UNIVERSITY OF CALIFORNIA

Los Angeles

The molecular signatures of bipolar disorder and lithium treatment

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Human Genetics

by

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2018

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Human Genetics

University of California, Los Angeles, 2018

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Bipolar disorder (BD) is a highly heritable mood disorder with a complex genetic architecture. It is commonly treated prophylactically with the mood stabilizer lithium, although treatment responses vary widely across patients. Both how BD genetic variants confer risk and the molecular mechanisms underlying lithium's therapeutic effects remain poorly understood. This dissertation begins with a review of recent findings from BD and lithium-response genetic studies and from BD and lithium treatment transcriptomic studies. This review will show that while presenting an opportunity to learn valuable information about underlying biology, gene expression studies investigating these phenotypes have had low sample sizes and inconsistent findings. Then, an original study attempting to fill this gap by exploring the whole blood transcriptome in a large BD case-control RNA sequencing sample is reported on. In this study, strong effects of lithium treatment and cell-type composition were revealed, pointing to potential therapeutic mechanisms of lithium, and underlining the importance of carefully correcting for these variables. To put these findings in the context of the current understanding of BD etiology

and lithium treatment mechanisms, a comparison was made with findings previously reported highlighting a list of high-confidence lithium-associated genes. A gene-set analysis comparing genes with differential expression to genes implicated from major psychiatric genome-wide association studies revealed that the observed gene expression changes were unrelated to genetic risk. The findings herein contribute to the current understanding of the BD transcriptome in whole blood and provide evidence for the mechanistic actions of lithium treatment.

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For my father, Britt Stevens Krebs.

He is the original reason for my desire to work in psychiatric genetics.

Since the beginning of my academic journey, I have hoped to contribute to work that aims at ultimately improving quality of life for individuals with mental illness.

I miss him every day.

For all people suffering from mental illness, especially those who have been failed repeatedly by our broken systems and reside untreated and unhoused.

All of us deserve healthcare, housing, justice, and dignity.

Let us never forget that our science is nothing without humanity.

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ACKNOWLEDGEMENTS

Thank you to the following individuals for their contributions to Chapter 4, a co-authored work currently in preparation for publication: Anil P.S. Ori, Annabel Vreeker, Timothy Wu, Rita M. Cantor, Marco P. Boks, Rene S. Kahn, Loes M. Olde Loohuis, and Roel A. Ophoff.

Thank you for support, guidance, and training, to my doctoral committee co-chairs and members: Roel Ophoff, Nelson Freimer, Chiara Sabatti, Eleazar Eskin, and Sriram Kosuri; to research faculty: Paivi Paijukanta, Rita Cantor, Jeanette Papp, Eric Sobel; and to program directors and advisers: Esteban Dell'Angelica, Gregory Payne, and Jeffrey Goldman. Thank you to Ophoff Lab members for providing an exceptional lab environment in which to learn and grow. Thank you to my classmates, friends, and family who continually teach me lessons in science, strength, and laughter. Thank you to everyone who helped us get Brewin' Talks and the Science Policy Group at UCLA off the ground. Thank you to my labor union, UAW 2865, for giving a powerful collective voice to me and my fellow graduate student workers in the fight for our workplace rights, protections, and benefits. Thank you to the Student Labor Advocacy Project for showing me what true student-worker solidarity looks like. Thank you to the American Foundation for Suicide Prevention for teaching me to harness hope and healing in the face of tragedy. Thank you to the Big Blue Bus operators who have taken me to and from campus countless times. And thank you to the tens of thousands of campus workers, including custodians, dining hall workers, landscapers, maintenance workers, bus drivers, and clerical workers, who keep the university running and without whom the academic pursuits of me and my colleagues would not be possible.

This work was supported by funding from the NIH Training Grant in Genomic Analysis and Interpretation T32HG002536 and from the NIMH funded R01 Grant R01MH090553.

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CHAPTER 1

Introduction

Bipolar disorder (BD) is a debilitating psychiatric disorder characterized clinically by oscillations in mood resulting in depressive and manic states interspersed between neutral, euthymic states[1]. The hallmark features of a depressive state are sadness, low energy, social withdrawal, hypersomnia, and low self-esteem, whereas the features of a manic state are expansive mood and behavior, hyperactivity, grandiosity, reduced need for sleep, and increased self-esteem. There is no diagnostic test for BD, and its phenotype is defined solely by its clinical features. According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), there are several clinical subtypes of BD that are distinguished based on the severity and duration of manic and depressive episodes[2]. BD type I is characterized by at least one full episode each of depression and mania. BD type II is characterized by at least one full episode of depression and at least one episode of hypomania, which is a less severe version of mania that does not impair function or interfere with daily life. Cyclothymic disorder is another type of BD characterized by less severe episodes of both depression and mania. Finally, there is a category of BD in the DSM-5 for other specified bipolar and related disorders that do not fulfill the diagnostic criteria for BD-I, BD-II, or cyclothymic disorder, and there are substance- and medication-induced bipolar and related disorders, in which bipolar symptoms should not recur once the causal element is removed.

In addition to episodic mood symptoms, patients with BD can experience cognitive disturbances[3] and psychosis. Although diagnostically distinct, there is extensive clinical overlap between BD, schizophrenia (SCZ), and major depressive disorder (MDD)[4,5]. Symptoms of a depressive episode (depressed mood, low energy, and withdrawn interests) can be observed in

all three disorders[6], as can the symptoms of a psychotic state (delusions, hallucinations, and disorganized thoughts)[7]. Accordingly, although BD is classified separately from psychotic disorders and affective disorders by the DSM-5, it is considered to be a part of the psychosis spectrum[8] and along the continuum of mood disorders characterized ranging in various proportions of depression and mania[9]. Furthermore, BD is often comorbid with other psychiatric disorders such as anxiety disorders, attention-deficit/hyperactivity disorders, and substance use disorders[10]. This clinical heterogeneity leads to problems of underdiagnosis and misdiagnosis, causing an estimated 5-10 years on average from disease onset to accurate diagnosis[11].

BD affects approximately 1% of the population worldwide[12,13]. Individuals with BD are far more likely than the general population to die by suicide[14], and the disease costs billions of dollars each year in health care costs, disability, and early loss of life[15,16]. Understanding the causes of BD, both inherited and environmental, is crucial for alleviating the problems with diagnosis and treatment of BD and ultimately for improving the quality of life for individuals with BD. With the past decade of advances in genomic technologies, much has been learned about the genetic architecture of BD, the ways in which genetic variants confer risk for BD, and gene expression patterns associated with BD, all of which have provided insight into elusive pathophysiology. In *Chapter 2* I discuss genetic and transcriptomic strategies for uncovering clues about BD-causing mechanisms and review findings from recent studies.

The management of patients with BD involves the acute treatment of manic or hypomanic episodes, the acute treatment of depressive episodes, along with maintenance care to prevent relapse and recurrence[17]. Lithium was the first medication to be approved by the US Food and Drug Administration for the treatment of acute mania in 1970[18], and since then 15 additional mood stabilizing or antipsychotic agents have been approved for the treatment of acute mania, bipolar depression, and/or maintenance therapy[1]. Lithium is the third smallest element and occurs naturally in the earth as a salt. It had been used for decades for its anti-gout

properties by the time it was first discovered to have sedative effects and subsequently used to treat mania in the 1940s[19]. It has a number of physiological effects that, unlike other psychopharmacological agents that target neuronal receptors, act mainly by altering intracellular second messenger systems via enzymatic inhibition in the phosphatidylinositol signaling and the glycogen synthase kinase 3 pathways[20]. It remains unclear which of these widespread effects are therapeutic, although theories exist that the molecular mechanisms of lithium converge to biological functions related to neuroprotection and neural plasticity, chronobiology, and stabilization of neuronal activity[21]. The therapeutic window of lithium dosage is very small, above which it becomes toxic and can lead to renal impairment, and therefore must be carefully monitored via regular serum concentration measurements[22,23]. Even with careful monitoring, patients undergoing long-term lithium treatment may experience a number of adverse side effects including hypothyroidism, gastrointestinal problems, polyuria, tremor, hyperparathyroidism, skin problems, among others, which contribute to non-adherence rates as high as 40%[22].

In addition to high non-adherence rates, there is significant interindividual variability in terms of therapeutic response to lithium treatment, with only about 30% of BD patients fully responding to this medication[21]. Family-based studies have suggested a genetic component underlying the lithium-response phenotype[24,25], and genome-wide association studies have provided some evidence for the role of common genetic variation in lithium response[26,27]. Despite issues of non-adherence and non-response, lithium remains the first-line treatment option for patients with BD due to its efficacy in preventing relapse and recurrence, its ability to treat acute mania, and its ability to reduce suicide risk and overall mortality in patients with BD[28,29]. Aside from providing insight into the elusive therapeutic mechanisms of lithium, studying the genetics of lithium response may eventually provide valuable information for clinicians and patients to avoid unnecessary treatments. In *Chapter 2* I review pharmacogenomics studies that

have explored the genetic basis of lithium response, and discuss transcriptomic studies that have examined the molecular signatures of lithium treatment.

The review in *Chapter 2* will show that gene expression studies investigating BD and lithium treatment phenotypes, while promising for uncovering mechanistic properties, have suffered from low sample sizes and had largely inconsistent findings. In *Chapter 3*, we present our own attempt to gain insight into the molecular mechanisms of BD and lithium treatment. In this original study, we explored the whole blood transcriptome in a large BD case-control RNA sequencing sample via differential gene expression and weighted gene co-expression network analyses. Results revealed little to no effects associated with BD diagnosis but pronounced effects related to lithium treatment and cell-type composition. We highlight functional pathways implicated in the cellular response to lithium, and underline the importance of carefully correcting for medication use and cell types in gene expression studies. We compared these results with findings previously reported and show a significant, concordant overlap with two previous studies of similar design. From these we announce a list of high-confidence genes that display altered expression in response to lithium treatment. A gene-set analysis comparing genes with differential expression to genes implicated from major psychiatric genome-wide association studies was also performed, revealing no enrichment of genetic signal in the lithium-use genes we discovered. We show how the above findings contribute to the current understanding of the BD transcriptome in whole blood, and provide evidence for the mechanistic actions of lithium treatment.

In *Chapter 4*, I conclude by putting these results in the context of the current understanding of BD etiology and lithium treatment mechanisms, and discuss future directions for this body of work.

