

Exon Array Biomarkers for the Differential Diagnosis of Schizophrenia and Bipolar Disorder

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Abstract

This study developed potential blood-based biomarker tests for diagnosing and differentiating schizophrenia (SZ), bipolar disorder type I (BD), and normal control (NC) subjects using mRNA gene expression signatures. A total of 90 subjects ($n = 30$ each for the three groups of subjects) provided blood samples at two visits. The Affymetrix exon microarray was used to profile the expression of over 1.4 million probesets. We selected potential biomarker panels using the temporal stability of the probesets and also back-tested them at two different visits for each subject. The 18-gene biomarker panels, using logistic regression modeling, correctly differentiated the three groups of subjects with high accuracy across the two different clinical visits (83–88% accuracy). The results are also consistent with the actual data and the “leave-one-out” analyses, indicating that the models should be predictive when applied to independent data cohorts. Many of the SZ and BD subjects were taking antipsychotic and mood stabilizer medications at the time of blood draw, raising the possibility that these drugs could have affected some of the

differential transcription signatures. Using an independent Illumina data set of gene expression data from antipsychotic medication-free SZ subjects, the 18-gene biomarker panels produced a receiver operating characteristic curve accuracy greater than 0.866 in patients that were less than 30 years of age and medication free. We confirmed select transcripts by quantitative PCR and the nCounter[®] System. The episodic nature of psychiatric disorders might lead to highly variable results depending on when blood is collected in relation to the severity of the disease/symptoms. We have found stable trait gene panel markers for lifelong psychiatric disorders that may have diagnostic utility in younger undiagnosed subjects where there is a critical unmet need. The study requires replication in subjects for ultimate proof of the utility of the differential diagnosis.

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Introduction

Schizophrenia (SZ) and bipolar disorder (BD) are chronic, severe, and disabling brain disorders that affect about 1 and 2%, respectively, of the US population aged 18 years and older. Despite moderately effective treat-

ments such as antipsychotic medications and psychosocial interventions, people with SZ and BD often do not receive timely treatment because the disorder is not correctly recognized. As a result, patients experience multiple hospitalizations and incur socioeconomic disadvantages that can last for decades.

Ideally, successful diagnostic tests could address the significant clinical problem of early identification and differential diagnosis and permit more timely initiation of treatments. Currently, there are no approved clinical diagnostic tests for psychiatric disorders, with diagnosis and treatment being based on the patient's report of symptoms along with clinical observations. Consequently, clinicians frequently fail to recognize initial presentations of SZ and BD.

These failures may lead to devastating consequences. Following an initial episode of psychosis among individuals aged 16–30 years, there is a 24-fold increase in the risk of death in the following year [1]. This study points towards a lack of treatment (61% did not receive any antipsychotic medication) after initial presentation with psychosis and even higher rates in those dying within 12 months of an initial episode of psychosis [1]. A panel of biomarkers would meet an unmet clinical need to help establish rapid and informative patient decisions. A clinical utility that might be derived from this work is establishing a biomarker panel for determining the psychosis risk state and which subjects may convert to SZ or BD [2]. An index of psychosis based upon a biomarker panel could be useful in evaluating a long-term response to treatments [3].

Conceivably, a panel of RNA biomarkers could address that need. A growing body of work has demonstrated the potential utility of RNA diagnostic tools with peripheral samples such as whole blood, peripheral blood mononuclear cells (PBMCs), and lymphoblastic cell lines in multiple studies of SZ and BD [4–12]. There have also been large studies that have used whole genome RNA expression to compare healthy controls and patients with disorders such as Alzheimer disease [13], autism [14], Down syndrome [15], epilepsy [16], Tourette syndrome [16], Huntington disease [17], Klinefelter syndrome [18], multiple sclerosis [19], smoking and major depression [20], panic disorder [21], posttraumatic stress disorder [22], and subjective social isolation (loneliness) [23]. The success in these studies suggests the possibility that similar approaches could be used to identify RNA profiles for the diagnosis of SZ and BD. Dysregulation of mRNA could potentially help to define sets of genes relevant to the diseases' pathophysiology and treatment or secondary to their causes.

We hypothesized that for SZ and BD there are unique and global sets of chronic, differentially expressed genes in blood for each disorder. Our hypothesis is based on the concept that circulating blood reflects the changing health of the body, i.e., the “sentinel principle” [24]. As blood circulates through the brain, communication occurs between cells in the blood and the brain [25]. Following a brain injury, neutrophils, macrophages, lymphocytes, and dendritic cells can extravasate into the brain from the blood [26]. These extravasated cells can induce changes in gene expression and proteins as reported in neuroimmunological studies [27, 28]. In prior work using blood-brain samples from the same subjects, about 20% of the transcriptome was expressed at comparable levels and significantly correlated in both tissues from the same subjects [29]. This correlation supports the idea that important biomarkers of either SZ or BD could be expressed peripherally, and these would also have a connection to the central modulation of neuroimmune responses. The advantages of a peripheral transcriptomic study are the ease of access to whole blood and the fact that immune genes are highly expressed. For example, multiple HLA region genes with genome-wide significance are expressed in peripheral blood samples, such as C4 [30] and HLA-DPA1 [31].

Although investigations of biomarkers for SZ and BD have been ongoing for at least a decade [7, 8, 32], there are no widely replicated studies using peripheral blood gene expression [11, 33–42]. A recent mega-analysis of blood samples comparing SZ cases and controls was conducted on a total of 578 subjects in 9 studies. The mega-analysis of the blood transcriptome showed that 220 genes reached a Bonferroni-corrected level of significance [35], indicating the utility of analysis of the blood transcriptome for finding differentially expressed genes. The stability of this potential dysregulation has not been tested across different time points.

The objective of the present study was to test subjects at multiple time points by enrolling SZ ($n = 30$), BD ($n = 30$), and normal control (NC; $n = 30$) subjects characterized at one clinical site to eliminate potential sources of variation. The expression levels of panels of genes were used to define with which disorder the patient was diagnosed with the highest likelihood. We have previously validated Tempus Blood RNA tubes and Affymetrix exon arrays in a study that measured the sources of variation for 8 healthy controls at 9 sequential blood draws every 6 h [29] for 54 h. The resulting data showed that about 20% of the transcripts measured on the Affymetrix exon array did not significantly change over the 9 blood draws

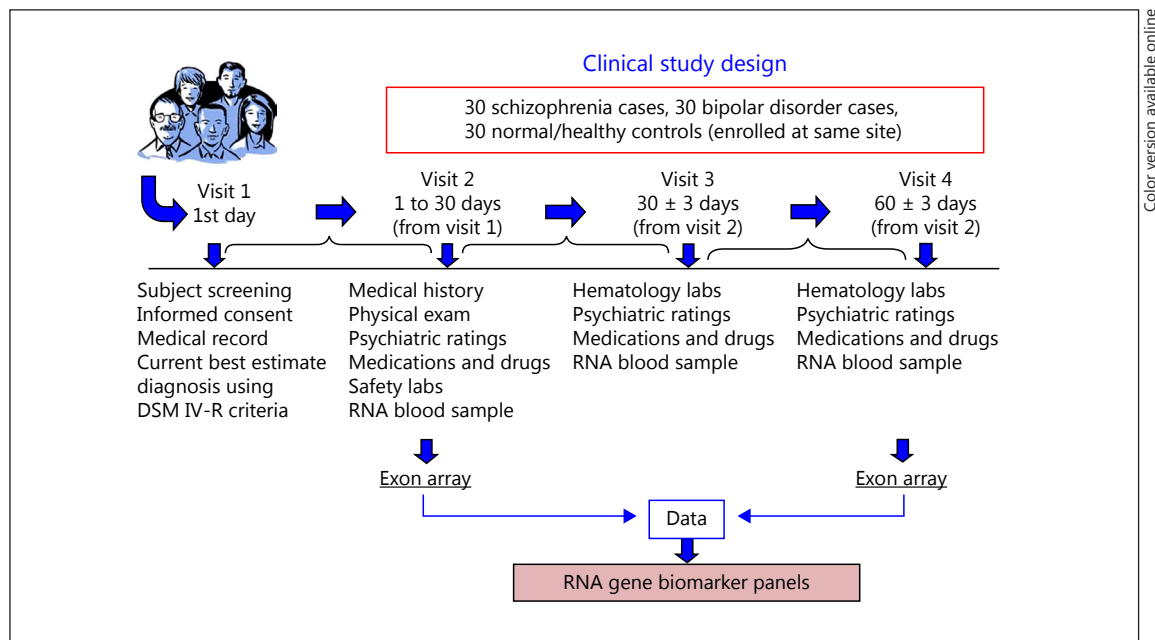


Fig. 1. Flowchart of the study: subject enrollment and RNA biomarker panel.

