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Differences in Intracellular Protein Levels in Monocytes and CD4+ Lymphocytes between Bipolar Depressed Patients and Healthy Controls: a Pilot Study with Tyramine-Based Signal-Amplified Flow Cytometry

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Abstract:
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Background: Molecular biomarkers for bipolar disorder (BD) that distinguish it from other manifestations of depressive symptoms remain unknown. The aim of this study was to determine if a very sensitive tyramine-based signal-amplification technology for flow cytometry (*CellPrint*™) could facilitate the identification of cell-specific analyte expression profiles of peripheral blood cells for bipolar depression (BPD) versus healthy controls (HCs).

Methods: The diagnosis of psychiatric disorders was ascertained with Mini International Neuropsychiatric Interview for DSM-5. Expression levels for eighteen protein analytes previously shown to be related to bipolar disorder were assessed with *CellPrint*™ in $CD4^+$ T cells and monocytes of bipolar patients and HCs. Implementation of protein-protein interaction (PPI) network and pathway analysis was subsequently used to identify new analytes and pathways for subsequent interrogations.

Results: Fourteen drug-naïve or –free patients with bipolar I or II depression and 17 healthy controls (HCs) were enrolled. The most distinguishable changes in analyte expression based on T tests included GSK3β, HMGB1, IRS2, phospho-GSK3αβ, phospho-RELA, and TSPO in $CD4^+$ T cells and calmodulin, GSK3β, IRS2, and phospho-HS1 in monocytes. Subsequent PPI and pathway analysis indicated that prolactin, leptin, BDNF, and interleukin-3 signal pathways were significantly different between bipolar patients and HCs.

Limitation: The sample size of the study was small and 2 patients were on medications.

Conclusion: In this pilot study, *CellPrint*™ was able to detect differences in cell-specific protein levels between BPD patients and HCs. A subsequent study including samples from patients with BPD, major depressive disorder, and HCs is warranted.

Key words: bipolar disorder, healthy controls, flow cytometry, peripheral blood mononuclear cells, intracellular proteins.

Introduction

Bipolar disorder (BD) is a highly prevalent, chronic and debilitating mental disorder (Blanco et al., 2017; Ferrari et al., 2016; Merikangas et al, 2007). Its pathophysiology remains unclear, especially during the depressive phase of the illness (Harrison et al. 2018; Young and Juruena, 2021), which hinders the development of effective treatment for bipolar depression (BPD) (Gao et al., 2015a, 2015b). More importantly, patients with BD spend much of their lives in a depressed state when they are symptomatic (Judd et al., 2002, 2003) and commonly seek care when they are depressed. Seeking care during the depression period increases the risk for misdiagnosis of bipolar depression (BPD) as major depressive disorder (MDD) also known as unipolar depression (UPD) (Altamura et al., 2015; Calabrese et al., 2003; Hirschfeld et al., 2003; Shen H et al., 2018). Misdiagnosis of BPD as UPD often results in inappropriate treatment with antidepressants (Gao et al., 2010; Matza et al., 2005; Stensland et al., 2010) and misses opportunities of trying mood stabilizers. Incorrect diagnosis and treatment of BD has been shown to degrade clinical course, decrease quality of life, and impose substantial economic costs to patients and society (Calabrese et al., 2003; Hirschfeld et al., 2003; Shen et al., 2018; Matza et al., 2005; Stensland et al., 2008, 2010). Therefore, there is an urgent unmet need to find biomarker(s) for differentiating BPD from MDD and other psychiatric disorders.

The effort of searching for biomarkers to differentiate BD from other psychiatric disorders, especially from MDD, has been ongoing for decades at different levels (Amare et al., 2020; Ambrosi et al., 2017; Cardoso de Almeida and Phillips, 2013; Coleman et al., 2020; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Fitzgerald and Watson 2018; Han et al., 2019; Kelberman et al., 2021; Le-Niculescu et al., 2009; Matsuo et al., 2019; Misty et al., 2018; Mitelman, 2019; Yasin et al., 2021). Blood-based research has mainly used plasma/serum to study circulating miRNAs, inflammatory cytokines, oxidative proteins,

neurotransmitters, and/or metabolites of patients with BD, MDD, and other psychiatric disorders relative to healthy controls (HCs) (Carvalho et al., 2020; Comes et al., 2018; Coppens et al., 2020; Haenisch et al., 2016; Huang et al., 2021; Jacoby et al. 2016b; Pan et al., 2018; Poletti et al., 2020; Roy et al., 2020; Wollenhaupt-Aguiar et al., 2020). However, the results from those studies have not been replicated.

The overlap of genes and proteins in BD and MDD (Amare et al., 2020; Coleman et al., 2020; Comes et al., 2018; Coppens et al., 2020; Lago et al., 2020) and similar pathological processes involved in mitochondria and immune system in BD and MDD (Felger 2018; Pfaffenseller et al., 2014; Resende et al., 2020) make the differentiation even more challenging. However, some subtle differences, especially at post-transcription levels may play an important role in finding biomarkers that are able to differentiate these two disorders (Lago et al., 2020). Flow cytometry simultaneously measure multiple extracellular and intracellular proteins, including protein expression level and protein post-translational modifications, in individual cells (Blundell et al., 2021) and has become a powerful tool for clinical and research use in different specialties of medicine (Bank et al., 2015; Delmonte and Fleisher, 2019; McKinnon, 2018; Pillai and Dorfman 2016; Soma et al. 2016; Suo et al., 2020) as well as in psychiatry (Barbosa et al., 2013; Brietzke et al, 2009; do Prado et al., 2013; Guloksuz et al., 2010; Largo et al., 2020; Miklowitz et al., 2016; Wieck et al., 2013).

CellPrint™ is a signal amplification technology for flow cytometry. Signal amplification is accomplished by tyramine-based catalyzed reporter deposition. The technology overcomes a number of weakness of traditional flow cytometry and is capable of quantitative measurement of subtle differences in analyte expression, including protein phosphorylation, and low abundant proteins on or inside individual cells (Clutter et al., 2010; Gong et al., 2015; Kaplan 2003a,

2003b; Kaplan and Smith 2000; Kaplan et al., 2001a, 2001b, 2001c, 2003, 2005, 2013, 2014; Karkmann et al., 1999; Levandoska et al., 2003; Myerson et al., 2006). The *CellPrint*™ platform has been utilized in a wide array of studies and diseases, including a pilot study showing that lower expression levels at baseline of certain analytes in BD patients predicted lithium treatment response (Gao et al., 2022), and lithium responders and non-responders showed divergent changes in almost all 28 analytes after lithium treatment (Gao et al., 2023).

As the first step of testing the utility of *CellPrint*™ in differentiating BPD from UPD, we chose to compare multiple intracellular protein between patients with BPD and healthy controls (HCs) in the current study. Previous studies found differences between patients with BD and heathy controls (HCs) in a small number of proteins from blood samples, primarily from plasma or serum (Akimoto et at., 2006; Barbosa et al., 2013; de Sousa et al., 2015; Jacoby et al., 2016; Ladeira et al., 2013; Li et al., 2007; Li et al., 2010; Miklowitz et al., 2016; Polter et al., 2010). Interestingly, some serum and intracellular markers with significant differences between BD patients and HCs also had significant differences between BD and MDD (Akimoto et al., 2006; Miklowitz et al., 2016; Pandey et al., 2010). Moreover, several studies, indicated that intracellular markers may be more robust than plasma markers for differentiating bipolar patients from HCs and thus more likely to provide a much needed diagnostic to differentiate BD from HCs and other psychiatric disorders (Barbosa et al., 2013; Lago et al., 2020; Miklowitz et al., 2016). Nevertheless, to date, only one study solely included patients with BPD (de Sousa et al., 2015). Therefore, comparing BPD patients with HCs in the current study will set a foundation of using *CellPrint*™ in identifying intracellular biomarkers to differentiate BPD from UPD.