CHAPTER 2

Review of bipolar disorder genomics and lithium pharmacogenomics

2.1 Introduction

BD is a chronic and recurrent disorder characterized by biphasic mood episodes of mania or hypomania and depression[1]. It affects approximately 1% of the world population[12] and is a major cause of functional impairment, disability, and mortality[15]. Time to diagnosis is often delayed[11], but when correctly diagnosed, BD symptoms can be managed. However, even with accurate diagnosis and treatment, episodes can be highly recurrent and the disease is associated with poor quality of life for patients[16,30]. In order to reduce time to diagnosis, to treat patients better, and to improve quality of life, it is crucial to understand the risk factors and biological mechanisms underlying BD. Risk for BD is partially environmental, and some environmental factors have been identified including childhood adversity[31], advanced paternal age[32], and others[33]. But the proportion of risk for BD due to genetic factors, or the heritability of BD (h^2), is very high, with estimates of up to 85%[34]. The familial clustering of response to lithium treatment suggests a genetic component to this phenotype as well[25], and it has even been purported that lithium response represents a distinct genetic subtype of BD with homogeneous clinical and molecular profiles[35]. Genetic inquiries about lithium response have the potential to improve diagnostics and management, and even provide insight into the etiology of BD. In this chapter, I review recent findings in BD and lithium-response genetics, review strategies for characterizing the mechanisms of genetic risk for these phenotypes, and discuss the findings of transcriptomic approaches.

2.2 Bipolar disorder genetics

Physicians have described the familial nature of psychiatric disorders since the early 1900s, when what we now know as bipolar disorder was still called *manic depressive illness*[36]. Indeed, early genetic epidemiological studies of BD demonstrated substantial heritability, spurring decades of linkage analysis studies[37]. These linkage studies, by scanning the genome for regions shared between family members, assumed that genetic risk for BD resided in one major locus or very few loci of the genome, and that causal variants followed a simple Mendelian pattern of inheritance and would thus segregate between affected and unaffected family members of a pedigree. They were altogether unsuccessful[37], which we now know is due to BD's complex genetic architecture and high degree of polygenicity[38], or heritability arising from a large number of loci each with small effect.

The polygenic nature of BD and other common disorders was not revealed until technologies allowed for genome-wide scans of common genetic variation. The most prevalent type of variation in the genome is the single nucleotide polymorphism (SNP). SNPs are single base-pair changes to DNA that are common in the population (usually with frequencies greater than 5%). Genome-wide association studies (GWAS), which compare frequencies of SNP alleles across traits in large samples, have definitively demonstrated the highly polygenic nature of BD[39-42]. The proportion of risk for BD due to common variation in the form of SNPs, also known as SNP-based heritability (h^2_{SNP}), is about 25% and is highly shared with SCZ (genetic correlation, $r_{g\,SNP} \approx 0.68$) and MDD ($r_{g\,SNP} \approx 0.47$)[43]. The largest BD GWAS to date, performed on 20,352 cases and 31,358 controls, discovered 31 SNPs in 30 loci that were associated with BD at genome-wide significance ($P < 5x10^{-8}$) including 20 novel loci[42]. Associated alleles, also called risk alleles, within these loci have small effects, with odds ratios < 1.2, hence the need for very large case-control samples for discovery. The estimated variance

explained by these alleles was only ~8%, meaning the locations of common genetic risk alleles associated with BD are still mostly unknown.

Risk alleles in a given individual can be weighted according to their measured GWAS effect and summed to form a polygenic risk score (PRS), which is essentially a measure of an individual's genetic risk for disease[44]. But because PRS scores calculated from even the largest BD GWAS only explain a small amount of variance in liability (8%), their use for the prediction of an individual's risk for BD is not yet powerful enough to discern between cases and controls and they therefore have limited clinical utility[42]. As BD GWAS sample sizes increase, more genetic risk variants will be discovered, more variance in genetic liability will be explained, and the predictive power of BD PRS scores should improve.

Although as of yet limited in their clinical utility, BD GWAS findings have shed light on the disease's etiology. Early BD GWAS were small and unconvincing, and it wasn't until the total sample reached over 10,000 that discovery and replication were successful[45]. There, in a collaborative effort combining data from three separate studies, two regions of association were reported on that included genes that are now widely accepted as being causal in BD etiology, *ankyrin 3* (*ANK3*) and *calcium voltage-gated channel subunit alpha-1C* (*CACNA1C*)[45]. The proteins encoded by these genes play important roles in neuronal function: ankyrins link integral neuronal membrane proteins like sodium channels to axonal cytoskeletons[46], and calcium channels mediate membrane potential and neuronal signaling[47]. The Psychiatric Genomics Consortium (PGC) was formed in 2007 as a way to pool samples gathered by different investigators and perform very large GWAS for psychiatric disorders in order to optimize power for genetic discoveries (www.med.unc.edu/pgc). The first BD GWAS performed by the PGC in 2011 was the largest until that point, with 7,481 cases, 9,250 controls, and over 45 thousand samples available for replication[39]. In this study, two loci were replicated at genome-wide significance, which include the genes *CACNA1C,* supporting previous findings, and *ODZ4*,

providing novel evidence for this gene that encodes a neuronally expressed transmembrane protein involved in axon guidance[39,48]. Since then, several additional large BD GWAS have been performed, confirming previously implicated susceptibility loci and suggesting many novel ones[49]. However, the strongest signal within many of these loci is in a non-coding variant, many loci contain multiple genes, and the causal variants remain largely unknown. Therefore the functional mechanisms of BD-associated variants remain largely elusive. Nevertheless, promising genes within loci associated with BD (e.g. *CACNA1C, GRIN2A, ANK3, TRANK1)* so far seem to implicate functional pathways related to ion channels, neurotransmitter channels, and other synaptic components[42].

As is the case with many complex genetic diseases, common variation only partially explains BD heritability ($h^2_{SNP} \approx .25$ compared with $h^2 \approx .75$)[43,50]. The additional risk that is unaccounted for may be explained by rare variants in the form of single nucleotide variants, small insertions and deletions, and larger copy number variants (CNVs) that span thousands to millions of base pairs and often encompass several genes. Rare (allele frequency < 1%), highly penetrant (convey high risk) CNVs have been found in other psychiatric disorders, particularly in SCZ and autism spectrum disorders, but the findings in BD have not been as clear[51]. A recent study analyzing large, rare CNVs found one CNV locus associated with BD, a duplication at 16p11.2 that is the most significant BD CNV to date, and found evidence confirming the hypothesis that CNVs contribute less to BD risk than to schizophrenia risk[52].

Smaller rare variants of just one to a few base pairs in length are more difficult to detect as they require exome or genome sequencing. These technologies are much more expensive to perform than the SNP arrays that detect common variants and large CNVs. Additionally, directly testing for differences in rare alleles like in GWAS is difficult because rare variants have such small allele frequencies. Therefore, rare variant studies in BD have either tested for high penetrant variant segregation in pedigrees (where alleles segregate with diagnosis in a

Mendelian fashion), or have tested groups of less penetrant variants aggregated by gene or functional set to increase power. Sequencing studies of BD pedigrees have not been successful at finding large effect variants with Mendelian patterns of inheritance[53-56]. However, studies have shown increased burdens of rare variants associated with BD, specifically in genes involved in neuronal excitability[57] and in genes likely to be relevant for BD[56], suggesting that the genetic risk for BD not arising from common variation comes from small effect rare variation.

2.3 Lithium-response genetics

Understanding the predictors for response to lithium in patients with BD is crucial not only for optimal management and improvement of quality of life but also for uncovering causal mechanisms of this apparent subtype of BD. Several clinical predictors are well-understood and remain the most useful factors in predicting response to lithium treatment, including course of illness, family history of BD, family history of lithium response, age of BD onset, number of hospitalizations, and classic clinical presentation of BD, that is BD without mixed states, rapid cycling, and psychotic features[58]. That lithium responders tend to have a family history of BD has been recognized for decades[59]. More recently, it has been suggested that lithium-responsiveness is itself familial. In one study, a total of 64 subjects were examined showing significantly higher rates of response in family members of responders (67%) than non-responders (35%; $P = 0.014$)[25]. In another study, out of 15 subjects with BD, patients who responded to lithium had a lithium-responsive parent and patients who responded to antipsychotics had a lithium non-responsive parent[24]. While small, these studies suggest that variation in the lithium-response phenotype may have a genetic basis, and a consortium, The International Consortium on Lithium Genetics (ConLiGen), has been set up to investigate this hypothesis in large samples[60]. It has even been argued that the homogenous phenotypic nature of lithium-responsiveness may represent a distinct heritable subtype of BD[35]. If the genetic

predictors for lithium response can be untangled, they have the potential to become a powerful tool in personalized medicine for patients with BD.

The evidence for lithium response clustering in families has indeed stimulated an era of genetic studies investigating this phenotype. As with BD and other complex traits, the results from candidate gene studies should be met with caution due to their limited sample sizes and biased nature[61]. Linkage studies have also been unsuccessful, suggesting that if genetic liability for lithium response exists, it is most likely comprised of common variants of small effect[62-64]. Estimates for the SNP heritability of lithium-responsive BD measured against healthy controls is about the same as the SNP heritability for the broader BD phenotype: between 0.25-0.29 depending on the definition of response used[26]. From these results it is unclear whether lithium-responsive BD is genetically distinct from non-lithium-responsive BD. Estimates for the SNP heritability of lithium responsive BD measured against non-lithium responsive BD have not yet estimated its SNP-based heritability to be statistically different from zero[26,27]. This could be due to a lack of power (they have been small with <2,563 cases) and, or it could be that lithium response is driven by non-heritable components. A recent study from ConLiGen, leveraging the sample size of the largest PGC SCZ GWAS (36,989 cases and 113,075 controls)[65] and the genetic overlap between SCZ and BD[43] investigated the relationship between SCZ PRS and lithium response in patients with BD[66]. In line with previous family studies that found an association between poor lithium response and a family history of SCZ[67], they found that individuals with BD who did not respond well to lithium had higher polygenic load for SCZ.

Despite the low sample sizes of lithium-response GWAS, two genome-wide significant loci have been discovered, although neither have been replicated[26,27]. The first of these loci, discovered with ConLiGen's dataset of 2,563 BD subjects evaluated for lithium response, is a region on chromosome 21 that includes two long noncoding RNAs of unknown biological function. The other, discovered comparing lithium responders (*N* = 1,639) with healthy controls

(*N* = 8,899) is a region on chromosome 2 that includes the gene *SESTD1*. The implication of this gene, which encodes a protein involved in phospholipid regulation, is in line with theorized mechanisms of therapeutic lithium action on phospholipid pathways[20]. Other GWAS have been performed on lithium response have been unsuccessful[68]. Overall, these results provide some evidence that lithium responders represent a more homogenous subtype of BD, however it remains to be determined if lithium response has distinct genetic liability. Larger sample sizes are needed to show this definitively.

