment for education and MADRS scores, we observed a significant time by treatment by pp38-MAPK interaction on DSST scores ($\chi^2 = 8.715$, $df = 2$, $p = 0.013$). In infliximab-treated patients, increases in pp38-MAPK levels were associated with increases in DSST scores, more strongly at week 12 (RR = 1.111, $df = 1$, $p = 0.011$), than week 2 (RR = 1.046, $df = 1$, $p = 0.084$) (Supplementary Fig. 2).

3.6. NEV biomarkers and neurostructural changes

We observed a significant treatment by time interaction for dlPFC ($\chi^2 = 4.408$, $df = 3$, $p = 0.036$) and vmPFC volumes ($\chi^2 = 4.467$, $df = 3$, $p = 0.035$), after adjustment for age, sex, ICV, and baseline volumes. Significant volumetric increases in the dlPFC and vmPFC were detected in infliximab-treated participants, relative to placebo-treated patients (Fig. 3). There was a trend characterizing an increase in hippocampal volume with infliximab vs. placebo over time ($\chi^2 = 3.623$, $df = 3$, $p = 0.057$), but no effect on ACC volume ($\chi^2 = 1.415$, $df = 3$, $p = 0.234$).

We assessed to what extent changes in NEV biomarkers mediated changes in brain volumes across multiple ROIs with infliximab (vs. placebo). Week-6 change in AP factor score was correlated with changes in dlPFC and vmPFC volumes at week 12 ($r = 0.476$, $p = 0.014$; $r = 0.471$, $p = 0.015$, respectively), whereas week-12 change in AP factor score was not ($r = 0.257$, $p = 0.226$; $r = 0.241$, $p = 0.257$, respectively). Change at neither week 6 nor 12 was associated with change in hippocampal volume ($r = 0.203$, $p = 0.319$, $r = 0.005$, $p = 0.981$).

We therefore used week-6 change in AP factor score, which were also when its scores were significantly different between treatments, in the mediation models. We observed that change in AP factor score at week 6 fully mediated the effects of infliximab on dlPFC volume at week 12 (Fig. 4). There was no exposure-mediator interaction ($p = 0.976$). The overall model, adjusted for relevant covariates, was significant ($F_{4,19} = 6.80$, $R^2 = 0.578$, $p = 0.001$). In contrast, week-6 change in AP factor score did not mediate change in vmPFC (indirect effect $\beta = 577.61$, 95% CI -6.511; 1438.41).

4. Discussion

Herein, we observed that biomarkers from the insulin signaling pathway, obtained from NEV, were associated with clinical and biological features of BD (i.e. cognitive dysfunction and neurostructural abnormalities). In addition, the current study suggests that treatment with the TNF- α infliximab engages the insulin cascade in neurons of individuals with BD, and results in neurostructural changes. The observation that NEV p-S312-IRS1 levels are associated with cognitive performance at baseline replicates findings from two previous studies (Kapogiannis et al., 2019b; Wijtenburg et al., 2019b). Moreover, a correlation between NEV biomarker and regional atrophy in Alzheimer's

disease has been reported (Mullins et al., 2017; Walker et al., 2020). Indeed, there has been increasing interest in the role of brain insulin signaling in cognitive function. Disrupted insulin signaling has been reported to influence pathways involved in neuroplasticity (Bosco et al., 2011; Trudeau et al., 2004). Imbalances in regulatory neurohormonal and related systems (i.e., pro-oxidant/anti-oxidant system, immune-inflammatory pathways) may alter pro-apoptotic intracellular signaling cascades thereby resulting in neuronal/glial loss and neuro-cognitive decline (Karunakaran and Park, 2013; Maritim et al., 2003; McIntyre et al., 2008; Reagan, 2012; Tran et al., 2012). The detrimental effects of insulin resistance on cognitive function has been consistently reported in clinical and non-clinical populations (Geijselaers et al., 2014; Gluck et al., 2013; Kenna et al., 2013; Nazaribadie et al., 2014; Samaras et al., 2014; Sanz et al., 2013; Yogi-Morren et al., 2014). In both adults with BD and offspring of parents with BD, comorbid T2DM, and/or overweight/obesity, were associated with decreased cognitive performance (Depp et al., 2014; Lackner et al., 2015; Mansur et al., 2019; McIntyre et al., 2017; Yim et al., 2012). The mediation analysis indicating that in individuals with BD NEV p-S312-IRS1 affects cognitive function through MRI measures reinforce the potential validity, and biological plausibility, of this finding. This is the first report of insulin signaling mediators in NEVs of individuals with BD, as surrogates of the state of the cascade in neurons, and the first to connect it with MRI measures and cognitive function in this population.