In addition, to provide a system-level interpretation of the analytes, we conducted protein-protein interaction (PPI) network analysis and pathway analysis. These analyses would also help

to identify additional potential molecules and pathways that may be important in distinguishing BD patients from HCs and/or patients with UPD.

Methods

Study design: This study was a cross-sectional study of drug-naïve (patients who never took a psychotropic at the time of blood collection) or drug-free (patients who did not take any psychotropic for at least 2 weeks before the blood collection) patients with bipolar I or II disorder in depressive phase versus HCs. Eighteen intracellular analytes in $CD4^+$ T cells and monocytes were analyzed with the *CellPrint*™ platform. The protocol was approved by the Institutional Review Board for Human Investigation of the University Hospitals Cleveland Medical Center. Written informed consent was obtained before beginning any study-specific procedures. Eligible subjects had one blood sample obtained. For medication-naïve patients, a medication(s) was started after obtaining the blood sample if a patient was willing to receive medication treatment. For medication-free patients, previous medication(s) was resumed or a new medication(s) was started after the collection of blood. The medication options were based on patient's previous treatment history and evidence-based treatment guidelines.

Participants

Healthy controls: As a healthy control, subjects must have met all of the following criteria: 1) Able to provide informed consent; 2) Male or female, at least 18 years of age; 3) Physically healthy**;** 4) No current and/or lifetime psychiatric disorder assessed with the MINI for DSM-5 (Mini Neuropsychiatric Interview for Diagnostic Statistical Manual edition-5) (Sheehan 2016); and 5) Willing to have blood drawn. Subjects with chronic medical conditions such as diabetes, coronary artery disease, immune diseases, infectious diseases and neurological disorders were not eligible. Subjects who had tested positive for illegal substances or

prescriptions medications for which they did not have a valid prescription or female patients with a positive pregnancy test were also excluded.

***Bipolar patients*:** The bipolar patients, in addition to being able to provide informed consent, male or female, at least 18 years of age, and willing to have blood drawn similar to the healthy controls, subjects must have met all of the following criteria: 1) Met current DSM-5 criteria for BP I or BP II disorder as assessed by the MINI for DSM-5; 2) Montgomery-Asberg Depression Rating Scale (MADRS) (Montgomery & Asberg 1979) total score ≥ 18 at screening visit/baseline evaluation and at the time of blood collection for those who were taking medication(s) and completed at least 2 weeks of washout period; 3) At least moderate impairment in work-, family-, or social-life measured with the Sheehan Disability Scale (SDS) (Leon et al., 1997) score of ≥ 4 in any of three subscales; 4) Had not taken any psychotropic medications within the past 2 weeks or were not responding to current medication(s) and willing and able to discontinue ongoing medication(s) for at least 2 weeks. For exclusion, like the heathy controls, bipolar subjects with chronic medical conditions, positive test for illegal substances or un-prescribed controlled medications, or current pregnancy were not eligible for the study. Patients with a substance use disorder within the last 3 months regardless of their medication status, rapid cycling course, current psychosis, or severe suicidal ideation judged by the study psychiatrist or suicide attempt in last 12 months were also not eligible. Patients who were taking a psychotropic medication(s), but lived alone or were unable to discontinue current medications were also excluded.

Procedures: After an IRB-approved informed consent form was executed, potential research participants were assessed by a research psychiatrist according to the inclusion and exclusion criteria. Psychiatric diagnoses were ascertained with extensive clinical interview (Gao

et al., 2008) and the MINI for DSM-5. Eligible subjects who were not taking psychotropic medications had a blood collection at the end of the first visit. Eligible subjects who were taking psychotropic medication(s) had their blood collection at the second visit, which took place when they washed out their medications for a minimum of 2 weeks. The speed of discontinuation of patients' medication(s) depended on the doses and half-life of medications the patients were taking. The study psychiatrist and research coordinator closely monitored their mood symptoms. Study staff contacted patients via phone at least once a week to assess their mood and mental status. If the symptoms of a patients got worse during the "washed out" period or "drug-free" period, an office visit with the study psychiatrist would be scheduled as needed and medication(s) might be resumed if clinically appropriate.

Assessments: The medical history including current and previous history of disease(s) related to all body systems was assessed with Cumulative Illness Rating Scale (Linn et al., 1968). Suicidality was assessed with Columbia Suicide-Severity Rating Scale (Posner et al 2007). Symptom severity was measured with Clinical Global Impression-Severity for Bipolar Disorder (Spearing et al., 2007) for overall bipolar illness severity; Montgomery Depression Rating Scale (MADRS) and 16 Quick Inventory of Depression Symptomatology-Self Report (Rush et al., 2003) for depression severity; Young Mania Rating Scale (Young et al., 1978) for manic symptoms severity; Hamilton anxiety score (Hamilton, 1959) for anxiety severity; and Snaith-Hamilton Pleasure Scale (Snaith et al., 1995) for feelings of anhedonia. Functional Impairment and Quality of Life were measured with Sheehan Disability Scale (Leon et al., 1997). Quality of Life Enjoyment and Satisfaction Questionnaire – Short Form (Endicott et al., 1991) was used to measure of the degree of enjoyment and satisfaction in various areas of daily living. A physical examination was conducted to ensure an overall physical health status.

Blood and urine sample collection: A fasting blood sample was obtained from all study subjects to measure fasting glucose, fasting insulin and fasting lipids, electrolytes, kidney and liver function, complete blood cell counts with differential, thyroid stimulating hormone, in addition to intracellular protein expression with *CellPrint*™. De-identified and coded blood samples were delivered to CellPrint Biotechnology within 3 hours of the venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll/hypaque discontinuous gradient centrifugation and cryopreserved for subsequent batch analysis. A urine sample was collected from all subjects for urine drug screen.

Antibodies and cytometric analyses

After sample accrual was completed, the frozen samples were thawed for cell-type specific molecular expression analysis with *CellPrint*™ flow cytometry. $CD4^+$ T cells and monocytes were identified using standard labeling procedures for flow cytometry as recommended by the antibody manufacturer (www.biolegend.com). *CellPrint*™ signal amplification was used to detect expression levels of specific intracellular analytes. The fundamental procedures of *CellPrint*™ signal amplification have been outlined previously (Kaplan, 2003a, 2003b, Kaplan and Smith, 2000; Kaplan et al., 2001a, 2001b). After *CellPrint*™ signal amplification, the median fluorescence intensities (MFI) were recorded for each of the analytes with a BD Accuri C6 flow cytometer. A median fluorescence ratio (MFR) was determined for each of the analytes by dividing the analyte MFI by a fluorescence minus one (FMO) negative control, which served to normalize the MFI for daily machine and technical variability. Cytometric analyses were accomplished by the technologists at CellPrint Biotechnology who were blind to the clinical status of the patients and blind to the purpose of the study.