In addition to small sample sizes, another challenge facing studies investigating this phenotype is the method of defining lithium response. Determining whether someone is responsive to lithium takes years to reliably establish and is muddled by non-adherence due to side effects, other medications used, and irregular clinical course[69,70]. Members of ConLiGen have developed a measure of lithium responsiveness called the Alda scale[25,71], which quantifies the degree of improvement in the course of treatment. This score measures the change in frequency and severity of mood symptoms, and is weighted by factors that determine if these changes are a result of the actual treatment as opposed to spontaneous improvement or the effect of an additional medication. In the largest ConLiGen GWAS, they considered both a dichotomous version of the Alda scale score and a continuous one[27]. The locus on chromosome 21 they report is associated with the continuously defined measure of lithium response, indicating that this measure may be better at capturing the supposed heritable lithium-responsive subtype of BD. Other studies have used measures of lithium response entirely different from the Alda scale[26,68]. This lack of consensus as to how to define the phenotype may lead to inconsistent results in future lithium-response genetics studies.

2.4 Functional genomics of bipolar disorder and lithium treatment

Characterizing genetic variation is just one way to understand the underlying biological processes involved in complex traits. Clues about molecular mechanisms can also be uncovered by investigating intermediate, molecular phenotypes that are partially heritable and partially regulated by environmental factors. Genomic technologies now allow for large-scale, unbiased measures of a slew of such molecular phenotypes including gene expression and splicing[72], DNA methylation[73], transcription machinery binding[74], chromatin accessibility[75], long-range DNA interactions[76], and more. The evidence from a decade of GWAS suggests that genetic variants conferring liability for complex traits act in part by altering one or more of these genomic characteristics[77]. Therefore, functional genomic investigations of BD provide the opportunity to learn about the ways in which BD risk variants ultimately lead to the phenotype and about more general genomic properties of the disease state.

Genome-wide scans of gene expression via microarray and sequencing technologies have made studying the transcriptome commonplace for complex traits and increasingly so in the context of pharmacological agents. Arguably the most common type of transcriptome analysis is a differential expression analysis (DEA), which is a search for individual genes with a difference in expression between two conditions, i.e. between cases and controls, or across a continuous trait. These differentially expressed genes (DEGs) may represent consequences of genetic liability, the biological state of the trait itself, or consequences of environmental conditions. Many studies of this type have been carried out for both BD and lithium treatment, and in Table 1 we present a summary of such studies[78-100]. This summary reveals a lack of consistency between study designs and findings, and perhaps most strikingly, shows the low numbers of samples investigated for BD and lithium ($N \leq 62$ BD subjects). We included select studies performed in SCZ and MDD because of their overlapping genetic risk with BD, which may be reflected in the transcriptome, and to illustrate what the results of a study of similar

First author	*Year*	*Tissue*	*Diagnosis*	*Condition tested*	*N samples*	*Platform*	*DEG cutoff*	*N DEGs*	*Pathways/ terms enriched in DEGs*
Elashoff[77]	2007	*Multiple brain regions	BD & HC	BD vs. HC	†284, 331	Microarray	meta *P* < 0.001	375	Energy metabolism, protein turnover, MHC antigen response, RNA processing, intracellular transport activity, stress response, and metallothionein
Matigian[78]	2007	LCLs	BD & MZ	BD vs MZ	3, 3	Microarray	FC > 1.3	82	Programmed cell death, protein metabolism, regulation of transcription, and Wnt signaling
Choi[79]	2011	Prefrontal cortex	BD & HC	BD vs. HC	40, 43	Microarray	FC > 1.3 & FDR < 0.05	367	-
Akula[80]	2014	Dorsolateral prefrontal cortex	BD & HC	BD vs. HC	11, 11	Sequencing	FDR < 0.05	5	‡Transmembrane receptor protein phosphatase activity, regulation of transmission of nerve impule, GTPase binding, regulation of cyclic nucleotide metabolic processes, and cell part morphogenesis
Beech[81]	2014	Whole blood	BD	LR vs. LNR	9, 19	Microarray	FDR < 0.1	62	-
Mostafavi[82]	2014	Whole blood	MDD & HC	MDD vs. HC	463, 459	Sequencing	*P* < 3.6E-6	0	§Interferon alpha/beta signaling
van Eijk[83]	2014	Whole blood	SCZ & HC	SCZ vs. HC	106, 96	Microarray	FDR < 0.05	525	-
Witt[84]	2014	Whole blood	BD & HC	EU vs. HC	11, 10	Microarray	FDR < 0.05	262	-
				MA vs. HC	11, 10	Microarray	FDR < 0.05	216	Human diseases, metabolism, ribosome
				EU vs. MA	11, 11	Microarray	FDR < 0.05	22	-
Xiao[85]	2014	BA9	BD & HC	BD vs. HC	7, 6	Sequencing	FC > 1.5	2,085	Morphogenesis, nervous system development, synaptic transmission, axon guidance, regulation of action potential, ion
		BA24	BD & HC	BD vs. HC	7, 6	Sequencing	FC > 1.5	1,643	Synaptic transmission, signaling, cellular homeostasis, morphogenesis, nervous system development, ion transport,
Cruceanu[86]	2015	Anterior cingulate cortex	BD & HC	BD vs. HC	13, 13	Sequencing	FDR ≤ 0.05	10	G-protein coupled receptor pathways
Madison[87]	2015	Fibroblasts	BD ped	AF vs. UAF	#6, 6	Sequencing	FC > 1.5 & P < 0.05	1	-
		iPSCs	BD ped	AF vs. UAF	#6, 6	Sequencing	FC > 1.5 & P < 0.05	0	-
		NPCs	BD ped	AF vs. UAF	#6, 6	Sequencing	FC > 1.5 & P < 0.05	18	‡Key neuronal processes
Mertens[88]	2015	Neurons	BD & HC	BD vs. HC	6, 4	Sequencing	FDR ≤ 0.1	45	Calcium ion signaling, neuroactive ligand-receptor interaction, PKA/PKC signaling, and action potential firing
Zhao[89]	2015	Cingulate cortex	SCZ & HC	SCZ vs. HC	31, 26	Sequencing	FDR ≤ 0.1	105	Circadian rhythm, prostate cancer, Natural killer cell mediated cytotoxicity, signaling pathways, etc.
			BD & HC	BD vs. HC	25, 26	Sequencing	FDR ≤ 0.1	153	GnRH signaling, taste transduction, vascular smooth muscle contraction, gap junction, Huntington's disease, chemokine signaling pathway, RNA polymerase, Phosphatidylinositol signaling system, apoptosis, etc.
Anand[90]	2016	Peripheral lymphocytes	BD	T vs. UT	22, 22	Microarray	FDR < 0.05	35	Interferon signaling, glucocorticoid, VDR/RXR, EGF and aldosterone receptor signaling, and PI3 kinase signaling
Breen[91]	2016	LCLs	BD	LR-T vs. LNR-T	8, 8	Sequencing	*P* < 0.05	244	DNA repair, protein deacetylation, cellular response to stress, nucleoplasm
			BD & HC	T vs. UT	23, 23	Sequencing	FDR < 0.05	2,803	-
Fromer[92]	2016	Dorsolateral prefrontal cortex	SCZ & HC	SCZ vs. HC	258, 279	Sequencing	FDR ≤ 0.05	693	-
Hess[93]	2016	Whole blood	SCZ & HC	SCZ vs. HC	300, 278	Microarray	mega FDR < 0.1	2,238	Innate immune and inflammatory signaling, cellular stress response, response to androgens, glycotic metabolism, cell survival and growth, DNA repair, mitochondrial function, etc.
Jansen[94]	2016	Whole blood	MDD & HC	C-MDD vs. HC	882, 331	Microarray	FDR < 0.1	129	Interleukin 6 signaling pathway, natural killer cell mediated cytotoxicity, apoptosis, immune response
Pacifico[95]	2016	Dorsal striatum	BD & HC	BD vs. HC	18, 17	Sequencing	FDR < 0.05	14	Immune response, inflammation, and oxidative phosphorylation
Peterson[96]	2016	LCLs	BD ped	AF vs. UAF	193, 593	Microarray	FDR < 0.05	0	-
Fries[97]	2017	LCLs	BD	T vs. UT	62, 62	Microarray	FDR < 0.05	236	Cell death
Kittel-Schneider[98]	2017	LCLs	BD & HC	T vs. UT	21, 21	Microarray	*P* < 0.05E-5	459	Apoptosis, protein transport, cell cycle, RNA processing, etc.
		Fibroblasts	BD & HC	BD vs. HC	10, 11	Microarray	*P* < 0.05E-4	296	Cell signaling, wound healing, cell adhesion, etc.
		LCLs	BD & HC	BD vs. HC	10, 11	Microarray	*P* < 0.05E-5	58	Leukocyte activation, apoptosis, immune response, etc.
Vizlin-Hodzic[99]	2017	iPSCs	BD	BD vs. HC	6, 4	Sequencing	FDR < 0.05	3	TREM1
		NSCs	BD	BD vs. HC	6, 4	Sequencing	FDR < 0.05	42	Inflammation, GABA receptor signaling, dopamine receptor signaling, and TREM1

Table 1. Review of BD and lithium previous studies with differential expression analyses.

(Table 1 legend cont.) *Multiple brain regions including frontal BA46, BA10, BA6, BA8, BA9, and cerebellum. †165 BD individuals (samples partially overlapping). ‡Enrichment analysis was performed on genes with nominal p-values ($P < 0.05$). §Enrichment analysis was performed on genes with small p-values (sets of top N genes, N = [30, 60, 100, 150, 300, 500]). #N = 2 samples with 3 replicates each. Abbreviations: AF, affected; BD, bipolar disorder; BD ped, BD pedigree; C-MDD, current major depressive disorder; EU, euthymic; FC, fold change; FDR, false discovery rate; HC, healthy control; iPSCs, induced pluripotent stem cells; LCLs, lymphoblastoid cell lines; LNR, lithium non-responder; LNR-T, lithium non-responder treated with lithium; LR, lithium-responder; LR-T, lithium responder treated with lithium; MA, manic; MDD, major depressive disorder; MZ, unaffected monozygotic twin; NPCs, neural progenitor cells; NSCs, neural stem cells; SCZ, schizophrenia; T, treated with lithium; UAF, unaffected; UT, untreated with lithium.