using Bonferroni correction during the 54-h period. The remaining 80% of the transcripts had changed significantly during this 54-h experiment [29]. Taken together, it is expected that at least 20% of transcripts will be stable over longer periods of time and do not significantly fluctuate by the time of day of the blood draw.

These data reinforce the purpose of the present study to test whether stable expression of genes occurs over months instead of days, so that they can be used as biomarkers for SZ and BD for a differential diagnosis from controls and each other. In this study, RNA expression was measured by the Affymetrix exon array 1.0 ST for biomarker screening. Exon arrays have been widely used for the study of genetic variation in coding regions [43–53]. Exon array findings correlate positively with RNA-Seq across most levels of transcript expression [54], and in some cases have less false-positive detection than RNA-Seq [55–57]. The analytical techniques for the exon arrays have been well established [56, 58–64].

Methods

Subject Enrollment

Subject enrollment occurred at a single clinical site at the University of Iowa. The University of Iowa and the Institutional Review Board approved the procedures in the study. The chronic SZ and BD type I outpatients aged 18–45 years provided consent for

the study. All subjects (SZ, $n = 30$; BD, $n = 30$; and NC, $n = 30$) met the DSM-IV-R criteria and completed the study. Clinical assessments included the Scale for the Assessment of Positive Symptoms and Scale for the Assessment of Negative Symptoms (SAPS, SANS), medications, and drugs for the SZ and BD subjects, as well as the Young Mania Rating Scale (YMRS) and Hamilton Rating Scale for Depression (HAM-D or HRSD) for the BD subjects. These neuropsychiatric assessment data were analyzed and are reported separately for state-biomarker relationships. The mental state examination for the NC subjects consisted of the Mini-Mental State Examination. The outline of the study is shown in Figure 1. The demographics of the SZ, BD, and NC subjects are shown in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000485800) for the subjects' age, sex, duration of illness, and ethnicity.

Whole blood samples were collected in Tempus Blood RNA tubes (Thermo Fisher Scientific) from the SZ, BD, and NC subjects at 3 visits spanning 3 months. For this report, the Tempus tubes from visits 2 and 4 were extracted and RNA gene expression was measured using Affymetrix exon arrays for both visits on all 90 subjects. High-quality RNA was extracted from the Tempus tubes using the manufacturer's protocol, and quality was assessed on an Agilent Bioanalyzer using the RNA integrity number.

Human Exon Array for Biomarker Profiling

There are advantages to using the Affymetrix exon arrays [65] compared to whole transcriptome shotgun sequencing (RNA-Seq). At the time of sample collection, the cost factor was favorable for future clinical biomarker trials that would require hundreds of arrays compared to the cost of RNA-Seq for the entire validation. The processing time and data storage requirements as well as more favorable for a study this size using exon arrays. Although we and oth-

ers have previously found that SNPs can affect probe hybridization and consequently alter expression [44, 51, 52, 66], those probesets with common SNPs were eliminated from the final data set. The exon arrays were run at the Functional Genomics Laboratory, University of California, using the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). The Functional Genomics Laboratory has run over 1,000 Affymetrix arrays with high-quality call rates.

Data Analysis

The Affymetrix exon array CEL files were imported into Partek Genomics using batch effect removal. The batch effect was based upon exon array scan dates, as usually 12 arrays were scanned in a single day. The mean intensity of probes was summarized at the probeset level. Probesets containing common SNPs were excluded from the import and downstream analysis. The resulting probesets were then median centered within each exon array sample individually ($n = 180$). A two-factor ANOVA was run for each probeset, using diagnosis, visit, and diagnosis $\times$ visit interaction. Visit was a repeated measure and was used to filter out genes that change significantly between visits. A false discovery rate (FDR) of 6×10^{-8} was established for diagnosis effect based upon 835,000 probesets. Three filters were used to select probesets from the ANOVA results that passed the FDR for diagnosis: (1) the most significant p values for BD compared to NC; (2) the most significant p values for BD compared to SZ; and (3) the most significant p values for SZ compared to NC. This resulted in a list of top probesets that was then reduced to probesets that mapped to known RefSeq genes. The top 100 RefSeq probesets for each of the three filters above were combined, and the resulting top 300 probesets were evaluated for biomarker signature.

Biomarker Signature

The modeling proceeded in four steps to select the most predictive panel of probesets out of the top 300 in each step for discriminating between groups:

- Step 1: NC versus BD + SZ
- Step 2: NC versus SZ
- Step 3: NC versus BD
- Step 4: SZ versus BD

Multivariate logistic regression modeling with forward stepwise selection (SAS PROC LOGISTIC) was used on the combined visit 2 and 4 data from the groups included in the step to select the probesets that were discriminating most strongly between the groups. We used forward stepwise regression to select probesets that differentiated two groups at a time (BD vs. SZ, BD vs. NC, and SZ vs. NC). A probeset was added to the model if the estimate was the most significant with $p < 0.001$ and the resulting ROC AUC also retained statistical significance. Forward selection stopped when potential probesets were no longer statistically significant or did not improve the ROC AUC by more than 1%. Processing for each step resulted in a subset of the 300 probesets where each probeset contributed to the model significantly and the panel represented the smallest number of probesets that had a very high diagnostic utility based on the ROC AUC.

Modeling for the diagnostic for each step was applied to the visit 2 data using the identified probesets. The optimal cut-point for discriminating between the groups based on the logistic model prediction was obtained by maximizing the Youden index J [67], where:

$$J = \text{True positive rate} - \text{FPR}.$$

Table 1. Probesets that were found to reliably discriminate BD, SZ, and NC were assigned to known RefSeq transcripts

Biomarker panel (comparisons)	Affymetrix exon microarray transcript ID	Gene
BD-NC	2661992	OXTR
BD-NC	3195034	PTGDS
BD-NC	3333247	FADS2
BD-NC	4048241	HLA-DRB5
BD-SZ	2661992	OXTR
BD-SZ	3333247	FADS2
BD-SZ	3554818	CRIP2
BD-SZ-NC	2418570	SLC44A5
BD-SZ-NC	2545092	HADHA
BD-SZ-NC	2647109	CPA3
BD-SZ-NC	2661992	OXTR
BD-SZ-NC	2739160	CCDC109B
BD-SZ-NC	2906720	TREML4
BD-SZ-NC	3063536	TRIM4
BD-SZ-NC	3195034	PTGDS
BD-SZ-NC	3667890	HPR
BD-SZ-NC	3846538	EEF2
BD-SZ-NC	3908149	ZMYND8
SZ-NC	2401347	TCEA3
SZ-NC	2418570	SLC44A5
SZ-NC	2660617	IL5RA
SZ-NC	3329099	GYTL1B
SZ-NC	3766893	DDX5

BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

The visit 2 prediction model was then applied to the visit 4 data to assess utility for the second set of data, which included stability over time.