Antibodies to 18 analytes related to BD in the literature were obtained from commercial sources, including BDNF (brain-derived neurotrophic factor), calmodulin (calcium-modulated protein), GSK3β (glycogen synthase kinase 3 beta), Fyn (a tyrosine kinase belongs to the Src family of tyrosine kinases including src, fyn, and yes), HMGB1 (high mobility group box 1 protein), Iba1 (Ionized calcium binding adaptor molecule 1), IRS2 (insulin receptor substrate 2), NLPR3 (NACHT, LRR and PYD domains-containing protein 3), NR3C1 (nuclear receptor subfamily 3, group C), phospho-Akt [phosphorylated Akt(Thr308)], phospho-Fyn/Yes [phosphorylated Fyn(Y530)/phosphorylated Yes(Y537)], phospho-GSK3αβ [phosphorylated Glycogen synthase kinase 3 alpha(Tyr279)beta(Tyr216)], phospho-HS1 [phosphorylated hematopoietic lineage cell-specific protein 1 (Tyr397)], phospho-RELA [phosphorylated nuclear factor NFκB p65(Ser536) subunit], phospho-Zap70 [phosphorylated ZAP70 (Tyr319)/Syk(Tyr352)], PPAR-γ (peroxisome proliferator-activated receptor gamma), TNFAIP3 (tumor necrosis factor, alpha-induced protein 3) and TSPO [(PBR) translocator protein]. The purchased antibodies were evaluated by the CellPrint Biotechnology team with its proprietary quality control methods. Only antibodies that passed the criteria were included in the study. The analysis interrogated a spectrum of pathways, kinases, and functions (Supplemental Table 1).

To test the reproducibility of analytes, a second analysis was conducted after propriety technical improvement. In the second analysis, eleven analytes in the first analysis (BDNF GSK3β, Fyn, IRS2, NLPR3, NR3C1, phospho-Akt, phospho-Fyn/Yes, phospho-RELA, TNFAIP3 and TSPO were included in addition to RELA and COMT (catechol-O-methyltransferase). The RELA and COMT were included in the first analysis.

Upon completing the flow cytometric analyses with *CellPrint*^{TM} amplification, the data were sent to the data management statistical analysis unit of the Mood Disorders Program. The

key to each subject (patient or healthy) was provided to the statistics team and used to assess any differences between the groups.

Rationale of Using Monocytes and $CD4^+$ lymphocytes

The rationale of using monocytes and $CD4^+$ lymphocytes in the current study was similar to our previous studies (Gao et al., 2022, 2023). The comparability of blood and brain and functional connections between brain and blood cells have been extensively investigated (Bei et al., 2009; Fiedorowicz et al., 2015; Liew et al., 2006; Rollins et al., 2010; Solmi et al., 2021; Spiliotaki et al., 2006; Sullivan et al., 2006; Tylee et al., 2013). Peripheral blood mononuclear cells (PBMC) have been used for diagnostic and pathologic studies of BD (Amoruso et al., 2015; Munkholm et al., 2015; So et al., 2007; Wieck et al., 2016]. Among the blood mononuclear cells, lymphocytes (Barbosa et al., 2014; Maecker et al., 2012; Milklowitz et al., 2016) and monocytes (Drexhage et al., 2011; Lambert et al., 2017; Milklowitz et al., 2016) have been extensively studied with flow cytometry for different purposes. Moreover, collection of blood sample is relatively easy and at low cost, and results from the PBMCs can be easily applied to routine clinical practice.

Raw data normalization

To normalize raw data, the fold change (FC) that was used to reflect the difference between patients and HCs was expressed as the ratio of averaged analyte MFR from HCs divided by patients and was calculated using MFR data with the equation:

$$\log_2\left(\frac{\textit{the average of MFR of healthy controls}}{\textit{the average of MFR of bipolar patients}}\right).$$

Therefore, a positive value of the $\log_2$(FC) is indicative of a higher expression level in HCs than in BPD patients, and a negative value of $\log_2$(FC) is indicative of a lower expression level in HCs than in BPD patients. Using $\log_2$ converts ratios below one to negative values and ratios

above one to positive values, making it easy to graphically visualize which analytes had decreased or increased expression between the groups. An analyte with a larger FC regardless of negative or positive indicates the analyte may have differential effect between patients and HCs.

Protein-to-protein interaction and pathway analyses

Network and pathway analyses of genomic, transcriptomic, and proteomic data have become essential to understand the psychopathology of complex psychiatric disorders (Kathuria et al., 2020; Oommen et al., 2021; Ziani et al., 2022; Zhang et al., 2021; Zuo et al., 2021;). Pathway analysis of proteomic data can directly interpret signal proteins in signal pathways, and network analysis of proteomic data can provide direct evidence of PPI among studied proteins (Wu et al., 2014). In the present study, for PPI network analysis, we used the FCs of all proteins in monocytes and $CD4^+$ lymphocytes and the BioGRID database, in which there are 8839 proteins and 67056 interactions (Oughtred et al., 2019), to generate PPI networks. Network-based analysis of diverse phenotypes demonstrate that the proteins that are implicated in similar phenotypes are clustered together in cellular networks (Zhou et al., 2014). In order to identify the highly connected modules (functional module) that are centered around the studied proteins, we used network propagation algorithms (Cowen et al., 2017) with the studied proteins as the seeds. For every pair of proteins in the study, the shortest path connecting the pair in the human PPI network was computed. The proteins on these paths were retained and the subnetworks of the PPI network that are induced by these proteins were utilized as functional modules for further analysis.

After the modules were identified, we performed pathway enrichment analysis on the induced subnetworks. For this purpose, we used a hypergeometric model to assess the

significance of the pathways in the Wiki Pathways dataset. Pathway enrichment analysis was conducted based on the Software of Emich (Chen et al. 2013).

Statistical Analysis

The categorical data were analyzed with Chi-square or Fisher Exact tests for any subgroup with a sample < 5, and continuous variables were analyzed by T-test. Demographics and historical correlates of patients and healthy controls were analyzed according to the nature of a variable. Patients who were not taking any medication at the screening time were identified as "drug-naïve". Patients who were taking a psychotropic medication(s) at the screening time and able to "wash out" their medication(s) for at least 2 weeks are labeled as "drug-free." The cytometric MFR data for the analytes in both monocyte and $CD4^+$ lymphocyte cell types between healthy controls and bipolar patients were analyzed with unpaired t-tests. As a pilot study, no adjustment for multiple comparisons was attempted. Also, the sample size calculation was not attempted because this was the first instance of using this technology in this population, and the nature of the study was exploratory and hypothesis-generating.

Results

Demographics

Fourteen patients (drug-naïve n=1, drug-free n=11, and 2 patients on medications) with BPD and 17 healthy subjects were included in the analysis. Among the drug-free patients, seven patients did not take any medication at the screening visit and four completed a washout period for at least 2 weeks. The two patients on medications were enrolled before the protocol was revised. Patients enrolled into the study were moderately depressed (Table 1). Compared to healthy controls, patients were less likely to be employed ($p \leq 0.05$) and had ≥4 year college education ($p \leq 0.05$), and more likely to be male ($p \leq 0.05$). However, the difference in age was

not significantly different although the subjects in the healthy control group were numerically younger.