	Anand	Breen 2	Choi	Fries	Kittel 1	Kittel 2	Kittel 3	Pacifico
Anand	35	2	0	0	2	0	1	0
Breen 2	2	1,504	25	62	71	15	2	0
Choi	0	25	347	3	7	8	2	0
Fries	0	62 (2)	3	236	15	4	0	0
Kittel 1	2	71 (3)	7	15 (4)	358	12	15	0
Kittel 2	0	15	8	4	12	209	2	0
Kittel 3	1	2	2	0	15 (1)	2	46	0
Pacifico	0	0	0	0	0	0	0	13

Table 2. Overlapping genes between select previous studies. Criteria for list inclusion was: BD or lithium conditions tested, > 10 samples, > 50 DEGs at FDR < 0.1, and differential expression analysis statistics available for download. Lists were filtered to include genes with false discovery rate (FDR) < 0.05. The exact degree of enrichment was not computed due to an unknown number of background genes in each study. Therefore instead, an arbitrary background of 12,000 genes was chosen and the significance rank of the top four overlaps was reported on in parentheses and cannot be considered to be the true rank of significance. The diagonal contains the number of genes in a given list.

design and size might look like for BD or lithium[83,84,93-95]. Upon checking for overlap of genes discovered from these studies (Table 2), the greatest degree of overlap was between two lists from the same study[99]: fibroblast BD versus control (N = 209) and LCL BD versus control (N = 46) with 15 genes shared[99]. The greatest degree of overlap between two lists from different studies was between treated versus untreated LCLs list[98] (N = 236) and the treated versus untreated LCLs list[92] (N = 1,504) with 62 genes shared. While this reinforces the findings of

these three studies and is reassuring given their similarity in design, overall the overlap between all eight lists was low. For the 28 pairwise comparisons made, the median number of genes shared between lists was two. This inconsistency is likely due to varying tissue types, designs, sample sizes, and analysis methods between studies.

Although the sharing of individual genes was low between these studies, several functional patterns emerge from pathway enrichment analyses. In the studies investigating differences between BD cases and healthy controls, terms related to metabolic processes, inflammation and immune response, apoptosis and cell death, and cell signaling are consistently enriched. Studies utilizing brain tissue or neuronal cell lines consistently show enrichment of neuronal-related functions, such as terms related to neurotransmitters and synapses. And in studies investigating lithium treatment, terms related to cell signaling are consistently enriched. These processes contribute to the current understanding of the cellular and molecular alterations associated with BD that cause internal neuronal dysfunction and alter neuronal interconnectivity, such as mitochondrial dysfunction, endoplasmic reticulum stress, neuroinflammation, oxidation, and apoptosis[101]. This neuronal dysfunction may reduce synaptic plasticity, alter circuitry and brain structure, and ultimately lead to the mood symptoms, changes in energy levels, and cognitive impairments associated with BD[102]. Likewise, lithium may restore some of these higher level mechanisms by reversing hyperexcitability and neuroinflammation in a neuroprotective manner[89]. These lines of research investigating the cellular and molecular signatures of BD and lithium are in their early stages. Larger sample sizes and *in vitro* innovations are needed to elucidate these mechanisms with higher reliability and at higher resolution. It is also unclear if these processes are causally linked to BD. Genetic findings should be incorporated with gene expression findings to determine if this is the case.

CHAPTER 3

Whole blood transcriptome analysis in bipolar disorder reveals strong lithium effect

3.1 Introduction

Bipolar disorder (BD) is a debilitating psychiatric disorder affecting approximately 1% of the population worldwide and presenting a major public health burden[13,15]. It is characterized clinically by oscillations in mood resulting in depressive and manic states interspersed between neutral, euthymic states, but its pathophysiological characteristics are not well understood[15]. It is clinically heterogeneous[2], frequently comorbid with other psychiatric disorders[10], and often difficult to diagnose[11]. Even when treated properly, recurrence and relapse are common[16]. The first-line treatment for BD is lithium, which is used not only for the long-term prevention of recurrent mood symptoms but also for the prevention of suicide for the treatment of acute mania[23,29]. However, only about 30% of BD patients fully respond to lithium, it has several adverse side effects, and its mechanisms of action are not well understood[21,22,103].

In order to improve diagnostics, treatments, and quality of life for individuals with BD, it is crucial to understand its risk factors and underlying biological mechanisms. Risk for BD is highly genetic, with heritability estimates as high as 85%[34], and common variation explaining up to a third of that[43]. Family studies suggest that response to lithium treatment has a genetic component as well, but GWAS sample sizes for lithium-response have been low and findings have therefore been inconsistent and sparse[24-27]. Investigating molecular phenotypes as an intermediate measure between genetic variation and clinical variation serves as an alternative strategy for uncovering disease mechanisms. A review of previous work exploring BD and lithium treatment transcriptomics can be found in *Chapter 2*. Briefly, studies have had low

sample sizes and a wide variety of designs and tissue types, and findings have been largely inconsistent (see Table 1).

Therefore, to explore the gene expression changes associated with BD and lithium use, we collected RNA sequencing data from peripheral whole blood in a large case-control cohort from The Netherlands, creating the largest transcriptomic datasets of BD and lithium treatment to date. Collection of whole blood, because of its accessibility, allows for larger sample collections than post-mortem brain tissue, thus improving power needed to detect expected subtle changes. We examined gene expression differences between groups both at the individual gene level and at the level of gene co-expression. While gene expression differences were minor between subjects with BD and controls, we identify widespread differences between subjects being treated with lithium and those not. These differences are partially but not entirely explained by differences in cell-type composition. Our results suggest there are nominal BD-related gene expression effects in blood but numerous effects related to lithium treatment. This work highlights the importance of accounting for medication use in psychiatric transcriptomic studies and provides insight into the molecular mechanisms of lithium's attenuating effects on mood disturbances.

3.2 Methods

3.2.1 *Participants*

Data were generated according to protocols approved by the respective local ethics committees at the University Medical Center Utrecht and the University of California Los Angeles. Patients were recruited via clinicians, the Dutch patients' association, pharmacies, and advertisements. Controls were recruited via advertisements and involvement in previously studies after having agreed to be re-contacted for new research. Participants were included upon the criteria of having at least three Dutch grandparents and being older than 18 years of age. Only patients

with a diagnosis of BD-I or BD-II and who were in a euthymic state were included. Control subjects did not have a diagnosis of BD or any psychotic or neurological disorder and had no first-degree relative with a diagnosis of BD or any psychotic disorder. Diagnosis was confirmed via assessment with the Structured Clinical Interview for DSM-IV (SCID) (www.scid4.org) by carefully trained staff members consisting of PhD students, research assistants, trainee psychiatrists, psychologists, and medical students, under the supervision of experienced psychiatrists. Participants were considered euthymic if they did not meet DSM-IV criteria for a mood episode in the last month according to the SCID. Of the recruited participants, peripheral whole blood was drawn and processed for genotyping and RNA sequencing from 240 controls and 240 cases, of whom 13 had a diagnosis of BD-II and 227 had a diagnosis of BD-I.

In addition to diagnosis assessments and blood sampling, subjects were assessed for medication use and tobacco use. Information about subjects' lithium use was gathered via self-report regarding treatment from their own physician in three ways: 1) in an online medical questionnaire that inquired about medication use, 2) during the on-site assessment where a list of current and lifetime medication use was discussed, and 3) in an assessment of a lithium satisfaction questionnaire. The data of these three measures was combined to accurately determine the current use of lithium of subjects with BD. Information regarding other medication use, response to lithium, and past lithium use was incomplete and less reliable. Nevertheless, this data showed that a majority of subjects being treated with lithium, since starting the medication, have experienced less frequent (N = 104, 68.4%) and less severe (N = 113, 74.3%) mood episodes, and are *satisfied* or *very satisfied* with the use of lithium (N = 113, 74.3%). Of the 152 subjects using lithium, 59 were using lithium with another mood stabilizer and 93 were using lithium as their only mood stabilizer. Without a more extensive lithium-response evaluation, *lithium use* in this study cannot be considered as *lithium response*, but we conclude

that it can be considered as a loose proxy. Data about antipsychotic use was too sparse for this to be considered as a phenotype in subsequent analyses.

3.2.2 *Sample preparation and RNA sequencing*

Whole blood was collected in PaxGene Blood RNA tubes and total RNA extracted using the PAXgene isolation kit (Qiagen) according to manufacturer's protocols. RNA integrity number (RIN) values were obtained using Agilent's NRA 6000 Nano kit and 2100 Bioanalyzer. RNA concentrations were determined using the Quant-iT RiboGreen RNA Assay kit. The UCLA Neuroscience Genomics Core subsequently performed RNA sequencing and prepared sample libraries using the TruSeq Stranded RNA plus Ribo-Zero Gold library prep kit to remove ribosomal and globin RNA to enrich for messenger and noncoding RNAs. Concentration of the sequencing library was determined on a TapeStation and a pool of barcoded libraries were layered on eight lanes of the Illumina flow cell bridge amplified to raw clusters. An average of 24.9 million paired-end reads of 75 bases in length per sample were obtained on an Illumina HiSeq 2500. The raw sequence data were processed for quality control (QC) using FastQC, after which all samples were deemed suitable for downstream analysis.

3.2.3 *RNA-seq alignment and gene expression quantification*

Reads were mapped to human reference genome hg19 using TopHat2[104] allowing for two mismatches yielding an average mapping rate of 96.0% per sample and an average concordant pair mapping rate of 89.8% per sample. Samples had an average of 33.9% duplicate reads. Picard Tools were used to obtain 18 different sequencing metrics such as number of reads, percent mapped reads, and number of coding bases, that were examined for QC and then processed for dimension reduction using principal component analysis. The first three principal components, which explain 75.9%, 16.9%, and 6.4% of variance, respectively, were used as

covariates in subsequent analyses. Known Ensembl gene levels were quantified using HTSeq in the union mode to obtain integral counts of reads that intersect the union of all transcripts of genes. Principal component analysis (PCA) of gene expression quantification was used for data visualization and additional QC, after which four samples were removed for apparent mix-up (Supplemental Methods). Thirty-two additional samples were excluded due to missing clinical information. Differential expression and co-expression analyses were therefore limited to a set of 444 subjects (240 cases and 204 controls).

3.2.4 *Normalization, covariate correction, and differential expression analysis*

Gene expression counts from HTSeq were filtered for genes having > 10 counts in 90% of samples, yielding 12,344 genes for subsequent analyses. Filtered counts were converted to log2-counts-per-million (log-cpm) to account for differences between samples in sequencing depth and to stabilize variances at high counts. Then, the mean-variance relationship was modelled with precision weights at the individual observation level using limma voom[105]. Briefly, voom non-parametrically estimates the mean-variance trend of the logged read counts and uses this to predict the variance of each log-cpm value. The predicted variance is then used as a weight, which is incorporated into the linear model procedure during differential expression analysis. These gene-wise weighted least-squares linear models are fitted to the normalized log-cpm values, taking into account the voom precision weights and the final covariate model, generating a coefficient for the effect of each variable on each gene's expression:

gene expression ~ trait of interest + covariates

Then, for each gene, the coefficient for the trait of interest is statistically tested for being significantly different from zero. *P*-values from this test were corrected for multiple testing using

the Benjamini-Hochberg false discovery rate (FDR) estimation, and a gene was considered to be differentially expressed if it had an FDR < 0.05. The final covariate model for differentially expressed genes (DEGs) between BD cases and controls included the following variables: age, sex, lithium use, tobacco use, assessment group, RIN, sequencing plate, and sequencing metric PCs 1 through 3. The final covariate model for DEGs between subjects being treated with lithium (i.e. lithium users) and non-lithium users included the following variables: BD diagnosis, age, sex, tobacco use, assessment group, RIN, sequencing plate, and sequencing metric PCs 1 through 3. An overview of covariates can be found in Supplementary Table 1. DEGs were checked for overlap and concordance with other datasets (Supplementary Methods). Fold changes (FC) reported are in $\log_2$ fold change units.