There were changes from baseline to endpoint in insulin signaling molecules, specifically from the alternative pathway (i.e. ERK1/2, JNK and p38-MAPK), following administration of infliximab, but not placebo. Previous studies have documented that infliximab treatment is associated with an increase in pERK1/2 and pp38-MAPK in diverse peripheral tissues (Petito et al., 2016; Roman et al., 2017; Waetzig et al., 2002). In contrast to our findings, TNF- α antagonism has been previously shown to reduce pJNK and p-S312-IRS1 levels (Araujo et al., 2007; Clemente et al., 2012; Stagakis et al., 2012), whereas we found an increase over time in these molecules. Previous NEV studies examining longitudinal changes in the insulin signaling pathway with experimental treatments showed that phosphorylation changes in downstream mediators of insulin signaling, albeit involving the canonical pathway, are consistently in the same direction, suggesting a co-ordinated change along the entire cascade (Athauda et al., 2019; Mustapic et al., 2019). Our present findings demonstrate that the same type of co-ordinated change may be occurring with interventions that primarily engage the alternative pathway. Such concerted changes may result from a combination of feed-forward mechanisms linking pERK1/2, pp38-MAPK and pJNK with each other and adaptive feed-back loops linking them with IRS-1 p-S312 (Athauda et al., 2019; Mustapic et al., 2019), suggesting that infliximab's engagement with these molecules lead to the activation of entire cascades rather than modification in individual molecules.

In both placebo- and infliximab-treated participants, activation of the insulin alternative pathway was associated with improvements in depressive symptoms severity. However, comparisons between responders and non-responders revealed differences in pJNK stimulation in the infliximab group. Interestingly, a moderating effect of change in a biomarker (i.e., pp38-MAPK) was detected on a cognitive outcome.

Therefore, infliximab's effects on the neuronal insulin alternative pathway could have had a neuroprotective effect. Indeed, treatment with infliximab resulted in volumetric increase of specific ROI s (i.e., vmPFC and dlPFC), and there was a temporal association between changes in NEV biomarkers of the alternative pathway and increases in dlPFC volume, suggesting a potential cause-effect relationship. Nonetheless, the clinical findings need to be interpreted with caution, given that there was no overall effect of treatment on depressive symptom severity and cognitive function, suggesting that these may be false positives and/or not causally related (Mansur et al., 2020; McIntyre et al., 2019).

Adding to the specificity of our findings, we did not find any significant association between infliximab and activation of the canonical pathway (i.e., pAkt, pGSK-3 β and pp70S6), suggesting dissociated effects on infliximab along the two arms of the cascade. In a previous study, we had seen the inverse dissociation, with mediators of the canonical pathway responding to an insulin sensitizing treatment (exenatide), but not those of the alternative pathway (Athauda et al., 2019). These two studies demonstrate the value of interrogating the state of intracellular cascades using NEV biomarkers depending on the particular treatment or physiological state involved. The finding itself was rather unexpected, since TNF- α has been shown to decrease phosphorylation of Akt (Hotamisligil et al., 1996). Previous preclinical studies have documented an increase in pAkt following treatment with infliximab (Araujo et al., 2007; Mendez-Garcia et al., 2018); similar results were reported by a study using peripheral blood mononuclear cells from human subjects with rheumatoid arthritis, albeit with a non-controlled design and a small sample size ($n = 7$) (Stagakis et al., 2012). Our data suggest that the canonical pathway is less likely to be involved in the neural effects of infliximab in BD, and that this agent might preferentially affect the alternative pathway.

This is the first study using NEVs harvested from peripheral blood as a source of biomarkers to investigate brain insulin signaling in individuals with BD. Accumulating evidence has demonstrated the utility and potential of this technique for the elucidation of pathophysiological mechanisms (Kapogiannis et al., 2015, 2019a; Mullins et al., 2017; Walker et al., 2020; Wijtenburg et al., 2019a) and target engagement in clinical trials, including of a protein restriction diet for prostate cancer (Eitan et al., 2016), exenatide for PD (Athauda et al., 2019) and intranasal insulin for AD (Mustapic et al., 2019). A conceptual limitation exists in that EV cargo is not identical to cell cargo since sorting mechanisms determine which molecules are loaded on EVs (Mathieu et al., 2019), a process that may depend upon the physiologic or disease state of the cell.

This study's strengths include the use of a therapeutic agent with a well-known biological target, a placebo comparator and a technique that allows for the indirect measurement of molecular mediators in neuronal cells, providing mechanistic evidence regarding the role of brain insulin signaling in the pathophysiology, and potentially in the treatment, of BD. Limitations include a relatively small sample size ($n = 55$), which makes the study unlikely to have been sufficiently powered to detect smaller effect sizes, and recruitment from a tertiary care clinic, which might limit its applicability to community samples. We also included participants receiving complex and mixed pharmacotherapy regimens; although we did not detect an association between specific agents and biomarkers levels, we could not completely rule out potential confounding effects. Limitations from the mediation analysis include that it is not sufficient, on its own, to determine a cause-effect relationship, nor it can rule out other potential causal models. Finally, this was a secondary, exploratory analysis, and results should be confirmed and replicated by subsequent studies.