Difference in protein expression levels between healthy controls and bipolar patients

One purpose of this pilot study was to implement the *CellPrint*™ platform as a hypothesis generating tool for BD diagnostics. Therefore, rather than adhering to strict p-value cutoffs for further study, we binned the differential expression levels into quartiles based on p-value and emphasized the lowest quartile which corresponds to the highest significance. Generally, analyte expression levels were lower in BPD patients than in HCs (Table 2). The differences in analyte expression with the lowest p-values (using quartiles as cutoffs) included GSK3β, HMGB1, IRS2, phospho-GSK3αβ, phospho-RELA, and TSPO in $CD4^+$ T cells, and calmodulin, GSK3β, IRS2, and phospho-HS1 in monocytes (Table 2). It is noteworthy that the two cell-types assessed, $CD4^+$ T cells and monocytes, showed overlapping but distinct differences in analyte expression profiles in the patient group. For instance, the difference between BPD patients and HCs in GSK3β was significantly different ($p < 0.01$) in monocytes, but only a trended significant in lymphocytes ($p=0.09$

After removing 2 patients who were on medications at the time of blood collection, the results in lymphocytes were similar to the first analysis, both protein levels of phospho-RELA and TSPO were still significant ($p \leq 0.05$) between patients and HCs. In monocytes, the protein levels of IRS2 and GSK3β remains significant ($p \leq 0.05$) between the groups, but the levels of phospho-HS1 and calmodulin became insignificant.

Of the 11 analytes included in the first and second analyses (Table 2), the pattern of differences in analytes between HCs and BPD were similar in both analyses, but a larger difference between patients and HCs in the second analysis in both lymphocytes and monocytes.

In lymphocytes, NLRP3 became significantly different between two groups in the second analysis (p=0.04) although the difference was not significantly different in the first analysis (Table 2). In monocytes, the differences in Fyn (p=0.04), phospho-Akt (p=0.02), and NLRP3 (p=0.05) also became significant in the second analysis.

Fold Change in Expression

To compare the magnitude of difference between the groups for each analyte, we calculated the fold change (FC) of each analyte from HCs versus BPD patients as described previously. The top quartile of FCs included calmodulin, GSK3β, HMGB1, NR3C1, and phospho-RELA in $CD4^+$ T cells, and calmodulin, GSK3β, phospho-HS1, and phospho-RELA in monocytes (Figure 1). The analytes with the largest FCs did not entirely overlap with expression level changes with the lowest p-values. For example, the p-value for the difference in calmodulin and NR3C1 in $CD4^+$ T cells and phospho-RELA in monocytes was not in the lowest quartile of p-values (Table 2) despite the fact that the FC for these analytes were among the largest. Likewise, some of the analytes with the lowest p-values did not have FCs that were in the top quartile. Examples include IRS2, phospho-GSK3αβ, and TSPO in $CD4^+$ T cells and IRS2 in monocytes (Figure 1 and Table 2).

Protein-protein interaction networks

The PPI network was explored with all proteins related to the 18 analytes in lymphocytes and monocytes. For analytes with modified (phosphorylated form) and unmodified forms, only the unmodified analytes/proteins were used for this analysis. Of the 18 analytes, Fyn and GSK3β had an unmodified form and one phosphorylated form so that 16 proteins were used to generate a PPI network in each cell type. Of the 16 proteins, 11 proteins were in the network of 41 proteins. The FC of each of 11 proteins between BPD patients and HCS was used to map the protein into

the network to reflect the magnitude of difference compared to other proteins in the network of lymphocytes (Figure 2A) and monocytes (Figure 2B) Akt, Calmodulin, Iba2, NLPR3, and TSPO were not in the network. In lymphocytes, only the difference in the phospho-RELA between the groups was significant (Table 2). In the monocytes, the differences between the groups in GSK3β and phospho-HS1 were significant (Table 2).

Pathway Enrichment Analysis

All 16 proteins in the study were used for functional pathway analysis. Pathway enrichment analyses found that genes coding GSK3β, IRS2, RELA, ZAP70, and FYN in the present study are involved in prolactin, leptin, BDNF, and interleukin-3 signal pathways that were significantly different between healthy controls and bipolar patients (Table 3). Among proteins analyzed in the study, only FYN was involved in all 4 pathways.

Overlap between PPI subnetworks in different signal pathways

Subnetwork PPI analyses were conducted similarly as the overall PPI network analysis. The proteins in the subnetwork were compared with proteins identified through the pathway enrichment analysis. We found that there were significant overlaps of studied proteins in the PPI subnetwork and proteins through functional pathway analysis (Figure 3). Among the studied proteins, only ZAP70 and Fyn were directly connected in the prolactin signal pathway (Figure 3B). Most studied proteins were indirectly connected to others in the same pathways through proteins that were not included in the study (Figure 3).

Discussion

The results presented in this pilot study suggest that the *CellPrint*™ technology is capable of detecting significant differences in some intracellular proteins in monocytes and $CD4^+$ lymphocytes between BPD patients and HCs (Table 2). Overall, the expression levels of proteins

measured in the current study were lower in patients with BPD than in HCs in both cell types (Figure 1). The differences in calmodulin, GSK3β, and phospho-RELA were the largest, suggesting that these 3 proteins should be considered for future studies to differentiate BPD patients from healthy people or patients with UPD. That a large number of proteins which were not included in the present study were recruited into the same pathways of studied proteins during the PPI subnetwork and pathway enrichment analyses suggests that there are additional proteins and pathways that may be probed to enhance the distinction between patients with BPD and HCs or patients with UPD.

BPD-specific changes in analyte levels measured in this study are consistent with results of some previous studies. In the current analysis, calmodulin was lower in monocytes of BPD patients. This result is consistent with a previous study which found that BD patients had dysregulation of the calmodulin system compared to HCs (Akimoto et al., 2006). TSPO and IRS2 were also found to be lower in BD patients. These result are consistent with the low density of TSPO in the platelet membrane of patients with BD and comorbid with separation anxiety disorder compared to HCs (Abelli et al., 2010), and brain or peripheral insulin resistance in patients with BD (Coello et al., 2019; Kemp et al., 2014; Mansur et al., 2021).

The result of GKS3β in our study was inconsistent with some previous studies. Two studies have found that GKS3β levels were similar in BD patients and HCs (de Sousa et al., 2015; Polter et al., 2010). One study only included BPD patients with moderate depressive symptom severity as the current study, but the levels of GKS3β were measured in platelets with enzyme immunoassay (de Sousa et al., 2015). Yet, in another study in bipolar inpatients with depression and/or manic/hypomanic symptoms who were medication-free for up to 2 weeks, GKS3β level was lower in patients with BD than in HCs (Pandey et al., 2010). The levels of

GKS3β in this study were measured in the cytosol and membrane of the platelets with the Western block technique. In contrast, patients with mania had significantly increased levels of GKS3β in peripheral blood mononuclear cells (PBMCs) compared to HCs (Li et al., 2010). Likely, the inconsistency among the studies might be due to study mood index, locations of tissues analyzed, and assay methodologies.