3.2.5 *Co-expression network analysis*

To determine networks of genes with correlated expression, weighted gene co-expression network analysis (WGCNA)[106] was performed using the WGCNA package in R. WGCNA defines a network of genes as nodes with edges between genes based on pairwise correlations between genes, and separates the network into modules of gene clusters with highly coordinated expression. To do this, first the 12,344 filtered and normalized genes were residualized adjusting for the following covariates: age, sex, tobacco use, assessment group, RIN, sequencing plate, and sequencing metric PCs 1 through 3. Then, a connectivity metric α_{ij} was calculated for each pair of genes i and j by taking the absolute value of the Pearson correlation coefficient between genes, and transforming that values to a power:

$$\alpha_{ij} = |r_{ij}|^{\beta}$$

The β parameter was chosen according to the approximate scale-free topology criterion described by Zhang and Horvath. We selected β = 7 using the soft thresholding approach and the resulting adjacency matrix of all pairwise correlations was calculated.

Modules of co-expressed genes were then defined as follows. First, network interconnectedness was determined by applying a topological overlap measure to the adjacency matrix. Average linkage hierarchical clustering was then derived from the topological overlap matrix, and modules were identified using the dynamic tree cut algorithm implemented in the WGCNA package. Then the gene expression profiles of each module q were summarized by calculating the module eigengene $E^{(q)}$, which is defined as the first principal component of the expression matrix of that module. Each gene was then assigned a continuous, fuzzy measure of module membership for each module, regardless of that gene's binary module assignment from the dynamic tree cut. Module membership $K_{cor,i}^{(q)}$ or K_{ME} is calculated by correlating the transformed expression of gene *i* with the module eigengene value for module q:

$$K_{\mathrm{ME}} := \mathrm{cor}(x_i, E^{(q)})$$

The module membership measure is between -1 and 1 and specifies how close node i is to the module q. Larger absolute value module memberships correspond to more similar gene-eigengene pairs.

To determine biologically significant modules, gene significance measures were assigned to each gene for each of our traits of interest, including BD diagnosis and lithium use, by calculating the absolute correlation between the trait and the expression profiles. Then a measure of module-trait significance was calculated by correlating module membership values with gene significance values. An association was considered significant if its *P*-value surpassed Bonferroni correction for testing multiple modules ($P < \alpha = 0.05/N_{\mathrm{modules}}$). Finally,

intramodular connectivity k_{IM} was calculated to determine the level of connectivity for the genes in modules significantly associated with traits of interest.

3.2.6 *Functional annotation*

The Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8)[107] was used for functional annotation of each gene list. We used three gene lists from the differential expression analysis: the 976 lithium DEGs at FDR < 0.05, the 754 up-regulated lithium DEGs at FDR < 0.05, and the 222 down-regulated lithium DEGs at FDR < 0.05. We also used gene lists from the five co-expression network analysis modules that were significantly associated with BD: M1 (N_{genes} = 2092), M7 (N_{genes} = 700), M9 (N_{genes} = 55), M11 (N_{genes} = 622), and M26 (N_{genes} = 484). The full set of 12,344 filtered and normalized genes used as input for differential expression and co-expression network analyses was used as background to determine overrepresentation in each of the gene lists. The functional annotation clustering tool was applied using unique Ensembl IDs and the following databases: SP_PIR_KEYWORDS, UP_SEQ_FEATURE, GOTERM_BP_FAT, GOTERM_CC_FAT, GOTERM_MF_FAT, BIOCARTA, KEGG_PATHWAY, INTERPRO, UCSC_TFBS. Cluster annotations were called significant if the enrichment was greater than 1.0 and at least 1 gene list in the annotation cluster survived Bonferroni correction (P < 0.05).

3.2.7 *Estimation of cell type proportions*

To estimate cell-type composition in our sample we employed the CIBERSORT online software (cibersort.stanford.edu)[108]. Briefly, CIBERSORT uses reference gene expression signatures to estimate the relative proportions of cell types in tissues with complex, heterogeneous cell composition via linear support vector regression. The reference dataset we used to deconvolve our mixture of whole blood cell types was the validated leukocyte gene signature matrix that is

provided with the CIBERSORT software, termed LM22[108]. It contains 547 genes whose expression discriminate between 22 different human hematopoietic cell phenotypes (Table 3), including seven T-cell types, naive and memory B cells, plasma cells, natural killer cells, and myeloid subsets.

LM22 reference cell types	
B cells memory	Neutrophils
B cells naive	NK cells activated
Dendritic cells activated	NK cells resting
Dendritic cells resting	Plasma cells
Eosinophils	T cells CD4 memory activated
Macrophages M0	T cells CD4 memory resting
Macrophages M1*	T cells CD4 naive
Macrophages M2	T cells CD8
Mast cells activated*	T cells follicular helper*
Mast cells resting	T cells gamma delta
Monocytes	T cells regulatory*

Table 3. Leukocyte reference cell types. *Estimated as zero in all samples.

To prepare our gene expression data for input to CIBERSORT, raw expression counts from HTSeq were converted to transcripts per million (TPM). Using the resulting matrix of TPM values for our 480 samples and the LM22 gene signature matrix as input, CIBERSORT was run online with 100 permutations and with quantile normalization disabled as recommended for RNA-seq data. The output matrix consisted of deconvolution results with relative fractions of cell types normalized to 1 across all cell subsets for each sample. These estimated cell-type proportions were then residualized using a linear regression model adjusting for the following covariates: sex, age, tobacco use, sequencing plate, RIN, and sequencing metric PCs 1 through 3. Then, residualized cell-type estimates were used to predict lithium use in a stepwise linear regression using the stepAIC function in the MASS package in R. The estimated cell-type

proportions were also appended to the table of technical and biological covariates and then used to re-run the differential expression analysis while accounting for cell-type heterogeneity in the sample.

3.2.8 *Enrichment of cell types in co-expression modules*

The enrichment of LM22 cell types in gene co-expression modules determined from WGCNA was calculated in two ways. First, the hypergeometric overlap between modules and cell type signature genes was calculated. The binary matrix of LM22 signature genes provided by Newman et al.[108], where 1 denotes that a gene was significantly differentially expressed in that particular cell type and 0 denotes that it was not, was used to extract lists of signature genes for each cell type, or genes with a value of 1. These lists are partially overlapping, with 262 genes being unique to a given list and 285 genes being shared between ≥ 2 lists (maximum 10). Then, using the GeneOverlap library in R, the hypergeometric overlap was calculated between each of these 22 cell type signature gene lists and each of the 27 module gene lists using the full set of 12,344 filtered and normalized genes as background.

Second, binary cell type signatures were used to predict module membership values in a linear model. We reasoned that this method might be more powerful than a strict overlap due to the fact that every gene has a module membership value for every module, regardless if it was assigned to that module. The gene co-expression network output, which consists of module membership values for each gene for each module, was limited to the set of LM22 signature genes that were expressed in our sample (N_{genes} = 331). These values were then used as an outcome in a linear model, with the binary matrix of LM22 signature genes as predictors. To avoid multiple testing penalties, only five regressions were run on the five modules that were associated with lithium: M1, M7, M9, M11, and M26.

3.2.9 *Integration of GWAS data*

Analyses were performed across five GWAS traits from publicly available datasets (bipolar disorder, lithium-response as a continuous trait, lithium-response as a dichotomous trait, schizophrenia, and self-reported depression) and 2 sets of DEGs (BD at FDR < 0.2 and lithium-use at FDR < 0.05). Differential expression log2 fold changes and FDR-corrected p-values for each of the 12,344 genes expressed at > 10 counts in 90% of samples were obtained from limma to integrate whole-blood gene expression signatures with GWAS data using Multi-marker Analysis of GenoMic Annotation (MAGMA v1.06)[109].

GWAS summary statistics were obtained for the following five GWAS traits:

1) SCZ[65]: 36,989 cases and 113,075 controls;
2) BD[42]: 20,352 cases and 31,358 controls;
3) 23andMe self-reported depression[110]: 75,607 cases and 231,747 controls;
4) Lithium-response, continuous A score (B score > 4 excluded)[27]: *N* = 2,098;
5) Lithium-response, dichotomous phenotype (total score > 7)[27]: *N* = 1,918.

The 1000 Genomes Project Phase 3 release European reference panel (*N* = 503) was used to model LD in all analyses[111]. Eight gene lists were used from two different DEG models along with a positive and negative control:

1) Lithium-use DEGs at FDR < 0.05: *N* = 897 genes;
2) Up-regulated ithium-use DEGs at FDR < 0.05: *N* = 680 genes;
3) Down-regulated lithium-use DEGs at FDR < 0.05: *N* = 217 genes;
4) BD DEGs at FDR < 0.2: *N* = 630 genes;
5) Up-regulated BD DEGs at FDR < 0.2: *N* = 389 genes;
6) Down-regulated BD DEGs at FDR < 0.2: *N* = 241 genes;
7) Positive control gene-set: the top 100 most significant genes from a random draw of *N* = 1,000 using the BD GWAS gene-level test statistics;

8) Negative control gene-set: a random draw of *N* = 1,000 genes using the BD GWAS gene-level test-statistics.

MAGMA was used to run *gene property* analyses, which uses a multiple regression framework to associate a continuous gene variable to GWAS gene level p-values. SNPs were mapped to genes using Ensembl gene IDs and NCBI build 37.3 gene boundaries +/- 10kb extensions using the -- annotate flag. For each phenotype, we generated gene-level p-values by computing the mean SNP association using the default gene model ('snp-wise=mean'). We only included SNP with MAF > 5% and dropped synonymous or duplicate SNPs after the first entry ('synonym-dup=drop-dup'). For each annotation, we then regressed gene-level GWAS test statistics on the corresponding gene annotation variable using the '--gene-covar' function while adjusting for gene size, SNP density, and LD-induced correlations ('--model correct=all'), which is estimated from an ancestry-matched 1KG reference panel. In all analyses, we included only genes for which we had both the gene variable and GWAS gene level test statistic available.