Further evaluation for each of the four panels included "leave-one-out" cross-validation where one subject was sequentially left out of the logistic model fit using the remaining subjects and then the predictability of the model for the excluded subject was assessed. This tested whether there were outliers in the data set that were driving the model.

Quantitative PCR

Transcripts were selected for quantitative PCR (qPCR) validation based upon significant differences using the ANOVA filter. We selected transcripts that represented a combination of the most

Table 2. Prediction model trained on combined visit 2 and 4 data and tested on each visit individually

Actual	Step 1		Step 2		Step 3		Step 4		Final call		
	NC	SZ + BD	NC	SZ	NC	BD	SZ	BD	NC	SZ	BD
<i>Training visit 2</i>											
30 NC	30	0	24	6	26	4			21	5	4
30 SZ	3	27	1	29			29	1	3	26	1
30 BD	1	29			1	29	1	29	1	1	28
<i>Testing visit 4</i>											
30 NC	28	2	23	7	28	2			22	6	2
30 SZ	1	29	0	30			29	1	1	28	1
30 BD	1	29			0	30	0	30	1	0	29

The confusion matrices show the numbers of subjects correctly and incorrectly classified for each iteration of the classification. Step 1: 11 genes diagnostically differentiating BD + SZ from NC. Step 2: 5 genes diagnostically differentiating SZ from NC. Step 3: 4 genes diagnostically differentiating BD from NC. Step 4: 3 genes diagnostically differentiating SZ from BD. Final call: summary of the step 1, 2, 3, and 4 classifications. BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

Table 3. The overall results show a stable clinical biomarker signature of mRNA expression across a 90-day test-retesting period with an accuracy of 88% on the retesting data

Predicted	Visit 2 actual			Visit 4 actual			Both actual		
	BD	SZ	NC	BD	SZ	NC	BD	SZ	NC
BD	28	1	4	29	1	2	57	2	6
SZ	1	26	5	0	28	6	1	54	11
NC	1	3	21	1	1	22	2	4	43
Accuracy		83%			88%			86%	
Sensitivity (SZ)		87%			93%			90%	
Sensitivity (BP)		93%			97%			95%	
Specificity (NC)		70%			73%			72%	

BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

significant ANOVA *p* values for SZ compared to NC and represented fold changes greater than 1.25. We initially selected qPCR to validate the exon array findings and later, after completing the entire biomarker panel analysis, used NanoString (see below) for validation. Standard SYBR Green qPCR methods previously described by the Functional Genomics Laboratory (University of California, Irvine) were used to confirm gene expression values derived from the exon array data set [31]. Briefly, in developing SYBR Green assays we use exon junction-crossing primers to eliminate any genomic DNA from amplification. We assess primers for amplification consistency by single dissociation peaks to represent a single region of cDNA amplification, and minimal primer-dimer formation that could interfere with the amplification signal. We require the primers to amplify genes in our samples at fewer than 35 cycles to be usable. We also run samples in triplicate, and use two housekeeping genes (*SDHA* and *HPRT1*) that have Ct within similar ranges to those of the genes being assayed.

NanoString Gene Expression Platform

A non-PCR-based approach to measuring RNA as a technical validation of the findings was used based upon NanoString technology, i.e., the nCounter® System (NanoString, Seattle, WA, USA) [68]. The NanoString platform requires a small quantity of RNA and provides digital counts of hybridization of mRNA to targets. The NanoString multiplex assay uses 100 ng of total RNA, and all of the RNA samples were processed at the UCI Genomics High-Throughput Facility. We selected a total of 44 custom NanoString probes designed to match the closest probeset on the Affymetrix exon array that was in the biomarker panel, and 6 NanoString probes for housekeeping genes (online suppl. Table 2). The resulting NanoString data were processed according to the manufacturer's suggested protocol as outlined (NanoString Technologies, 2008–2012 #127). Each data point was preprocessed by the 6 positive controls (PC), the 8 negative controls (NC), and the 5 housekeeping (HK) genes as follows:

Table 4. The overall results fitting a “leave-one-out” validation model to the remaining subjects show a stable clinical biomarker signature of mRNA expression across a 90-day test-retesting period with an accuracy greater than 87% for the retesting data at each of the model classification steps

	BD + SZ vs. NC (11-gene panel)	SZ vs. NC (5-gene panel)	BD vs. NC (4-gene panel)	SZ vs. BD (3-gene panel)
<i>Actual visit 2 data</i>				
Sensitivity (SZ)	93%	97%	–	97%
Sensitivity (BP)		–	97%	97%
Specificity (NC)	100%	80%	87%	–
Accuracy	96%	88%	92%	97%
<i>“Leave-one-out” cross-validation</i>				
Sensitivity (SZ)	90%	93%	–	90%
Sensitivity (BP)		–	93%	93%
Specificity (NC)	83%	80%	80%	–
Accuracy	88%	87%	87%	92%

BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

Calculate for each subject/visit, the $\Sigma_1^6 PC$ (sum of the positive controls), the $\bar{X}_{NC}$ (mean of the negative controls), and the $\Sigma_1^5 HK$ (sum of the housekeeping genes).

Calculate across all subjects/visits, the $\bar{X}_{PC} = \text{Sample } n_{1,180}(\Sigma_1^6 PC)/180$ (mean of the positive control sums), and the $\bar{X}_{HK} = \text{Sample } n_{1,180}(\Sigma_1^5 HK)/180$ (mean of the housekeeping gene sums).

To normalize the gene expression data point (GEDP) for each subject/visit,

$$\text{Normalized GEDP} = \left(\text{GEDP} \times \left(\frac{\Sigma_1^6 PC}{\bar{X}_{PC}} \right) - \left(\bar{X}(NC) \right) \right) / \left(\frac{\Sigma_1^5 HK}{\bar{X}_{HK}} \right)$$

that is, multiply GEDP by (sum of positive controls/mean of positive control sums), subtract the mean of the negative controls, and divide by (sum of housekeeping genes/mean of housekeeping gene sums). If the normalized GEDP was negative, it was set to 0. The normalized GEDPs for the NanoString data were then analyzed for diagnostic accuracy using a model fitting approach. A potential gene probeset was selected by including all probesets that had $p < 0.1$ in a univariate logistic regression fit of the probeset on at least one of the diagnosis comparisons (NC vs. [SZ + BD], NC vs. SZ, NC vs. BD, SZ vs. BD) for the visit 2 data. The nominal p value selected was based upon prior significance in the exon array.

Results

The top 300 probesets from the Affymetrix exon microarray based upon ANOVA significance (online suppl. Table 3) were evaluated for biomarker signature (as described in Methods) for differentiating BD, SZ, and NC subjects. The resulting biomarker signature was com-

Table 5. The diagnostic algorithm uses four individual steps, shown in each column

BD + SZ vs. NC (11-gene panel)	SZ vs. NC (5-gene panel)	BD vs. NC (4-gene panel)	SZ vs. BD (3-gene panel)
AUC = 0.994 ($p < 0.0001$)	AUC = 0.954 ($p < 0.0001$)	AUC = 0.974 ($p < 0.0001$)	AUC = 0.998 ($p < 0.0001$)
BD, bipolar disorder; SZ, schizophrenia; NC, normal control.			

posed of 23 probesets that condensed into 18 known RefSeq genes (biomarker panel; Table 1). The diagnostic logistic model was built in four steps, using the visit 2 transcripts shown in Table 1.

The resulting logistic predictive model based on visit 2 was then applied to the visit 4 data. The summary of individual steps in the construction of the biomarker gene panels is shown in Table 2. The diagnostic algorithm uses a four-step decision model: step 1, BD and SZ versus NC; step 2, SZ versus NC; step 3, BD versus NC; and step 4, SZ versus BD.