Also, the lower level of phospho-RELA in BD patients in our study (Table 2) was inconsistent with a previous study in pediatric patients with BD (Miklowitz et al., 2016). In this pediatric study, adolescents with bipolar disorder, type I, II, or not otherwise specified and depression and/or manic/hypomanic symptoms were included. A mean score on Children's Depression Rating Scale-Revised (CDRS-R) of 40.9 points and YMRS score of 11.4 points suggest that most of patients were depressed. In addition, a majority of patients if not all were on psychotropic medication(s). In contrast to our current study analyzing multiple proteins, this study only analyzed phospho-RELA in PBMCs, monocytes, and lymphocytes with single color flow cytometry. The differences in age, bipolar types, depression severity, mediation use, and type of flow cytometry between our study and the pediatric study might cause the inconsistency of the result in phospho-RELA.

Some previous studies of the molecular basis of BD have assessed serum levels of cytokines. However, serum cytokine levels have not yet been found to distinguish BD patients (Barbosa et al., 2013; Miklowitz et al., 2016). Therefore, it is important to continue the search for analyte expression unique to BPD. One important aspect of the current study was the analysis of intracellular analytes. Intracellular analytes are notoriously difficult to assess by flow cytometry due to issues caused by low abundance and poor signal to noise. *CellPrint*™ enhances the ability to study intracellular analytes by improving signal to noise. Thus, the assessment of cells from

BD patients with *CellPrint*™ may provide new opportunities for biomarker discovery based on expression signatures of intracellular analytes. That intracellular markers were more robust than plasma markers in differentiating bipolar patients from HCs (Barbosa et al., 2013; Miklowitz et al., 2016) support this speculation.

The PPI network and subnetwork and pathway enrichment analyses suggested that in addition to the proteins measured in the current study, there are many proteins and pathways that may have differences between patients with BPD and HCs (Table 3, Figure 2 and 3). PIK3R1 (phosphatidylinositol 3-kinase receptor 1), STAT3 (signal transducer and activation of transcription 3), CRB2 (Crumbs homolog-2), SHC1 (SHC-transforming protein 1), PTPN11 (protein tyrosine phosphatase non-receptor type 11), and RAF1 (Raf proto-oncogene, serine/threonine kinase) were also in all 4 pathways (Table 3, Figure 3), but were not included in the current study. These proteins may be considered for future studies.

Both RELA and GSK3β are directly involved in some network/pathways and their levels are significantly different between patients and HCs in at least one cell type (Table 2, Figure 1 & 3). Therefore, both proteins should be considered in the future studies of BPD. In contrast, Fyn is involved in multiple networks and pathways (Figure 3), but the differences in the levels of Fyn protein expression in both cell types between patients and HCs were small and not significant (Table 2, Figure 1). Therefore, using the Fyn protein levels in monocytes and lymphocytes to differentiate bipolar patients from healthy controls may not be productive.

Although the primary aim of this study was to determine if the enhanced sensitivity provided by *CellPrint*™ analysis could help detect differences between BPD patients and HCs, our ultimate goal is to study the utility of *CellPrint*™ in differentiating BPD from UPD. As mentioned in the Introduction, BPD patients are commonly misdiagnosed and mistreated as UPD

(Calabrese et al., 2003; Cegla-Scvartzman et al., 2021; Hirschfeld et al., 2003; Gao et al., 2010; Shen et al., 2018; Stensland et al., 2010). Along with the results from previous studies including UPD patients, bipolar patients and HCs (Akimoto et al., 2006; Miklowitz et al., 2016; Pandey et al., 2010), the results from the current study suggest that enhanced flow cytometry like *CellPrint*™ may be able to detect significant differences in multiple intracellular proteins in monocytes and lymphocytes between BPD and UPD. The differences in analytes between BPD patients and HCs in different cell types (Table 2 and Figure 1) support the notion that molecular biomarkers for BPD may be generated by using cell-specific composite biomarkers. These findings provide a conceptual and experimental foundation for future studies designed to apply *CellPrint*™ to compare cell-specific intracellular analyte levels between BPD and UPD. The results from our second analysis suggest that propriety technological improvement of *CellPrint*™ can further increase the sensitivity of detecting differences in multiple intracellular proteins between comparison groups. Although more studies with large sample sizes are necessary to confirm and expand the preliminary findings in the current study, enhanced flow cytometry is likely to help the field in searching biomarkers that differentiate BD from UPD and/or other psychiatric disorders.

Limitations

First, the sample size of this study is small and we did not adjust significant level for multiple comparisons. Since we were not intent to make any inference, all significant results should be viewed as preliminary and hypothesis-generating, and only be considered for design of future studies. Second, we had two patients with ongoing medications and four were medication-free only for 2 or more weeks before their blood samples were collected. Since there is no consensus on how long medication(s) needs to be stopped before blood collection, different

studies used different medication-free durations before blood collection (de Sousa et al., 2015; Li et al., 2010; Miklowitz et al., 2016; Polter et al., 2010). Previously, global gene expression in leukocytes of patients with BD was observed after 1-2 weeks of lithium treatment and the expression levels of some genes did not return to normal at 2-weeks of post-lithium treatment (Kikuchi et al., 2019; Watanabe et al., 2014), suggesting that a 2-week medication-free duration before blood collection in our study might not be long enough for those who were on medication(s) at the screening visit although no patient was on lithium. However, a duration of washing out ongoing medications longer than 2 weeks without a protocol-defined treatment(s) may have ethical and legal challenges. In routine clinical practice, stopping all mediations, even just a week or two, for a diagnostic test is also not practical, especially for patients who presents severe risk to harm self or others. Future studies need include patients at the different mood states and different symptom severities to determine if identifiable biomarkers are trait, state, or both. If the biomarkers are trait, patients can be tested at any time without stopping their medication(s). Also, different studies were conducted in patients with different ongoing medications (Barbosa et al., 2013; Ladeira et al., 2013; Li et al., 2007; Miklowitz et al., 2016). Removing the 2 patients with ongoing medications in the current study did not totally change the results, but did affect the results of some proteins, suggesting that the potential interference of ongoing treatment(s) on the levels of proteins in the current and previous studies could occur. Moreover, different mood states at the time of blood collection could further complicate the comparison between our study and previous ones (Akimoto et at 2006; Barbosa et al., 2013; de Sousa et al., 2015; Jacoby et al., 2016; Ladeira et al., 2013; Li et al., 2007; Li et al., 2010; Miklowitz et al., 2016; Polter et al., 2010). Third, we included patients with bipolar I or II depression. It remains unclear how much impact the subtypes have on the difference between

BPD patients and HCs. However, patients with bipolar II disorder spend much more time in depression than those with bipolar I disorder (Judd et al., 2002, 2003). The inclusion of two types of bipolar depression may make the results more generalizable.

Conclusions

This study showed that tyramine-based signal-amplified flow cytometry *CellPrint*™ technology was able to detect significant differences in certain protein levels in PBMCs between BPD patients and HCs. A subsequent study including BPD, MDD, and HCs with a larger sample size is warranted. Proteins including GSK3β, phospho-RELA, calmodulin and TPSO should be considered candidates for potential diagnostic biomarkers of BPD.

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Legends to Figures

Figure 1. Log_2 (Fold Change) in analytes between healthy controls and bipolar patients in $CD4^+$ lymphocytes (brown bars) and monocytes (blue bars). A positive value of the FC is indicative of a higher expression level in healthy controls than in patients. A negative value of the FC is indicative of a lower expression level in healthy controls than in patients.