3.3 Results

3.3.1 *Sample description*

Subjects comprised of individuals of Dutch ancestry from the Netherlands from which whole blood was drawn and a clinical evaluation was gathered. The sample consisted of 240 cases with a diagnosis of BD (94.6% BD-I and 5.4% BD-II) and 240 controls. Of the BD cases, 152 were being treated with lithium at the time of assessment. RNA from all 480 blood samples was prepared using the TruSeq Stranded RNA plus Ribo-Zero Gold library kit then sequenced on the Illumina HiSeq 2500, yielding an average of 24.9 million paired end reads of 75 bases in length per individual. Thirty-six RNA-seq samples were excluded due to missing clinical information or apparent sample mix-up. Differential expression and co-expression network analyses were therefore limited to a set of 444 subjects (240 cases and 204 controls; Table 4).

	Case	*Control*		*Lithium user*	*Non-lithium user*	
	N (%)		*P*	*N* (%)		*P*
Diagnosis	240	204	-	152 (100%)	88 (30.1%)	<2.20E-16
Female sex	131 (54.6%)	119 (58.3%)	0.444	90 (59.2%)	160 (54.8%)	0.420
Lithium use	152 (63.3%)	0 (0%)	<2.20E-16	152	292	-
Tobacco use	74 (30.8%)	39 (19.1%)	6.14E-03	48 (31.5%)	65 (22.3%)	0.0387
	Mean (SD)			Mean (SD)		
Age	50.3 (12.4)	43.4 (14.8)	1.95E-07	48.0 (13.1)	46.7 (14.4)	0.309

Table 4. Sample demographics. P-values for categorical variables were calculated using Fisher's exact test. P-values for continuous variables were calculated using Student's t-test. SD, standard deviation.

3.3.2 *Minimal changes in bipolar disorder gene expression*

To explore the transcriptomic signatures of BD, we first evaluated whether subjects with BD harbor transcriptional differences on a per gene level compared with controls. Gene expression counts were normalized to log counts per million (log-cpm) to account for sequence depth, then the mean-variance relationship was accounted for with limma voom[105]. Finally, known clinical and technical covariates, including lithium use, were corrected for in gene-wise linear models. Of the 12,344 genes tested, only six were differentially expressed in BD after correcting for multiple testing (FDR < 0.05; Figure 1). The differences in expression were very small, with absolute fold changes ranging from 0.116 to 0.437. While the number of identified differentially expressed genes (DEGs) was too small to perform functional enrichment analysis, we did find that three of the six genes (*COG4*, *DOCK3*, and *BBS9*) were expressed in GTEx frontal cortex tissue (median TPM > 1) and show relatively stable expression across brain cell types except for *DOCK3*, which is enriched in neurons (fold change relative to other cell types = 6.823; Table 5). Four of the genes were present in the Stanley Genomics brain gene expression database, and two of these were found to be differentially expressed in BD individuals in at least one study, *COG4* and *DOCK3*, although the latter was altered in the opposite direction. *COG4* was also reported as differentially expressed in a schizophrenia mega-analysis of nine whole blood

microarray datasets [94]. Using polygenic risk scores (PRS) for BD as the differential expression trait of interest rather than the dichotomous case-control phenotype did not yield any significant genes, while PRS did significantly differ between BD cases and controls (P = 3.85 x 10^{-6}; Supplementary Figure 1; Supplementary Methods).

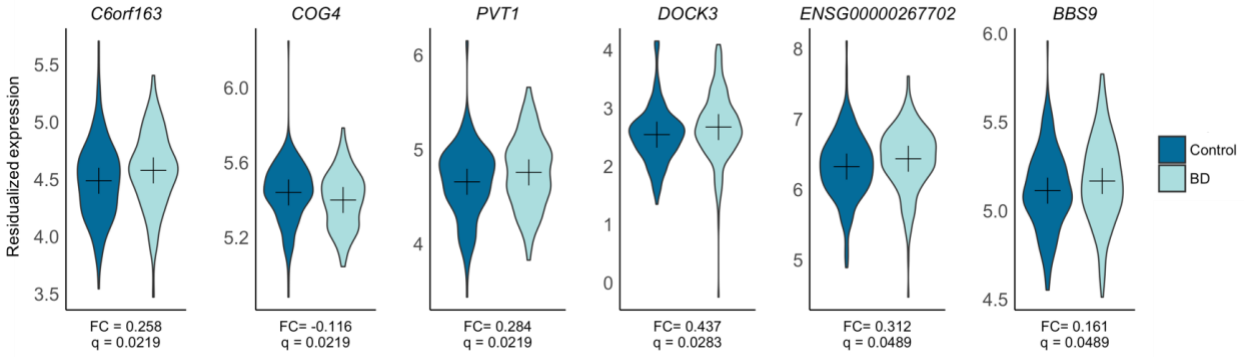


Figure 1. Genes significantly differentially expressed in BD. q, FDR-adjusted P < 0.05.

			Mean expression in Zhang in FPKM (FC relative to the other five cell types)						
Ensembl ID	*Gene symbol*	*Median expression in GTEx frontal cortex (TPM)*	*Fetal astrocytes*	*Mature astrocytes*	*Neurons*	*Oligoden-drocytes*	*Microglia/ macrophage*	*Endothelial*	*Stanley Genomics differential expression results for BD*
ENSG00000203872	*C6orf163*	0.2999	-	-	-	-	-	-	NS
ENSG00000103051	*COG4*	27.44	1.621 (1.159)	2.953 (2.607)	1.095 (0.728)	1.414 (0.982)	0.928 (0.603)	0.606 (0.378)	NS in combined analysis, down-regulated in three studies
ENSG00000249859	*PVT1*	0.5569	-	-	-	-	-	-	NS
ENSG00000088538	*DOCK3*	37.62	1.953 (0.742)	1.727 (0.645)	8.723 (6.823)	1.891 (0.715)	0.516 (0.177)	0.305 (0.103)	NS in combined analysis, down-regulated in one study
ENSG00000267702	-	0.04648	-	-	-	-	-	-	-
ENSG00000122507	*BBS9*	4.311	10.113 (1.385)	7.621 (0.977)	5.721 (0.699)	9.392 (1.262)	4.03 (0.473)	9.739 (1.320)	-

Table 5. BD DEG expression in brain tissue and cell types and results from Stanley Genomics analyses. TPM, transcripts per million; FPKM, fragments per kilobase million; NS, not significant; -, not present.

3.3.3 *Widespread subtle gene expression changes in lithium users*

To investigate the effects of lithium on gene expression, we analyzed differences in the expression of individual genes between subjects undergoing lithium treatment (N = 152) and subjects not undergoing lithium treatment (N = 292). Following the same differential expression

pipeline as above, we found 976 genes with small differences (|FC| mean = 0.201, max = 0.820, SD = 0.100) in gene expression between lithium users and non-lithium users (Figure 2A). These genes were enriched for biological terms related to Ca2+ signaling and other signaling pathways, and immunity (Figure 2B). To distinguish between up- and down-regulated gene pathways, we stratified genes by their direction of change in expression. The 754 up-regulated genes were annotated for many of the same terms as the full set but with greater enrichment scores, indicating that the up-regulated genes are driving the enrichment scores in the full set (Figure 2B). Of the 976 lithium-use DEGs, 804 were expressed in GTEx frontal cortex samples (TPM > 1), and 488, 553, 503, 478, 512, and 403 were expressed in neurons, fetal astrocytes, mature astrocytes, oligodendrocytes, microglia/ macrophages, and endothelia, respectively (FPKM > 1). However, none of these gene sets were significantly enriched (hypergeometric P > 0.05).

Because our study is the largest of its kind to date, a replication dataset was unavailable. Nevertheless, the 976 lithium-use DEGs were tested for overlap with lists of DEGs from similar studies found in the literature (Table 6). While these studies vary widely in their design, tissue type, and sample size, and even though there is no study with the same design, tissue type, and size as ours, we were able to find a significant overlap between our 976 lithium-use DEGs and the lists from two studies. In the first study, DEGs were detected by comparing peripheral monocyte gene expression in subjects before and after lithium monotherapy. Of the 35 DEGs discovered, 18 were shared with the current study (hypergeometric $P = 4.66 \times 10^{-12}$), and all 18 were concordant in direction (Figure 3A). In the second study, DEGs were detected by comparing LCL gene expression before and after lithium treatment *in vitro*. Of the 1,504 DEGs discovered, 134 were shared with our study (hypergeometric $P = 9.23 \times 10^{-3}$), and 84.6% of these were concordant in direction (Figure 3B). There were two genes shared between all three

lists, *RFX2* and *SLC29A1*. We announce genes in these overlapping lists as high confidence lithium-associated genes.