The 18-gene biomarker panels, using logistic regression modeling, correctly differentiated the three groups of subjects (SZ, BD type I, and NC) with high accuracy at visit 2 and visit 4. The visit 2 cut-point probabilities for the SZ-NC comparison were significantly correlated with the visit 4 cut-point probabilities ($p < 0.0001$) with $r = 0.74$ (95% CI 0.59–0.83) showing temporal stability (Table 3).

The initial model was developed for selecting stable probesets across visits and incorporated all subjects and visits to select the most informative probesets. To test that no single subject was overly influential in determining the model, the initial probesets were evaluated by a “leave-one-out” method, whereby a new model is fit to the remaining subjects, and the left-out subject is identified. “Leave-one-out” cross-validation is a model validation technique for assessing how the results of a statistical analysis will generalize to an independent data set. It is mainly used in settings where the goal is prediction to estimate how accurately a predictive model will perform in practice. This cross-validation was applied to the visit 2 data from each of the four probeset panels (Table 4). The results are very consistent with the actual data, and the “leave-one-out” analyses indicate that the models should be predictive when applied to independent data cohorts.

The AUC for each step was greater than 0.95, which is an indication of the high combined sensitivity and specificity of the classification into three groups (Table 5). When analyzing the same 18-gene biomarker panel and

Table 6. The 18-gene signature was extracted from an independent data set of gene expression data and analyzed by the same logistic regression equations as derived from the fit to our original data

Subjects	NC, <i>n</i>	SZ, <i>n</i>	ROC AUC	Sensitivity	Specificity
Antipsychotic free (all subjects)	22	15	0.642	80.00%	59.10%
Antipsychotic free (subjects aged <30 years)	14	8	0.866	100.00%	71.40%

Using this data set from antipsychotic-free SZ subjects, the 18-gene diagnostics produced a ROC accuracy of 0.866 in medication-free patients below 30 years of age. The classification of antipsychotic-free patients with SZ was made using data from the Illumina HT-8 array [6]. SZ, schizophrenia; NC, normal control.

including the white blood cell counts as a covariate, the analysis slightly improved the diagnostic predictability of SZ versus NC, and of BP and SZ versus NC (data not shown). The data were not normalized to blood counts (CBC measurements) for our main data analysis reported in this paper, but this model confirmed that potential differences in white blood cell counts did not change our results as reported.

To account for potential medication effects (since many of the SZ and BD subjects were on stable dosages of antipsychotic or mood stabilizer medications at the time of blood draw), we analyzed the possibility that these medications could drive some of the differential transcription signatures. A subset of unmedicated BD ($n = 3$) and SZ ($n = 1$) cases were analyzed using the 18-gene diagnostics, which produced 100% accuracy in these 4 cases.

In addition, to test this possible explanation for the gene signature, we reanalyzed an even larger cohort of antipsychotic-free SZ patients using the Illumina gene expression microarray data sets [6]. The genome-wide RNA expression profiling was obtained with the Illumina HumanRef-8 V3 arrays for batch 1 and HumanRef-12 V3 arrays for batch 2 using Illumina's standard protocol at the Illumina facility of the University of California, Los Angeles. The raw microarray data were available at gene expression omnibus (GEO) under accession No. GSE38485. Data set 1 includes antipsychotic-free SZ patients ($n = 15$) and healthy controls ($n = 22$). The 18-gene signature was extracted from the Illumina data set and analyzed by the same logistic regression equations as derived from the fit to our original data. Using this independent Illumina data set of gene expression data from the antipsychotic-free SZ subjects, the 18-gene diagnostics produced a ROC accuracy of 0.866 in medication-free patients below 30 years of age (Table 6). These results were achieved with different gene expression technologies, different blood collection tubes, and different mRNA ex-

Table 7. qPCR results for candidate gene expression differences between SZ and NC

Exon array (gene symbol)	<i>p</i> value (SZ vs. NC)	Ratio (SZ vs. NC)	qPCR	<i>p</i> value (SZ vs. NC)	Ratio (SZ vs. NC)
EDIL3	1.04E-13	0.446	EDIL3	0.01418	0.425
NRCAM	1.37E-02	0.525	NRCAM	0.06127	0.663
PTGDS	1.41E-14	0.722	PTGDS	0.04731	0.695
DSC2	6.86E-04	1.592	DSC2	0.00005	2.021
NRG1	1.14E-02	2.062	NRG1	0.01021	1.979
ITGA2B	3.71E-02	2.088	ITGA2B	0.00007	2.269
ITGB3	2.65E-02	2.601	ITGB3	0.00005	2.065

qPCR, quantitative PCR; SZ, schizophrenia; NC, normal control.

traction techniques. The details on the methods and analysis of the antipsychotic-free patients are in the online supplementary Appendix. The duration of illness might allow us to test for more trait effects than state effects in the data set instead of using an arbitrary age cutoff for young subjects of below 30 years. Duration of illness is not entirely accurate, due to retrospective subject recall, which is why we chose age as our cut-point. Age and duration of illness are significantly collinear for BD subjects ($r = 0.65$; $p < 0.001$) and for SZ subjects ($r = 0.62$; $p < 0.001$). Further, we did not have access to the Illumina duration of illness data for comparison.

qPCR Validation of the Exon Array

The transcripts were validated for SZ and NC using the qPCR methods described by Morgan et al. [31]. The transcripts for qPCR were selected based upon the differential expression results from the SZ-NC comparisons and upon visual inspection of exon expression differences across probesets that map to RefSeq transcripts. The qPCR results showed concordance ($r = 0.86$) between

Table 8. The NanoString data set was analyzed by ANOVA, and the following 23 probesets representing 11 genes passed the initial ANOVA filter ($p < 0.1$)

2537112_SH3YL1 (30)	3554838_CRIP2 (39)	2661997_OXTR (43)	2723770_TBC1D1 (55)
2537128_SH3YL1 (61)	3554839_CRIP2 (58)	2906726_TREML4 (45)	4048243_HLA-DRB5 (57)
2647127_CPA3 (35)	3554833_CRIP2 (68)	2906736_TREML4 (50)	3195045_PTGDS (59)
2647122_CPA3 (47)	2418615_SLC44A5 (40)	2906735_TREML4 (53)	2401364_TCEA3 (64)
2647124_CPA3 (65)	2418581_SLC44A5 (46)	2906733_TREML4 (62)	2401362_TCEA3 (70)
2647119_CPA3 (69)	2418590_SLC44A5 (51)	3063538_TRIM4 (52)	

The format is: "Affymetrix Exon Array Probeset ID_Gene Symbol (variable number entered into regression)."

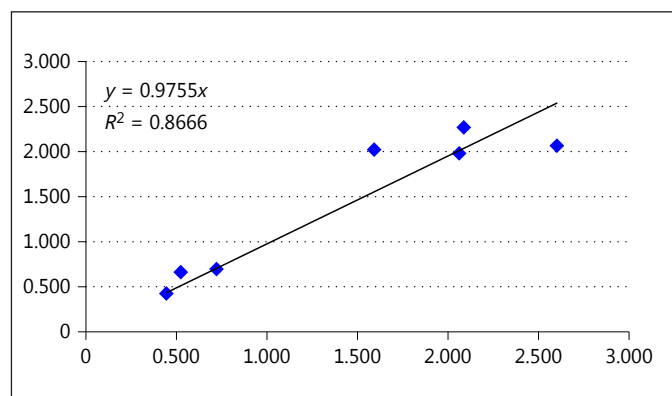


Fig. 2. The fold change ratio (SZ compared to NC) for the exon microarray results was compared to the fold change ratio (SZ compared to NC) of quantitative PCR. The fold changes were highly correlated. SZ, schizophrenia; NC, normal control.

exon array expression differences and qPCR (Table 7; Fig. 2). We used NanoString to test the complete panel of 18 biomarker genes.