Abbreviation: BDNF: Brain-derived neurotrophic factor; **Calmodulin**: calcium-modulated protein; **Fyn**: a tyrosine kinase belongs to the Src family of tyrosine kinases including src, fyn, and yes; **GSK3β**: Glycogen synthase kinase 3 beta; **HMGB1**: High mobility group box 1 protein; **Iba1:** Ionized calcium binding adaptor molecule 1; **IRS2**: insulin receptor substrate 2; **NLPR3**: NACHT, LRR and PYD domains-containing protein 3; **NR3C1**: nuclear receptor subfamily 3, group C; **Phospho-Akt:** phosphorylated Akt(Thr308);): **phospho-Fyn/Yes**: phosphorylated Fyn(Y530)/phosphorylated Yes(Y537); **phospho-GSK3αβ**: phosphorylated Glycogen synthase kinase 3 alpha(Tyr279)beta(Tyr216); **phospho-HS1**: phosphorylated hematopoietic lineage cell-specific protein 1 (Tyr397); **phospho-RELA**: Phosphorylated nuclear factor NFκB p65(Ser536) subunit. **Phospho-Zap70:** Phosphorylated Zap70 (Tyr319)/Syk(Tyr352). **PPAR-γ**: Peroxisome proliferator-activated receptor gamma; **TNFAIP3:** Tumor necrosis factor, alpha-induced protein 3: **TSPO (PBR)**: translocator protein.

Figure 2. PPI Network of 11 Proteins in the study that are mapped to BioGRID PPI in lymphocytes (Figure 2A) and monocytes (Figure 2B). **Note:** The color of the nodes represents the $\log_2$ (fold change) of the healthy controls versus patients in lymphocytes. The white nodes are not measured in the study but they are on the shortest paths between pairs of proteins that are included in the study.

Abbreviation: **BDNF**: Brain-derived neurotrophic factor; **Fyn**: a tyrosine kinase belongs to the Src family of tyrosine kinases including src, fyn, and yes**; GSK3B**: Glycogen synthase kinase 3 beta; **HMGB1**: High mobility group box 1 protein; **IRS2**: insulin receptor substrate 2; **NR3C1**: nuclear receptor subfamily 3, group C: **HCLS1**: hematopoietic lineage cell-specific protein 1; **RELA**: nuclear factor NFκB p65 subunit. **PPARG**: Peroxisome proliferator-activated receptor gamma; **TNFAIP3:** Tumor necrosis factor, alpha-induced protein 3: **ZAP70:** a zeta-chain-associated protein kinase.

Figure 3. Protein-protein subnetworks in BDNF, Prolactin, Leptin, and Interleukin-3 signal pathways based on the 11 studied proteins

Abbreviation: **BDNF**: Brain-derived neurotrophic factor; **Fyn**: a tyrosine kinase belongs to the Src family of tyrosine kinases including src, fyn, and yes**; GSK3B**: Glycogen synthase kinase 3 beta; **IRS2**: insulin receptor substrate 2; **RELA**: nuclear factor NFκB p65 subunit. **ZAP70:** a zeta-chain-associated protein kinase.

Figure 1

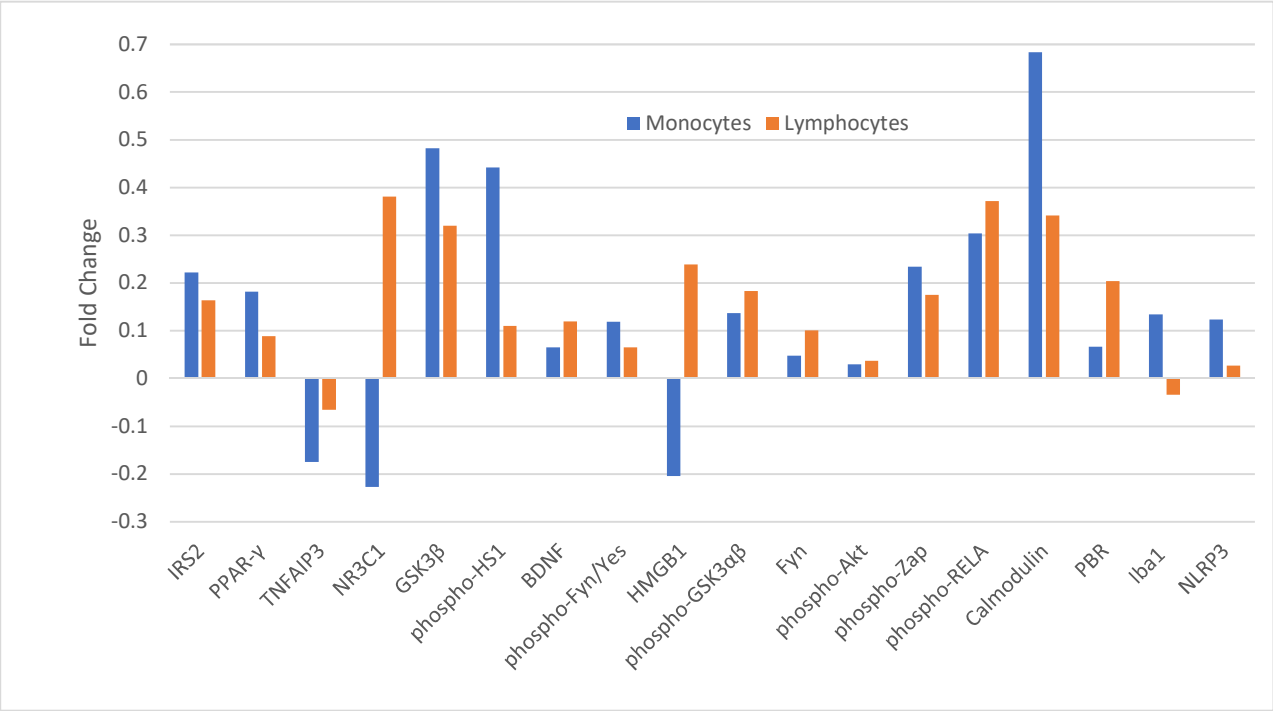
Monocytes
Lymphocytes
Fold Change
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
IRS2
PPAR-γ
TNFAIP3
NR3C1
GSK3β
phospho-HS1
BDNF
phospho-Fyn/Yes
HMGB1
phospho-GSK3αβ
Fyn
phospho-Akt
phospho-Zap
phospho-RELA
Calmodulin
PBR
Iba1
NLRP3