Moreover, our approach to NEV isolation has some limitations. We used immunoprecipitation targeting L1CAM, which has been widely accepted as a neuronal marker suitable for positive selection of NEVs due to its high expression by brain neurons (e.g. see recent publications by multiple groups (Jia et al., 2019; Kapogiannis et al., 2019b; Pulliam

et al., 2019; Shi et al., 2016)); however it is also widely recognized that L1CAM is not exclusive to neurons (see <https://www.proteinatlas.org/ENSG00000198910-L1CAM/tissue>). Given that insulin cascade signaling is also not specific to neurons, the potential contribution of non-neuronal but L1CAM⁺ EVs to the isolated EV subpopulation raises the possibility that the effects of infliximab on biomarkers cannot be solely attributable to neurons.

In conclusion, the results of this NEV biomarker study based on a randomized, placebo-controlled clinical trial indicates that biomarkers of brain insulin resistance are associated with relevant clinical and biological features of BD. In addition, the concerted changes in NEV cargo and brain structure resulting from treatment with infliximab observed support the hypothesis that brain insulin signaling is a relevant pathophysiological mechanism, and potential target for interventions in BD. This is the first study using NEVs to investigate the brain insulin signaling pathways in neurons of living individuals with BD. The evidence described herein can inform disease models of neuropsychiatric disorders centered on and/or involving brain insulin signaling, in addition to providing further insights for potential therapeutic interventions.

Declaration of competing interest

Dr Mansur reported receiving funding from the Academic Scholar Awards, Department of Psychiatry, University of Toronto, outside the submitted work. Dr McIntyre reported receiving grants from Stanley Medical Research Institute during the conduct of the study; receiving grants from the Canadian Institutes of Health Research/Global Alliance for Chronic Diseases/Chinese National Natural Research Foundation outside the submitted work; and receiving speaking/consultation fees from Lundbeck, Janssen, Shire, Purdue, Pfizer, Otsuka, Allergan, Takeda, Neurocrine, Sunovion, and Minerva outside the submitted work. Ms Subramaniapillai reported receiving grants from Stanley Medical Research Institute during the conduct of the study. Dr Brietzke reported receiving personal fees from Daiichi Sankyo and Lundbeck and receiving grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico, São Paulo Research Foundation, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Southeastern Ontario Academic Medical Organization, Queen's University School of Medicine and L'Oreal forWomen in Science Award outside the submitted work. Dr Suppes reported in the last 36 months receiving grants from Stanley Medical Research Institute during the conduct of the study and from the National Institute on Drug Abuse, the National Institutes of Health, National Institute of Mental Health, Palo Alto Health Sciences, Pathway Genomics, and VA Cooperative Studies Program; receiving personal and nonfinancial support from CMEology, Global Medical Education, and Sunovion Pharmaceuticals Inc; and receiving personal fees from Allergan Inc, Hogrefe Publishing, Jones and Bartlett, Medscape Education, and UpToDate. Dr. Raison is director of Clinical and Translational Research for Usona Institute and serves as a consultant to Usona Institute, Emory Healthcare, Alkermes and Novartis, outside the submitted work. Dr. Fagiolini is /has been a consultant and/or a speaker and/or has received research grants from Allergan, Angelini, Apsen, Boehringer Ingelheim, Daiichi Sankyo Brasil Farmacêutica, Doc Generici, FB-Health, Italfarmaco, Janssen, Lundbeck, Mylan, Otsuka, Pfizer, Recordati, Sanofi Aventis, Sunovion, Vifor, outside the submitted work. The remaining authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jpsychires.2021.08.001>.

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Author statement

Drs. Mansur and Kapogiannis had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Concept and design: Mansur, Lee, Rosenblatt, Brietzke, Suppes, McIntyre, Kapogiannis.

Acquisition, analysis, or interpretation of clinical data: Mansur, Subramaniapillai, Lee, Iacobucci, Rodrigues, Cosgrove, Kramer, Suppes, McIntyre.

Acquisition, analysis, or interpretation of biomarker data: Mansur, Delgado-Peraza, Chawla, Nogueras-Ortiz, McIntyre, Kapogiannis.

Drafting of the manuscript: Mansur, Delgado-Peraza, McIntyre, Kapogiannis.

Critical revision of the manuscript for important intellectual content: Rosenblatt, Brietzke, Suppes, Raison, Fagiolini, Rasgon.

Statistical analysis: Mansur.

Obtained funding: Mansur, McIntyre, Kapogiannis.

Administrative, technical, or material support: Subramaniapillai, Lee, Cosgrove, McIntyre, Kapogiannis.

Supervision: McIntyre, Kapogiannis.

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