NanoString Platform Accuracy for Diagnostic Classification

The NanoString platform was chosen to validate the biomarker panel using the entire panel as well as probesets mapping close to the original exon array probesets. With the NanoString data, the best multivariate logistic regression model for diagnosis at visit 2 was obtained by stepwise backward elimination from the full 23-probeset multivariate model to a reduced model with all included probesets significant at $p < 0.05$ or $p < 0.1$ to maintain an AUC above 0.9 (Table 8).

A cut-point for each reduced diagnostic model was found which optimized both sensitivity and specificity based on the visit 2 data, which reduced the useful probeset number to 12 (Table 9). The diagnostic model and

Table 9. Summary of NanoString cut-point diagnostic accuracy

Significant probesets ¹	NC vs. SZ/BD	NC vs. SZ	NC vs. BD	SZ vs. BD
2537112_SH3YL1 (30)	x	x	x	x
2537128_SH3YL1 (61)		x	x	x
2647124_CPA3 (65)	x	x	x	x
2647119_CPA3 (69)	x	x	x	x
3554833_CRIP2 (68)	x		x	
2418590_SLC44A5 (51)	x	x		
2661997_OXTR (43)			x	x
2906733_TREML4 (62)		x		
3063538_TRIM4 (52)	x	x	x	
2723770_TBC1D1 (55)				x
3195045_PTGDS (59)	x	x	x	x
2401362_TCEA3 (70)				x
AUC	0.913	0.990	0.953	0.905
Visit 2 sensitivity	98.3%	96.6%	89.3%	86.2%
Visit 2 specificity	75.0%	96.4%	92.9%	82.1%
Visit 4 sensitivity	87.5%	75.0%	75.0%	57.1%
Visit 4 specificity	60.0%	80.0%	76.0%	85.7%
Visit 2/4 agreement	77.6%	76.0%	75.5%	64.2%

BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

¹ Format as explained in Table 8.

cut-point were then applied to the visit 4 data to estimate the visit 4 sensitivity and specificity as well as the agreement between the visit 2 and the visit 4 diagnostic predictions.

Comparison of Affymetrix Exon Array with NanoString Results

The prediction accuracies for diagnosis were compared between the Affymetrix exon array and the NanoString platform. In general, for the exon array, the model performed equally well for visit 2 and visit 4 data due to the data driving the selection algorithm from the original platform. The visit 2/visit 4 agreement for the Af-

Table 10. Comparison of Affymetrix and NanoString results showing that the Affymetrix exon array had higher sensitivity and specificity for prediction

NanoString probeset ID	Genes included in each predictive model							
	NC vs. SZ/BD		NC vs. SZ		NC vs. BD		SZ vs. BD	
	Affymetrix ID	Nano-String	Affymetrix ID	Nano-String	Affymetrix ID	Nano-String	Affymetrix ID	Nano-String
2537112_SH3YL1 (30)		x		x		x		x
2537128_SH3YL1 (61)				x		x		x
2647124_CPA3 (65)		x		x		x		x
2647119_CPA3 (69)	2647109	x		x		x		x
3554833_CRIP2 (68)		x				x	3554818	
2418590_SLC44A5 (51)	2418570	x	2418570	x				
2661997_OXTR (43)	2661992				2661992	x	2661992	x
2906733_TREML4 (62)	2906720			x				
3063538_TRIM4 (52)	3063536	x		x		x		
2723770_TBC1D1 (55)								x
3195045_PTGDS (59)	3195034	x		x	3195034	x		x
2401362_TCEA3 (70)			2401347					x
3667896,97_HPR	3667890							
3908171_ZMYND8	3908149							
2545100_HADHA	2545092							
2739191_CCDC109B	2739160							
3846545_EEF2	3846538							
3333251,56,58,62,69,70,74_FADS2					3333247		3333247	
4048243,52_HLA-DRB5					4048241			
3329128_GYTL1B			3329099					
2660633,41_IL5RA			2660617					
3766938_DDX5			3766893					
AUC ¹	0.994	0.913	0.954	0.990	0.974	0.953	0.998	0.905
Visit 2 sensitivity	93.3%	98.3%	96.7%	96.6%	96.7%	89.3%	96.7%	86.2%
Visit 2 specificity	100%	75.0%	83.3%	96.4%	86.7%	92.9%	100%	82.1%
Visit 4 sensitivity	96.7%	87.5%	100%	75.0%	93.3%	75.0%	96.7%	57.1%
Visit 4 specificity	93.3%	60.0%	80%	80.0%	100.0%	76.0%	100%	85.7%
Visit 2/4 agreement	93.3%	77.6%	86.7%	76.0%	88.3%	75.5%	100%	64.2%

BD, bipolar disorder; SZ, schizophrenia; NC, normal control. ¹ The AUC was for combined visit 2 and 4 modeling, and each visit was evaluated for predictions individually.

Table 11. Overlap between a mega-analysis of dysregulated genes in blood [35] and the current study

Gene symbol	Gene product	Mean difference	FDR <i>q</i> value	Gene symbol	<i>p</i> value (SZ vs. NC)	Ratio (SZ vs. NC)
FAM118A	family with sequence similarity 118, member A	-0.37	5.90E-04	FAM118A	4.17E-13	0.58
MRPL42	mitochondrial ribosomal protein L42	0.27	1.90E-02	MRPL42	0.000535	1.24
PHF14	PHD finger protein 14	0.26	2.50E-02	PHF14	3.09E-14	0.49
PHIP	pleckstrin homology domain interacting protein	0.29	9.10E-03	PHIP	4.66E-29	0.59
PLB1	phospholipase B1	0.26	3.40E-02	PLB1	2.70E-13	1.52
SLC22A4	solute carrier family 22 (organic cation/zwitterion transporter), member 4	0.34	3.30E-03	SLC22A4	0.039619	1.14
STX2	syntaxin 2	0.28	1.30E-02	STX2	1.49E-05	0.81
TFB1M	transcription factor B1, mitochondrial	-0.5	3.60E-02	TFB1M	1.11E-05	0.79
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	-0.31	3.70E-03	TNFRSF21	1.96E-13	0.53

FDR, false discovery rate; SZ, schizophrenia; NC, normal control.

fymetrix exon array was significantly higher than for the NanoString reproducibility diagnosis classification (two-tailed paired *t* test, *p* = 0.046). These results show that the test-retest reproducibility of the algorithm using the same subjects was best when using the original platform (Table 10), although there was general agreement across platforms.

In comparing the qPCR, exon array, and NanoString results, 5 of 7 genes tested by qPCR passed with significant *p* values, while all genes were concordant with fold changes between the exon array and qPCR results. The biomarker panel was developed more than 2 years after the initial qPCR approach to validate the findings on the exon array. Therefore, only 7 genes were selected by qPCR for confirmation of the exon array findings. Instead of qPCR, the panel of NanoString markers was used for validation of the panel of biomarkers. The NanoString data set confirmed that the best prediction was based upon 10 NanoString genes of the original 18 exon array genes.

Bioinformatic Analysis of the Biomarker Panel

A recent mega-analysis of differentially expressed genes in SZ across 9 studies was conducted in blood-based transcriptomics [35]. There were 1,624 genes that survived the FDR that were compared to the top 122 genes identified by ANOVA in the present study. The formula for the expected number of genes was calculated as:

$$\text{Expected gene number overlaps}[(p_1 p_2) \times 24,000] \\ = \text{where } p_1 \text{ is } \frac{\text{observed genes in list 1}}{24,000} \text{ and } p_2 \text{ is } \frac{\text{observed genes in list 2}}{24,000}.$$

This formula yields an expected number of 8.2 genes, while the actually observed number was 9 genes; we conclude that the overlap was not exceeding chance level. Interestingly, 2 mitochondrial genes were found among these top 9, and they agreed in fold change direction across the two studies (Table 11): (1) mitochondrial ribosomal protein L42 (MRPL42) and (2) transcription factor B1, mitochondrial (TFB1M).