Figure 2A

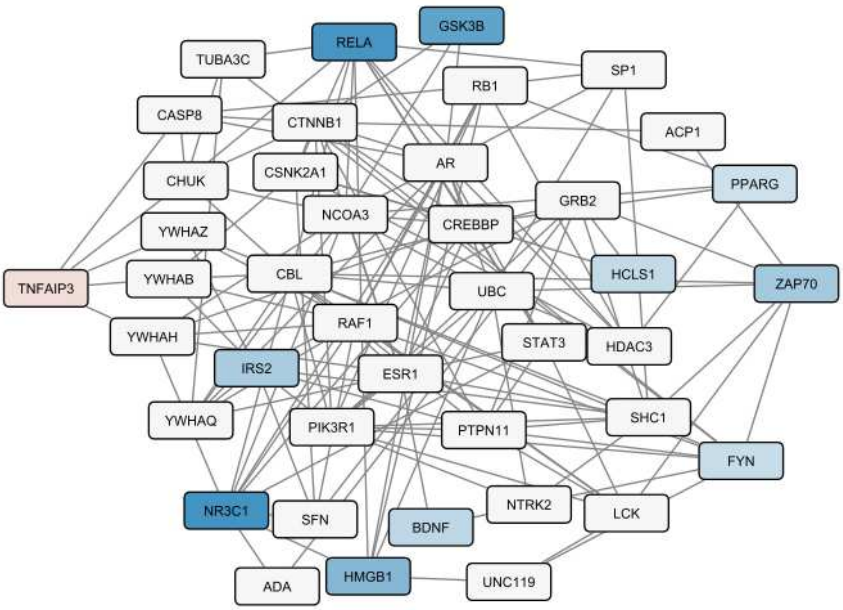

Figure 2B

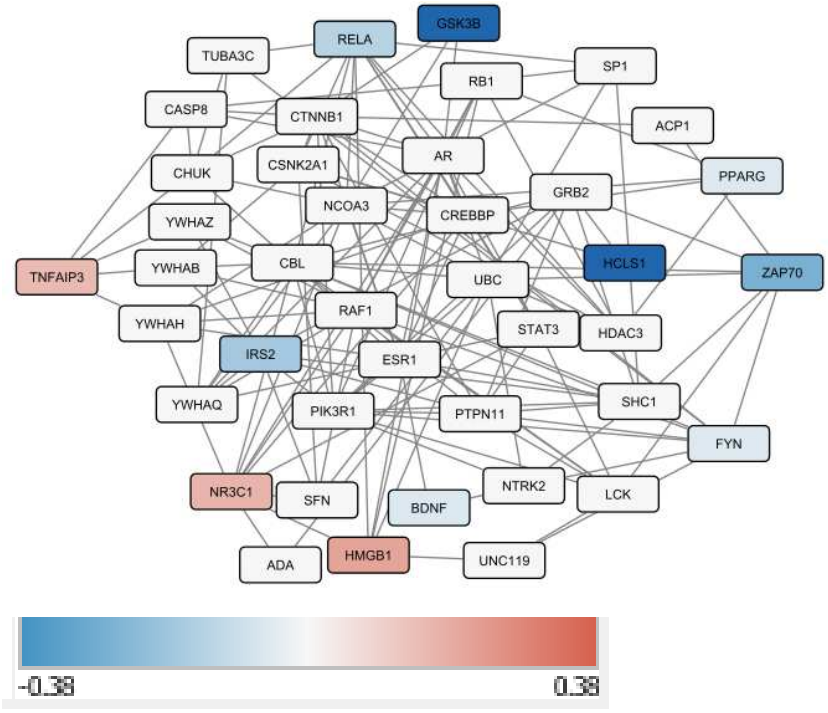

Figure 3

Figure 3A. BDNF Signaling Pathway

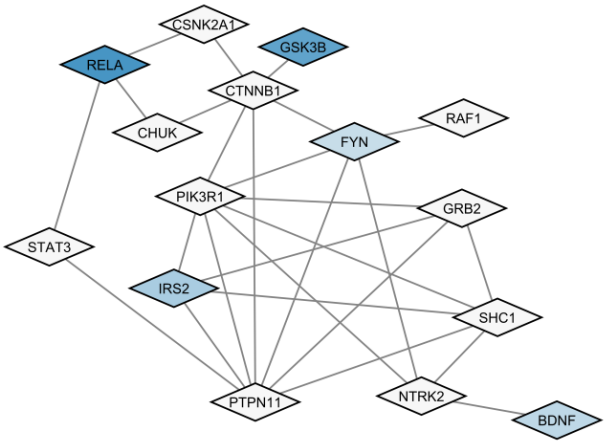

Figure 3B. Prolactin Signaling Pathway

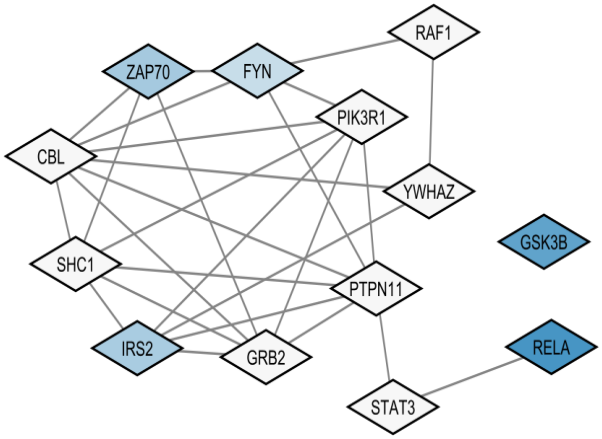

Figure 3C. Leptin Signaling Pathway

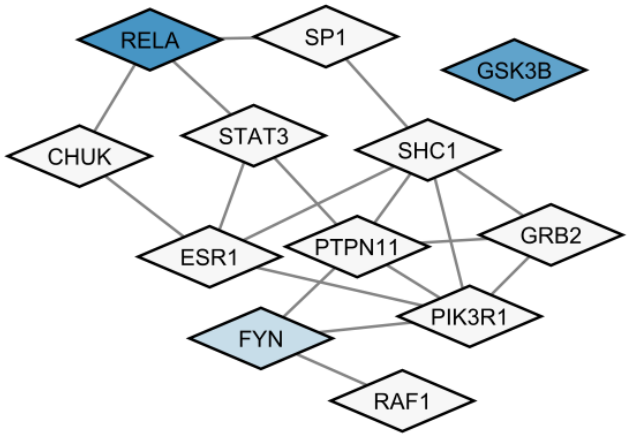

Figure 3D. Interleukin-3 Signaling Pathway

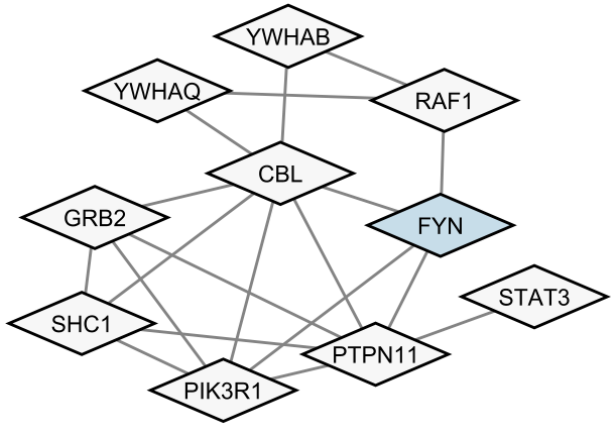

Table 1 Demographics and Baseline Symptom Severity

	Healthy Controls (n= 17)		Bipolar Patients (n= 14)	
	N	**%**	**N**	**%**
Gender				
Male	3	17.6	5	35.7
Race				
White	9	52.9	7	50.0
Marital Status				
Single/ Never Married	9	52.9	9	64.3
Married	5	29.4	3	21.4
Divorced/ Separated	2	11.7	1	7.1
Widowed	1	5.9	1	7.1
Education Level				
Some High School	0	0.0	1	7.1
Graduated High School/GED	3	18.0	1	7.1
Some College	4	23.5	8	57.1
≥ 4 year college	10	58.8	4	28.6
Employment Status				
Unemployed	3	17.6	7	50.0
Employed Part- or Full-Time	14	82.4	5	35.7
Homemaker/retired/Disabled	0	0.0	2	14.3
Baseline	**Mean**	**Std. Dev**	**Mean**	**Std. Dev**
Age	32.7	11.7	38.5	14.2
MADRS	0.65	1.00	21.36	8.12
YMRS	0.29	0.77	8.36	7.73
HAM-A	1.00	1.66	17.21	6.27
QIDS-SR-16	1.88	1.58	12.29	5.11
SHAPS	16.35	4.09	29.54	9.62
SDS	0.00	0.00	15.92	9.00
Q-LES-Q	63.31	4.94	40.0	12.22
IFS	17.53	3.83	36.21	7.51
CIRS	0.65	0.86	5.57	2.28
CGI-S-Mania	0.00	0.00	1.86	1.03
CGI-S-Depression	0.00	0.00	3.21	0.89
CGI-S-Overall	0.00	0.00	3.00	1.04

Abbreviations: CGI-S: Clincal global Impression – Severity; CIRS: Cumulative Illness Rating Scale; GED: General Educational Development; HAM-A: Hamilton Axiety Rating Scale; IFS, Iwoa Fatigue Scale; MADRS: Montgomery-Asberg Depression Rating Scale; QIDS-SR-16; the 16 item Quick Inventory of Depression Symptomatlory Self Report; Q-LES-Q: Quality of Life Enjoyment and Sastisfaction Questionnaire; SHAPS: Snaith-Hamilton Pleasure Scale; SAS: Sheehan Disability Scale; YMRS, Young Mania Rating Scale.

Table 2. Comparions of Intracelluar Levels of 18 Anaytes in Monocytes and Lymphocytes between Patients with Bipolar Disorder and Healthy Controls

	Lymphocytes					**Monocytes**				
	Bipolar Disorder (BD)		Healthy Control (HC)		BD vs. HC	Bipolar Disorder (BD)		Healthy Control (HC)		BD vs. HC
	Mean	SD	Mean	SD	p-value	Mean	SD	Mean	SD	p-value
BDNF*	118.2	23.8	128.4	21.3	0.24	115.1	23.6	120.4	23.8	0.56
Calmodulin	51.1	19.7	64.7	35.5	0.20	32.1	14.8	51.5	34.6	***0.05***
Fyn*	101.3	16.0	108.6	18.1	0.26	97.3	17.8	100.6	19.1	0.64
GSK3β*	48.2	19.2	60.2	16.7	0.09	44.5	15.8	62.1	18.2	***0.01***
HMGB1	85.5	21.2	100.9	20.7	0.06	49.5	17.1	43.0	17.4	0.32
Iba1	4.2	1.0	4.1	0.8	0.78	22.9	5.9	25.2	5.8	0.32
IRS2*	102.3	18.8	114.6	16.2	0.07	102.8	16.8	119.9	22.2	***0.03***
NLRP3*	101.2	20.5	103.2	14.6	0.78	87.9	19.5	95.8	15.7	0.25