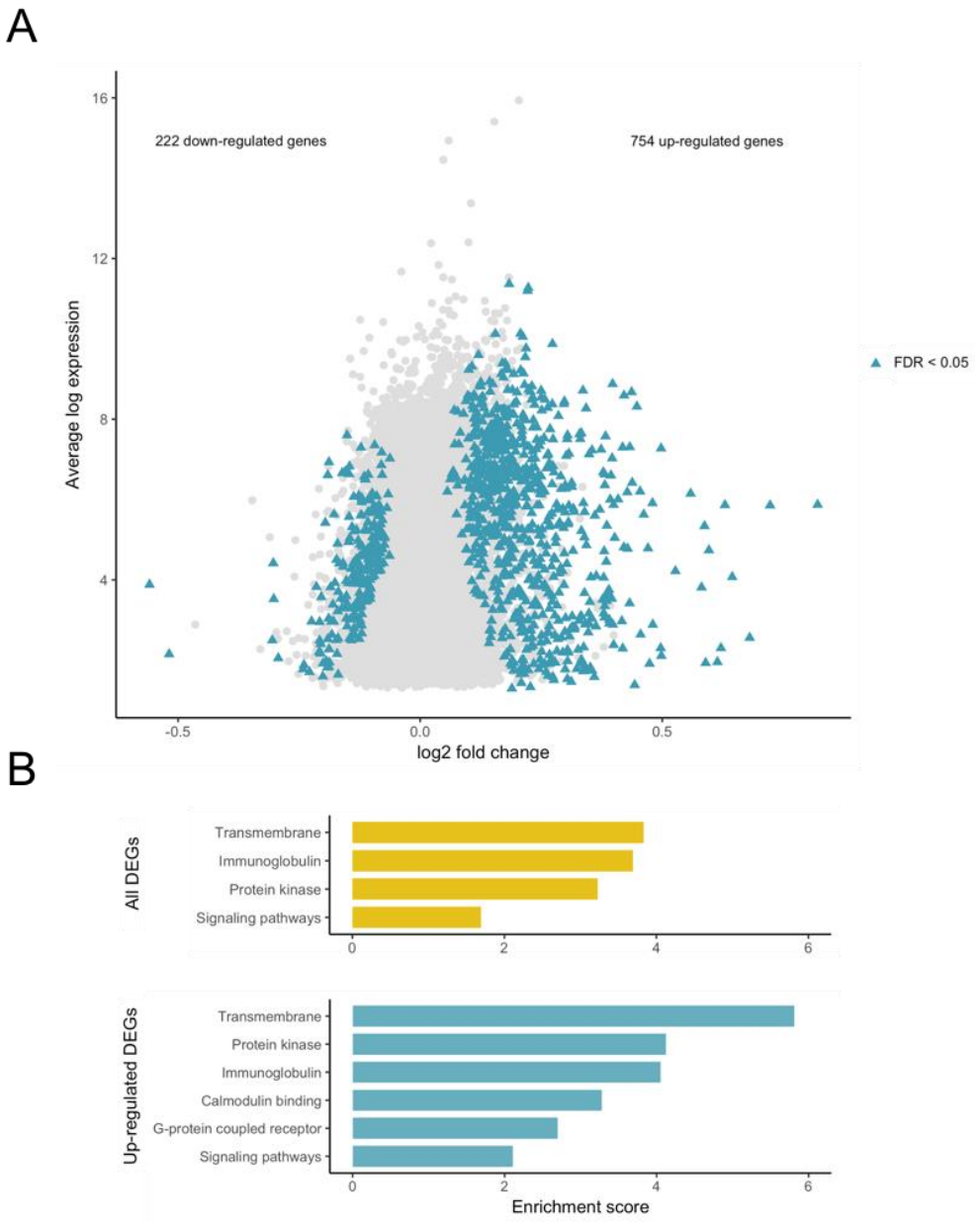


Figure 2. Genes significantly differentially expressed in lithium users. (A) 976 genes differentially expressed between lithium users and non-lithium users (shown as blue triangles, FDR-adjusted $P < 0.05$; all other genes tested shown as light gray circles). (B) DAVID[107] functional annotation cluster enrichment of all 976 DEGs (upper) and 754 up-regulated DEGs (lower). Enrichment scores increase when the gene list is limited to up-regulated genes only. Clusters were considered significant if the enrichment score > 1 and at least one term in the cluster survived Bonferroni correction for multiple testing.

Study description							Genes used		Intersection with current study				
First author	*Year*	*Tissue*	*Diagnosis*	*Conditions tested*	*N samples*	*Platform*	*FDR*	*N DEGs*	*P*	*N*	*% concordant*	*r*	*P*
Anand	2016	Peripheral lymphocytes	BD	T vs. UT	22, 22	Microarray	0.05	35	4.66E-12	18	100	0.35	0.151
Beech	2014	Whole blood	BD	LR vs. LNR	9, 19	Microarray	0.1	62	0.991	1	-	-	-
Breen	2016	LCLs	BD & HC	T vs. UT	23, 23	Sequencing	0.005	1504	9.23E-03	134	84.6	0.550	2.20E-16
Choi	2011	Prefrontal cortex	BD & HC	BD vs. HC	40, 43	Microarray	0.05	379	0.998	13	-	-	-
Fries	2017	LCLs	BD	T vs. UT	62, 62	Microarray	0.05	236	6.62E-02	24	-	-	-
Fromer	2016	Prefrontal cortex	SCZ & HC	SCZ vs. HC	258, 279	Sequencing	0.05	693	0.976	41	-	-	-
Hess	2016	Whole blood	SCZ & HC	SCZ vs. HC	300, 278	Microarray	0.05	1613	0.852	109	-	-	-
Jansen	2016	Whole blood	MDD & HC	C-MDD vs. HC	882, 331	Microarray	0.1	142	0.480	10	-	-	-
Kittel-Schneider	2017	LCLs	BD & HC	T vs. UT	21, 21	Microarray	*	459	0.862	2	-	-	-
		Fibroblasts	BD & HC	BD vs. HC	10, 11	Microarray	*	296	0.971	18	-	-	-
		LCLs	BD & HC	BD vs. HC	10, 11	Microarray	*	58	1.00	4	-	-	-
van Eijk	2014	Whole blood	SCZ & HC	BD vs. HC	106, 96	Microarray	0.05	525	0.120	34	-	-	-
Zhao	2015	Cingulate cortex	SCZ & HC	BD vs. HC	31, 26	Sequencing	0.1	105	0.368	9	-	-	-
			BD & HC	BD vs. HC	25, 26	Sequencing	0.1	153	0.973	6	-	-	-

Table 6. Overlap between lithium-use DEGs and previous studies. DEG lists from previous studies were included upon the following criteria: >10 samples, > 50 DEGs at FDR < 0.1, and data available for download. *Bonferroni-Holm $P < 0.05$

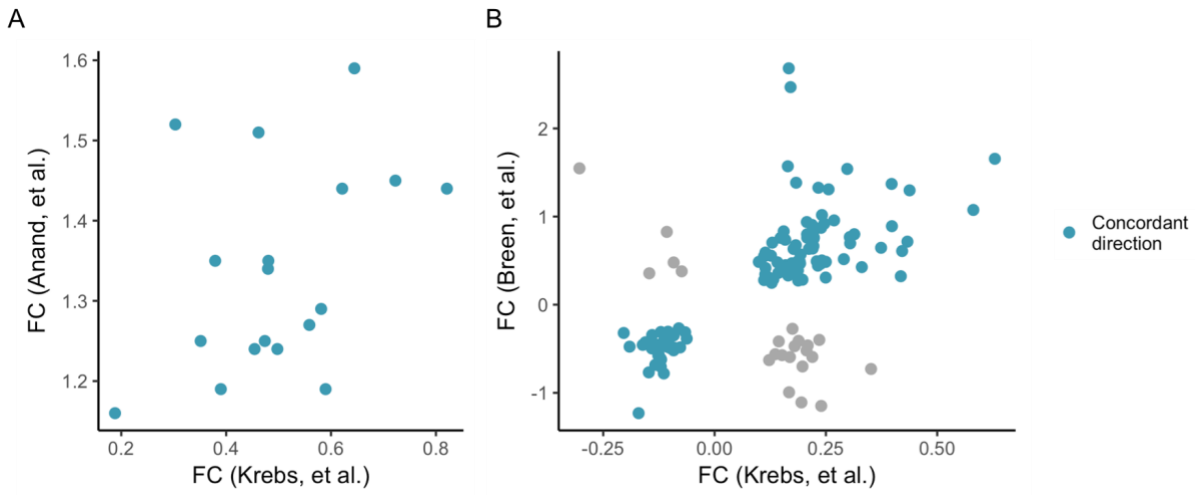


Figure 3. Comparison of fold changes of overlapping DEGs in the current study (Krebs, et al.) overlapping with Anand, et al.[91] (Left) and with Breen, et al.[92] (Right). Genes whose effects are concordant in direction are colored in blue.

3.3.4 *Modules of co-expressed genes are associated with lithium use*

We hypothesized that in addition to individual genes demonstrating altered expression, the coordinated expression of genes may be disrupted in BD, reflecting widespread regulatory effects or coordination of specific biological pathways. We therefore constructed a network of co-expressed genes in the entire sample using WGCNA and assessed the detected modules for association with BD. This network consisted of 27 modules ranging in size from 48 to 2,760 genes (mean N_{genes} = 441). By evaluating the correlation of module membership values with gene significance for BD diagnosis, we quantified the association of each module with BD. After Bonferroni multiple testing correction, five modules were significantly associated with lithium-use, but no modules were associated with BD or any other clinical or technical variable (Supplementary Table 2).

Of the five modules associated with lithium use, three shared significant overlap with lithium-use DEGs (Table 7). M26 was most significantly associated with lithium ($P = 2.00 \times 10^{-4}$; Figure 4A) but was not significantly enriched for lithium DEGs. M1 was also associated with lithium ($P = 9.04 \times 10^{-4}$; Figure 4B) and had the most significant enrichment of DEGs (431 of 2,092 genes in the module were DEGs; hypergeometric $P = 2.03 \times 10^{-97}$). Functional annotation clustering of the genes in M1 showed an enrichment of terms related to cell signaling, immunity, and glycophosphatidylonositol anchor.

Module preservation analysis was also performed to assess differences in network density and connectivity between groups, but showed full preservation indicating that networks constructed in separate groups maintain their underlying structure (Supplementary Methods and Supplementary Figure 2).

			Correlation with lithium use		*Overlap with DEGs*	
Module	*N genes*	*Functional annotation cluster term(s)*	*r*	*P*	*N genes*	*P*
M1	2,092	Transmembrane, GPI anchor, immunoglobulin	0.156	9.40E-04	431	2.03E-97
M7	700	Helicase activity, ATP binding, metabolism, DNA replication, endoplasmic reticulum, proteasome, protein biosynthesis	-0.165	4.50E-04	22	1.00
M9	55	G-protein coupled receptor	0.153	1.15E-03	17	6.15E-07
M11	622	-	0.17	3.12E-04	102	4.93E-13
M26	484	Nucleic acid binding, splicing	-0.175	2.00E-04	17	1.00

Table 7. Co-expression module association with lithium use. Functional annotation cluster enrichment determined using DAVID[107]. Correlation with lithium use calculated by correlating gene module membership values with gene significance values for lithium use. Overlap was calculated by testing for hypergeometric overlap between the list of lithium-use DEGs and the list of genes within each module. GPI, glycophosphaditylinositol.

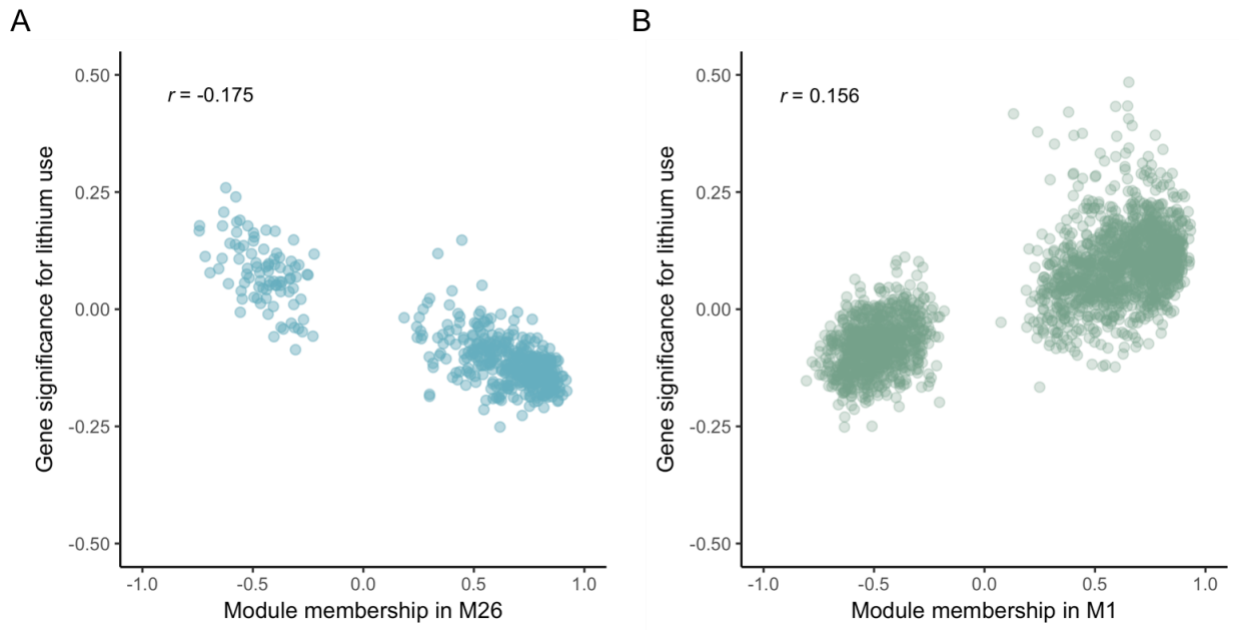


Figure 4. Module correlation with lithium use for (A) M26 and (B) M1. Module membership is the correlation of each gene's expression with the module eigengene, which is the first principal component of the module. Gene significance for lithium use is the correlation of each gene's expression with lithium use.