Regarding genes implicated in BD or SZ from a database of genome-wide association study (GWAS)-curated genes (GWASdb v2 [69]) there were 2,189 unique genes meeting this criterion (*p* < 0.001). In our list of 122 differentially expressed genes for BD and SZ that were temporally stable, there was an overlap of 22 genes with the GWASdb genes (Table 12), resulting in a 2-fold enrichment, as only 11 genes were expected (*p* = 0.029, one-tailed Pearson).

An IPA (Ingenuity Pathway Analysis) of the top 122 genes for the BD and SZ biomarker panel showed some overlap in a network related to 2 different proto-oncogenes: (1) REL and (2) MKL2. The REL gene (REL proto-oncogene, NF-κB subunit) encodes a protein that belongs to the Rel homology domain/immunoglobulin-like fold, plexin, transcription factor (RHD/IPT) family. This proto-oncogene plays a role in the survival and proliferation of B lymphocytes. SNPs in this gene are associated with susceptibility to ulcerative colitis and rheumatoid arthritis. The direct REL gene targets were differentially expressed in BD and SZ (Fig. 3). Opposite fold change genes related to REL were seen in BD and SZ; for instance, ATP11A was up-regulated in SZ (1.68) and downregulated in BD (-1.63),

Table 12. Overlap between GWASdb v2 [69] and the current study of differentially expressed genes in BD and SZ

Overlap BD and GWASdb v2	Overlap SZ and GWASdb v2
CNTNAP2	CDC42BPB
TBC1D1	KANK1
EDIL3	PRICKLE1
LRRC16A	SLC44A5
TCEA3	FCER2
NRCAM	AK5
PAX5	DSP
EXO1	DNAH6
JUP	CNTNAP2
	C22orf34
	NPAS2
	SUMF1
	PAX5
	LAPTM4B
	EXO1
	LRRC16A
	ADARB2

GWASdb contains lists of genes that have been associated with either or both disorders by GWAS. There was an enrichment of the overlap of genes found to be differentially expressed and associated with disorder. GWAS, genome-wide association study; BD, bipolar disorder; SZ, schizophrenia.

while an opposite relationship was seen for PHF14 (down-regulated in SZ [−2.01] and upregulated in BD [2.95]).

MKL2 is a proto-oncogene which is widely distributed, highly expressed in brain regions such as the dentate gyrus (Allen Institute Brain Science [70]), and associated with microcephaly [71]. The MKL2 gene consists of multiple exons many of which are downregulated in SZ and not significantly changed in BD. The exon levels in blood for MKL2 were differentially expressed in SZ, although not in the top 300 genes (Fig. 4). The downstream effects on MKL2-regulated genes in the top biomarker classification genes were in the expected directions, e.g., there were decreased fold changes seen in genes regulated by MKL2 in SZ (ACTA2, FADS2, and CPA3), while the same set was upregulated in BD.

Two additional genes in the biomarker panels, i.e., PTGDS (prostaglandin D₂ [PGD₂] synthase) and FADS2 (fatty acid desaturase 2), were also found to be candidate genes in the literature. PTGDS expression was reduced in BD PBMCs [72], also in the present study ($p = 0.000271$; fold change −1.23), and it was down in SZ as well ($p = 9.04E-12$; fold change −1.52). In BD and SZ there appears to be a downregulation of PTGDS, which also was ranked

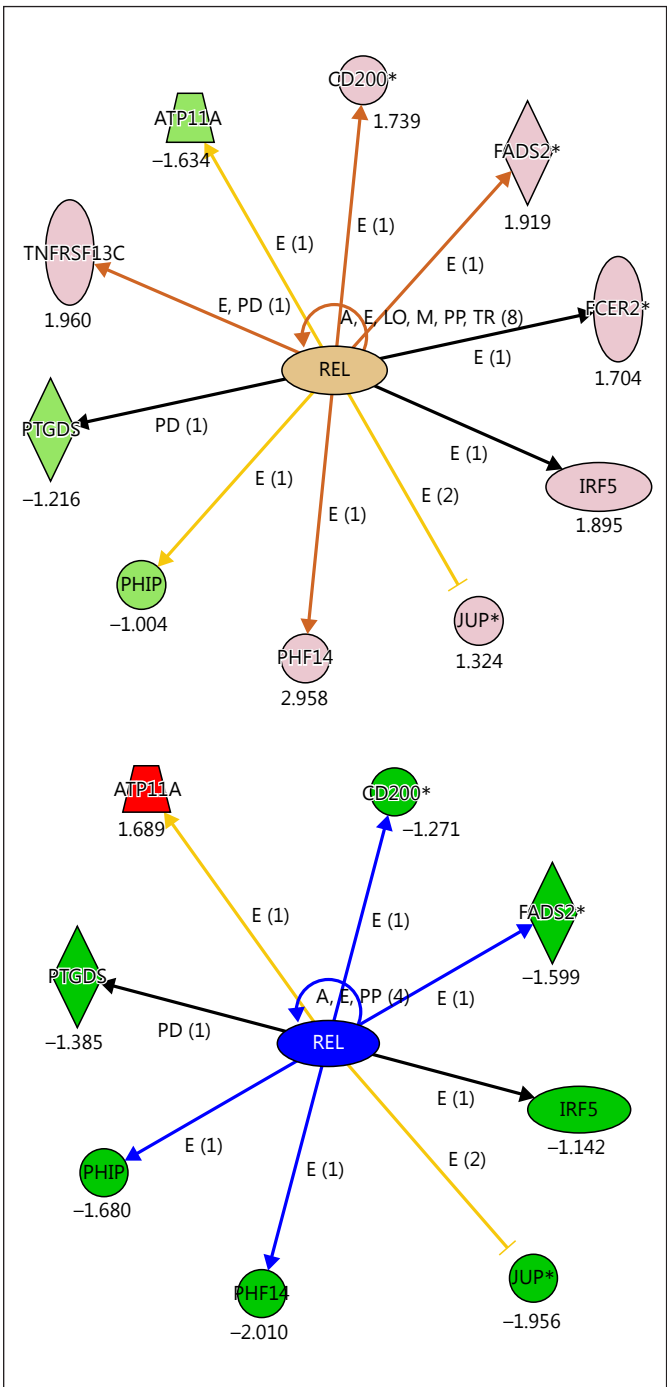


Fig. 3. The REL transcription factor is a central hub for genes in the top 122 differentially expressed list. The top panel shows the relationship for BD compared to NC, the bottom panel shows the relationship for SZ compared to NC. Many of the genes are oppositely regulated across BD and SZ. BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