NR3C1*	18.0	13.5	23.4	13.3	0.29	12.9	5.9	11.0	2.3	0.29
phospho-Akt*	101.7	23.2	104.3	17.1	0.74	99.0	23.0	101.1	20.4	0.80
phospho-Fyn/Yes*	37.1	16.5	38.8	6.7	0.73	54.1	20.5	58.7	12.9	0.49
phospho-GSK3αβ	3.2	0.6	3.6	0.7	0.08	3.6	0.6	3.9	1.0	0.24
phospho-HS1	19.5	7.8	21.1	8.1	0.60	40.3	19.2	54.8	16.3	***0.04***
phospho-RELA*	69.0	30.3	89.3	20.4	***0.05***	18.9	10.3	23.4	8.6	0.23
phospho-ZAP70	7.9	2.0	9.0	3.5	0.33	42.3	13.8	49.7	11.5	0.13
PPAR-γ	52.7	19.9	56.0	15.9	0.62	57.7	19.4	65.4	18.6	0.29
TNFAIP3*	99.9	22.5	95.4	16.1	0.55	83.1	29.9	73.6	18.6	0.33
TSPO*	104.0	19.9	119.8	18.6	***0.04***	89.2	15.2	93.4	17.6	0.49

Note: * Analytes were included in both first and second analysis.
Abbreviation: BDNF: Brain-derived neurotrophic factor; **Calmodulin**: calcium-modulated protein; **GSK3β**: Glycogen synthase kinase 3 beta; **Fyn**: a tyrosine kinase belongs to the Src family of tyrosine kinases including src, fyn, and yes; **HMGB1**: High mobility group box 1 protein; **Iba1:** Ionized calcium binding adaptor molecule 1; **IRS2**: insulin receptor substrate 2; **NLPR3**: NACHT, LRR and PYD domains-containing protein 3; **NR3C1**: glucocorticoid receptor; **Phospho-Akt:** phosphorylated Akt(Thr308);): **phospho-Fyn/Yes**: phosphorylated Fyn(Y530)/phosphorylated Yes(Y537); **phospho-GSK3αβ**: phosphorylated Glycogen synthase kinase 3

alpha(Tyr279)beta(Tyr216); **phospho-HS1**: phosphorylated hematopoietic lineage cell-specific protein 1 (Tyr397); **phospho-RELA**: Phosphorylated nuclear factor NFκB p65(Ser536) subunit. **Phospho-Zap70:** Phosphorylated ZAP70 (Tyr319)/Syk(Tyr352). **PPAR-γ**: Peroxisome proliferator-activated receptor gamma; **TNFAIP3:** Tumor necrosis factor, alpha-induced protein 3: **TSPO (PBR)**: translocator protein**.**

Table 3. Results of genes coding the 11 proteins in the study based on pathway enrichment analysis on the protein-protein networks of the 11 proteins

Pathway	Genes	P-value
Prolactin Signaling Pathway	**GSK3β**;**IRS2**;**RELA**;**ZAP70**;**FYN**;GRB2;RAF1; SHC1;STAT3; PTPN11; PIK3R1; CBL; YWHAZ;	E-20
Leptin signaling pathway	**GSK3β**; **FYN**; **RELA;** CHUK;SHC1;SP1;STAT3; PTPN11; GRB2; PIK3R1; RAF1; ESR1;	E-18
Brain-Derived Neurotrophic Factor (BDNF) signaling pathway	**GSK3β**; **BDNF**; **IRS2**; **RELA**; **FYN**;NTRK2;CHUK;CSNK2A1;SHC1; STAT3; PTPN11;PIK3R1; CTNNB1; GRB2;RAF1	E-13
IL-3 Signaling Pathway	**FYN**; YWHAQ;SHC1;YWHAB;STAT3; PTPN11;GRB2;PIK3R1;CBL;RAF1	E-12

Note: The bold genes are tested in the current study.
Abbreviations: BDNF: brain-derived neurotrophic factor**; GSK3β**: Glycogen synthase kinase 3 beta: **IRS2:** insulin receptor substrate; **RELA:** nuclear factor NF-kappa-B p65 subunit**;** **ZAP70:** a zeta-chain-associated protein kinase.