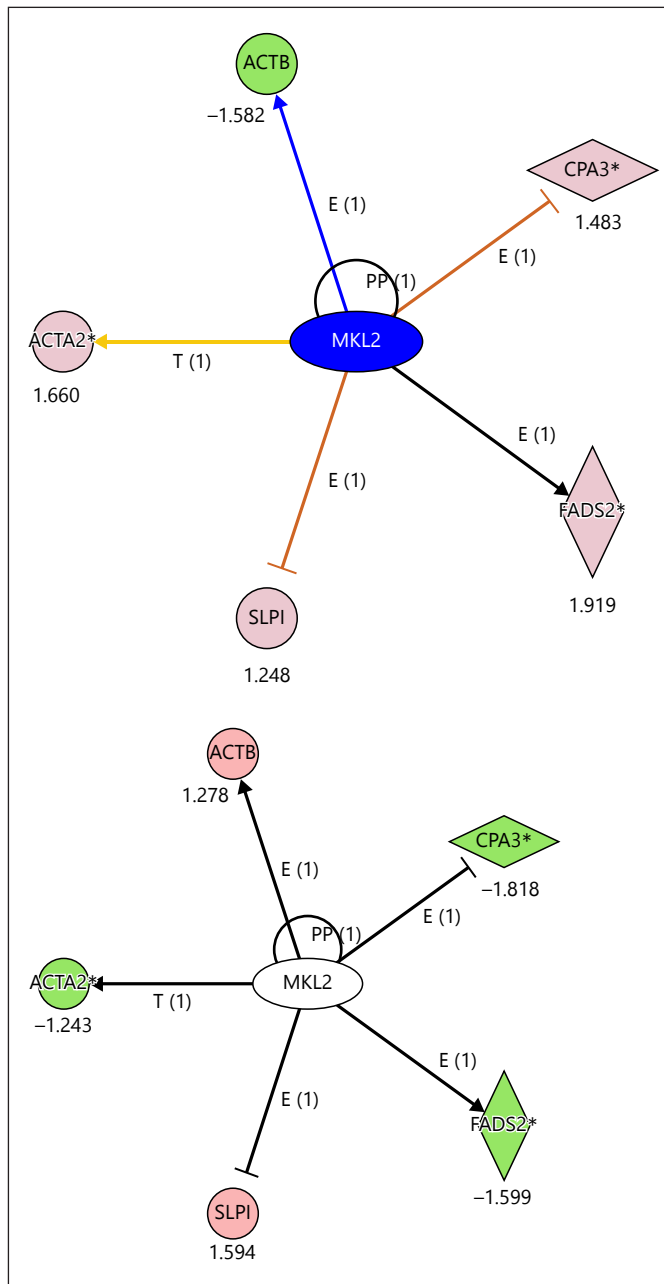


Fig. 4. The MKL2 proto-oncogene is a central hub for genes in the top 122 differentially expressed list. The top panel shows the relationship for BD compared to NC, the bottom panel shows the relationship for SZ compared to NC. Many of the genes are oppositely regulated across BD and SZ. BD, bipolar disorder; SZ, schizophrenia; NC, normal control.

high in the convergent functional genomics paradigm for anxiety [73]. FADS2 expression was decreased in SZ in this study ($p = 9.20\text{E-}09$; fold change -1.80) and increased in BD ($p = 2.79\text{E-}06$; fold change 1.6).

The FADS2 and PTGDS genes work in the biosynthesis of the fatty acid pathway and converge on key molecules in BD such as arachidonic acid. HADHA was significantly increased in BD ($p = 4.74\text{E-}08$; fold change 1.67) and was not changed in SZ. HADHA is closely related to FADS2 and HADHA in the fatty acid pathway (Fig. 5) and along with PTGDS participates in the biosynthesis and degradation of unsaturated fatty acids, an important pathway implicated in BD and SZ.

Discussion

To determine stable temporal biomarkers, this study evaluated whole blood gene expression at two different time points using the same subjects (SZ, BD, and NC) for differential diagnosis. The diagnostic algorithm used logistic regression modeling and a total of 18 uniquely expressed exons within known mRNA transcripts. The model discriminated SZ and BD from each other, as well as both from healthy controls in four steps. The upper limit of accuracy achieved in this biomarker study was 88%, using an independent visit of the same patients. When using the “leave-one-out” evaluation algorithm, the results were very consistent with the actual data; thus, the “leave-one-out” analyses indicated that the models were not driven by outliers and that they should be predictive when applied to independent data cohorts.

It is expected that the application of these panels to first-episode or prodromal subjects may improve prediction for those subjects that ultimately convert to either illness, as well as for the millions of patients worldwide that have not received any clear diagnosis of their ongoing disorder. This will require an additional validation study of the biomarker signatures with a larger cohort in a follow-on project.

The differences in expression of 3 genes (PTGDS, FADS2, and HADHA) related to polyunsaturated fatty acid (PUFA) and prostaglandin biosynthesis were used for the final biomarker panels to differentiate between SZ, BD, and NC. Previously, these genes have been associated with psychiatric disorders such as BD, major affective disorder, SZ, and anxiety. PTGDS is involved in the synthesis of PGD_2 from PGH_2 , the cyclooxygenase-mediated product of arachidonic acid which is a PUFA [4]. PTGDS is a top anxiety gene modulated by changes in PUFA (omega-3 fatty acid docosahexaenoic acid) [73] on the convergent functional genomics scale. Increased expression of FADS2 has been found in SZ and BD brains post

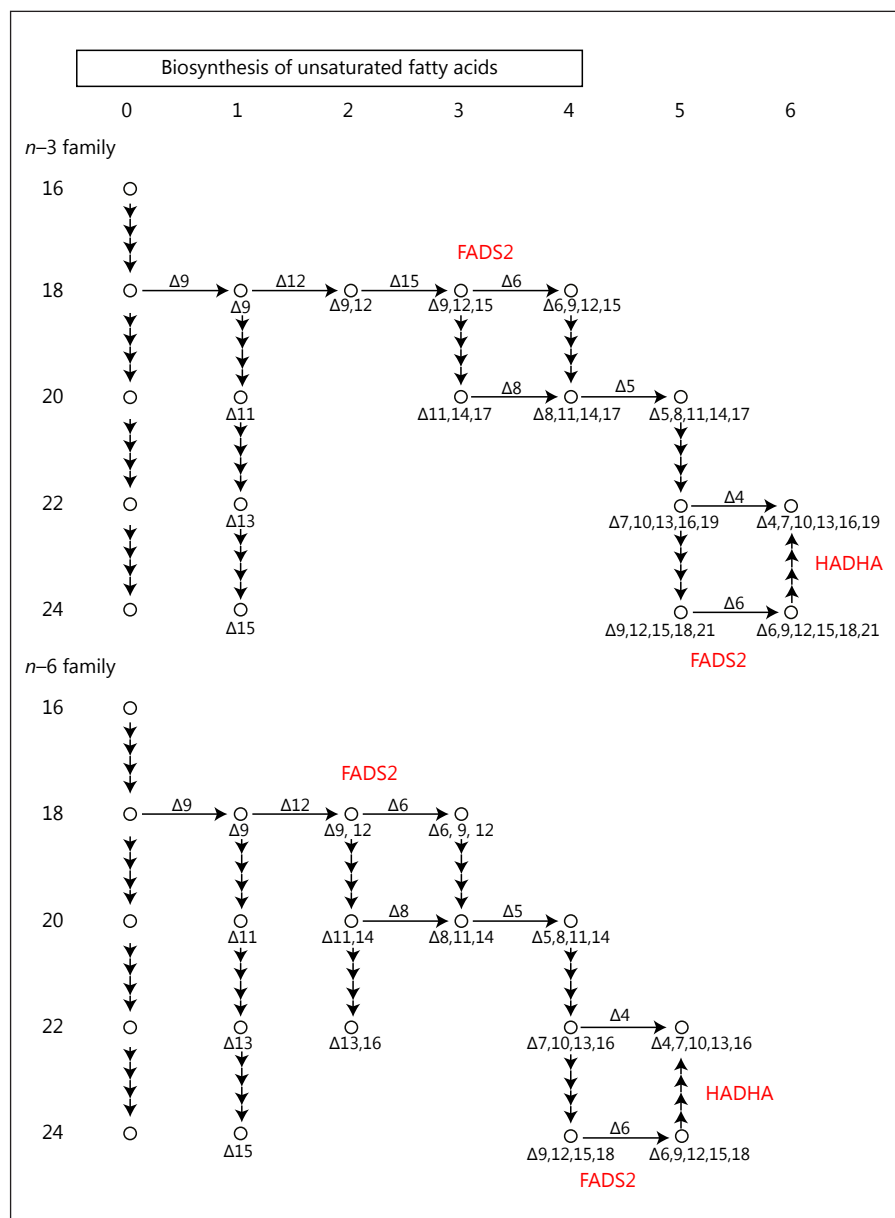


Fig. 5. Modified KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway labels for FADS2 and HADHA participate in the conversion of polyunsaturated fatty acids and fatty acid β -oxidation.

mortem [74, 75]. FADS2 activity is increased in BD and is associated with suicidal behavior [76].

In the present study, we found an increased expression of FADS2 in BD, in agreement with the FADS2 findings reported. The increased activity of FADS2 could reduce PUFA levels of both arachidonic acid and eicosapentaenoic acid by promoting conversion to longer-chain fatty acids, shown in both the *n*-3 and the *n*-6 pathway (Fig. 5). Thus, PUFA supplementation with *n*-3 fatty acids in mood disorder was effective in reducing mood symptoms in 4 out of 7 well-controlled studies [76]. The expression

data for FADS2, while interesting, could be subject to dietary influence, such as amounts and types of daily dietary intake of fatty acids, the timing of intake, and also medication effects on these genes. Further, genetics plays a significant role, especially in modulating levels of fatty acids and FADS2 expression. Another limitation to the assessment of these genes as representing actual pathophysiological markers is that, potentially, stress could modulate the biomarker panel genes. Many patients with BD and SZ experience higher levels of stress than controls, which might explain differences in immune cell activation and

prostaglandin synthesis. We examined our biomarker panel of 18 genes in our unpublished stress data set (M. Martin and M.P.V.) using the same exon array approach and Tempus tube approach on healthy volunteers who underwent sleep deprivation and 9 repeated blood draws over 54 h, i.e., every 6 h. We checked our results for the healthy controls and found that 4 transcripts that passed Bonferroni corrections were affected by time of day and, potentially, stress induction as well (DDX5, EEF2, HADHA, and CCDC109B). However, these 4 genes did not vary in the present study, even though in the stress data set these genes varied by time of day. Taken together, although these 4 genes were dysregulated as a consequence of time of day and sleep deprivation, these genes in the present study were stable across 8 weeks of time, and would have been expected to show some fluctuation with stress levels or time of day.

Several design points could be potential limitations as well. First, the study was not designed to test for treatment effects or treatment response. Although we have some psychiatric ratings available for the subjects, a feasible approach would be to identify treatment responders versus nonresponders using a within-subject design, comparing the severity of psychotic symptoms before and after 8 weeks of treatment, and correlating stably expressed biomarkers with response to treatment. This alternative approach would yield useful information, and is planned for a follow-on analysis of the data to explore biomarkers of treatment response. An inspection of the “treatment responders” in the study showed that the majority of patients did not change their medications, were clinically stable, and maintained similar psychiatric ratings across the 8 weeks. Further, for differentiating diseased from nondiseased groups, we adopted an outpatient case-control analysis. Another approach that could yield potential data in a future test of the biomarker panel is whether unaffected siblings of psychiatric patients could be reliably differentiated by the panel. This approach could be utilized as well in future studies. The current biomarker panel could also be used on subjects with major depressive disorder to examine if there is some coherence to a subclassification into more psychotic (in the SZ group) or more affective disorder (in the BD group) for aiding in potential treatments for these patients. These directions will go far in providing useful information to clinicians, researchers, and patients to guide our understanding of these illnesses.

A tremendous effort has been expended into GWAS of SZ [77] and BD [78], and it is recognized that there is a large number of common variants contributing to the polygenic susceptibility for these disorders. Estimates

from several hundred genes of small effect size, as published in the largest international genetic study of SZ [79], to the possibility that thousands of genes are involved in the pathogenesis of SZ were made [80]. We have highlighted several genes that contribute towards this susceptibility using transcription analysis (Table 12), finding some overlap between GWAS variants and differential gene expression. Our present biomarker results overlap with a genetic database of genes implicated in either BD or SZ or both from a database of GWAS-curated genes (GWASdb v2 [69]). We found a significant overlap with our list of 122 differentially expressed genes for BD and SZ that were temporally stable (Table 12). The overlap of 22 genes suggests that peripheral biomarkers of either SZ or BD may have some genetic component that could drive differential expression. This leaves open the possibility that a number of serum proteins [81–85] and transcripts will advance as biomarkers.

Additionally, 2 upstream transcription factors merit further investigation, i.e., REL and MKL2, which appear to oppositely regulate genes in BD and SZ. The downstream targets of the genes were used to differentially diagnose subjects with BD and SZ. These proto-oncogenes have not previously been associated with psychiatric disorders. The expression of these 2 transcription factors (REL and MKL2) was studied in the cerebral cortex and shown to be expressed in microglia and endothelial cell types at higher levels than in neurons and astrocytes [86]. This raises the possibility that blood transcriptomics is mirroring some of the microglial/endothelial cell activity in the brain. It is known that peripheral cells can interact with brain microglia and endothelial cells.

There are prior reports supporting the directions of gene expression changes found in this biomarker study. For example, there was a consistent increase in expression of IL5RA (interleukin 5 receptor, alpha) in lithium-treated subjects with BD in PBMCs that passed a strict FDR [87]. In the final biomarker panel, we also found a trend for an increase in IL5RA expression in BD (p value for BD = 0.056; fold change 1.16); however, IL5RA expression in SZ was significantly decreased and passing the FDR (p value for SZ vs. NC = $2.65\text{E}-08$; fold change -1.59). Regarding PLB1 (phospholipase B1) in PBMCs, lithium decreased its expression (-1.17 fold change), passing the FDR [87] in PBMCs, while in the present study, PLB1 was significantly decreased in BD by -1.19 and was not significantly increased in SZ ($p = 0.06$; fold change 1.16). It is unlikely that some expression in the biomarker panel might be due to lithium treatment, since only 2 BD subjects were lithium treated in this study. An-

other ramification of using biomarkers is to provide a method for monitoring drug efficacy and other appropriate early psychosocial interventions for mental disorders. In a planned separate analysis, we will further correlate the phenotypic neuropsychiatric rating data obtained from each patient along with the gene expression data.

Over 100,000 adolescent Americans suffer from symptoms of psychosis each year, as well as millions of patients worldwide that have not received any clear differential diagnosis of their ongoing disorder; yet, currently, there are no biomarker tests that are FDA approved to classify SZ or BD. There is a serious need for “objective” clinical laboratory tests for an early diagnosis of these mental disorders, since today these disorders may typically take months or even years to reach a diagnosis and for patients to receive effective treatment. The lag in treatment is associated with an increase in suicide rates and recurrent episodes of psychosis and mood dysregulation. There is a large increase in deaths reported among first-episode psychotic subjects due to lack of treatment after the first year of illness [1]. Thus, it is important to have objective biomarkers to help implement treatment at an early stage. One estimate of the direct and indirect annual costs in the USA for SZ is USD 174 billion [88], with an additional cost of USD 151 billion for BD [89]. Biomarker signatures could lead to faster and more accurate diagnoses, reducing the duration of untreated psychosis, suicidality, and

cognitive decline and adding to an understanding of the shared and unique pathophysiologies of each disorder. The blood test results that are described in this paper, if further validated in a larger number of subjects, will offer molecular diagnostic support for psychiatrists’ clinical evaluation with rapid clinical laboratory test results.

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Statement of Ethics

The University of Iowa and the Institutional Review Board approved the procedures in the study. The chronic SZ and BD type I outpatients aged 18–45 years provided consent for the study.

Disclosure Statement

A SBIR Phase I project “Gene expression exon array biomarkers to diagnose schizophrenia” (R43 MH090806) was awarded (T.W.O.) for developing a blood-based commercial biomarker test for psychiatric disorders. The authors (M.P.V. and T.W.O.) are officers of Laguna Diagnostic, LLC, and co-inventors of a patent application to commercialize the results presented.

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