3.3.5 *Estimated neutrophil composition is associated with lithium use*

We then sought to determine if variation in our sample could be explained by differences in blood cell-type composition. To deconvolve cellular heterogeneity, we applied CIBERSORT[108] to our gene expression quantifications using a reference panel of 22 blood cell-type signatures. The resulting estimated cell-type proportions (Figure 5A) were then examined for their relationship with lithium use in BD cases only. Each cell type was residualized for demographic and technical variables then used to predict lithium use in a stepwise linear model. One cell type significantly predicted lithium use within the BD cases, neutrophils (P = 0.0236), which are elevated in individuals being treated with lithium (Figure 5B). Indeed, 16 of 60 signature neutrophil genes were also lithium-use DEGs (hypergeometric $P = 4.45 \times 10^{-6}$).

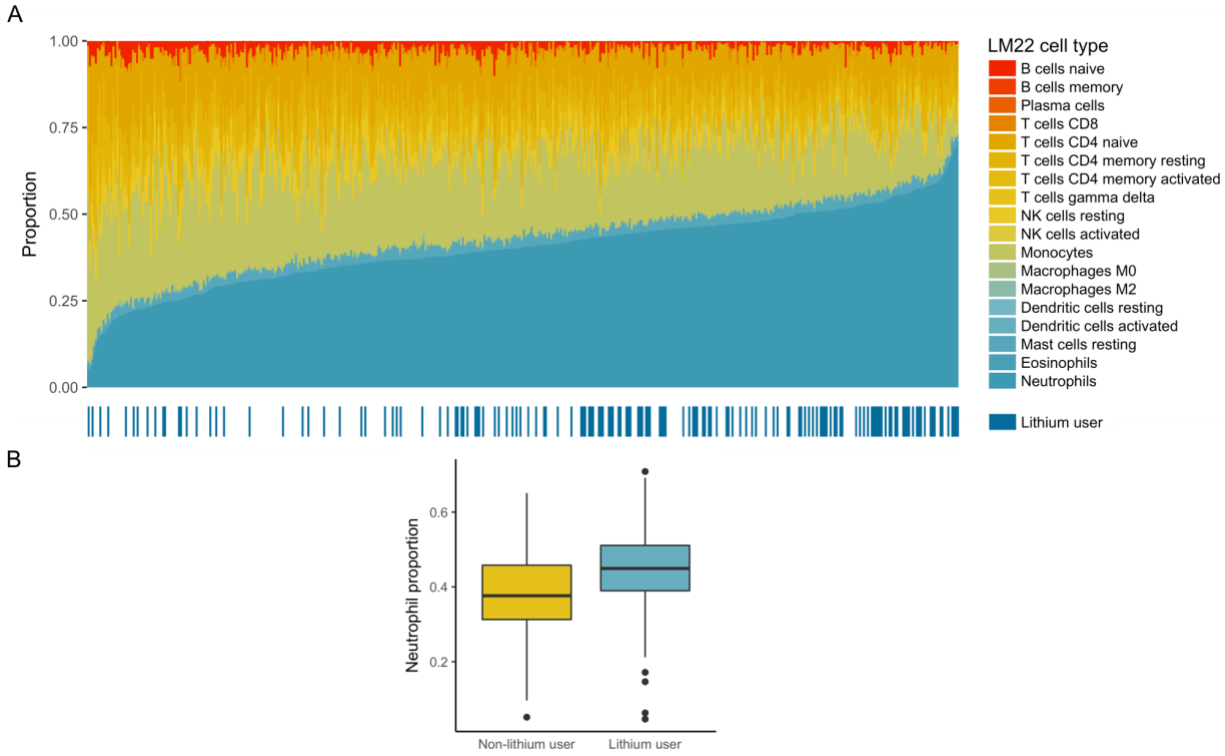


Figure 5. Estimated neutrophil composition association with lithium use. (A) Leukocyte cell type proportions per sample as estimated from gene expression, sorted by neutrophil proportions. Lithium users, as shown in the bar on the bottom, cluster on the right-hand side where neutrophil proportions are higher. (B) Lithium users have higher estimated neutrophil proportions (P = 0.0236).

3.3.6 *Estimated cell type proportions partially explain lithium-associated changes in gene expression*

Because estimated neutrophil composition was associated with lithium use, and because cell-type composition affects gene expression profiles[108], we next sought to determine how cell-type composition contributed to lithium-associated gene expression changes in our sample. To do so, we included the estimated cell-type proportions as covariates in addition to the ones used previously and re-evaluated differential expression in lithium users. The number of genes showing differential expression in subjects undergoing lithium treatment decreased from 976 in the model without cell-type estimates to 233 in the model with cell-type estimates (FDR < 0.05; Figure 6A), of which 194 (83.2%) were significant in the original model and concordant in direction of effect (Figure 6B). No functional annotation cluster terms remained significant after correcting for multiple testing. The number of genes differentially expressed between BD cases and controls decreased to zero after accounting for estimated cell-type proportions.

3.3.7 *Lithium-associated co-expression module M1 is enriched for neutrophil gene expression signatures*

We then sought to determine if the various lithium-associated modules of co-expressed genes reflected biologic signatures of distinct populations of blood cell types. We did this in two ways. First, a hypergeometric overlap between lithium-associated module gene lists and cell-type signature gene lists revealed a significant overlap between module M1 with monocyte and neutrophil signature genes and M9 with eosinophil and activated mast cell signature genes (Figure 7A, left). Second, the expression of cell-type signature genes was used to predict module membership values in a linear model for each of the five lithium-associated modules. Neutrophils, monocytes, and eosinophils were again implicated (Figure 7A, right). In both of

these analyses, the most significant cell type-module relationship was M1 with neutrophil estimates (hypergeometric $P = 5.68 \times 10^{-21}$, linear model $P < 2.20 \times 10^{-16}$). Indeed, neutrophil signature genes had higher M1 membership values (Figure 7B).

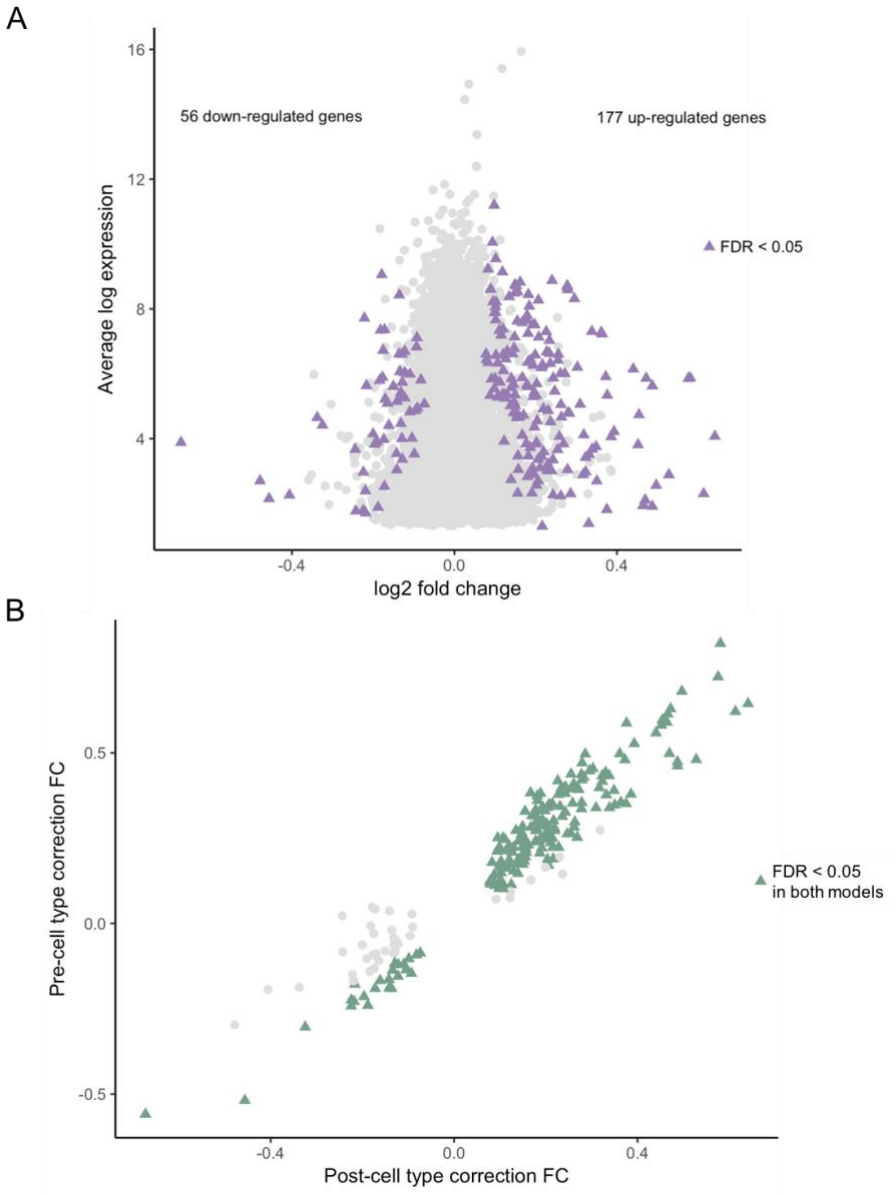


Figure 6. Estimated cell type proportions partially explain lithium-associated changes in gene expression. (A) After correcting for estimated cell type proportions, 233 DEGs remain significant (shown as purple triangles, FDR < 0.05; all other genes tested shown as light gray circles). (B) Comparison of $\log_2$ fold changes (FC) before (y-axis) and after (x-axis) correcting for cell type proportion estimates. Of the 233 genes significant after correcting for cell type estimates, 194 (83.2%) were significant in the original model without correcting for cell type estimates and concordant in the direction of effect (shown as green triangles, FDR < 0.05).

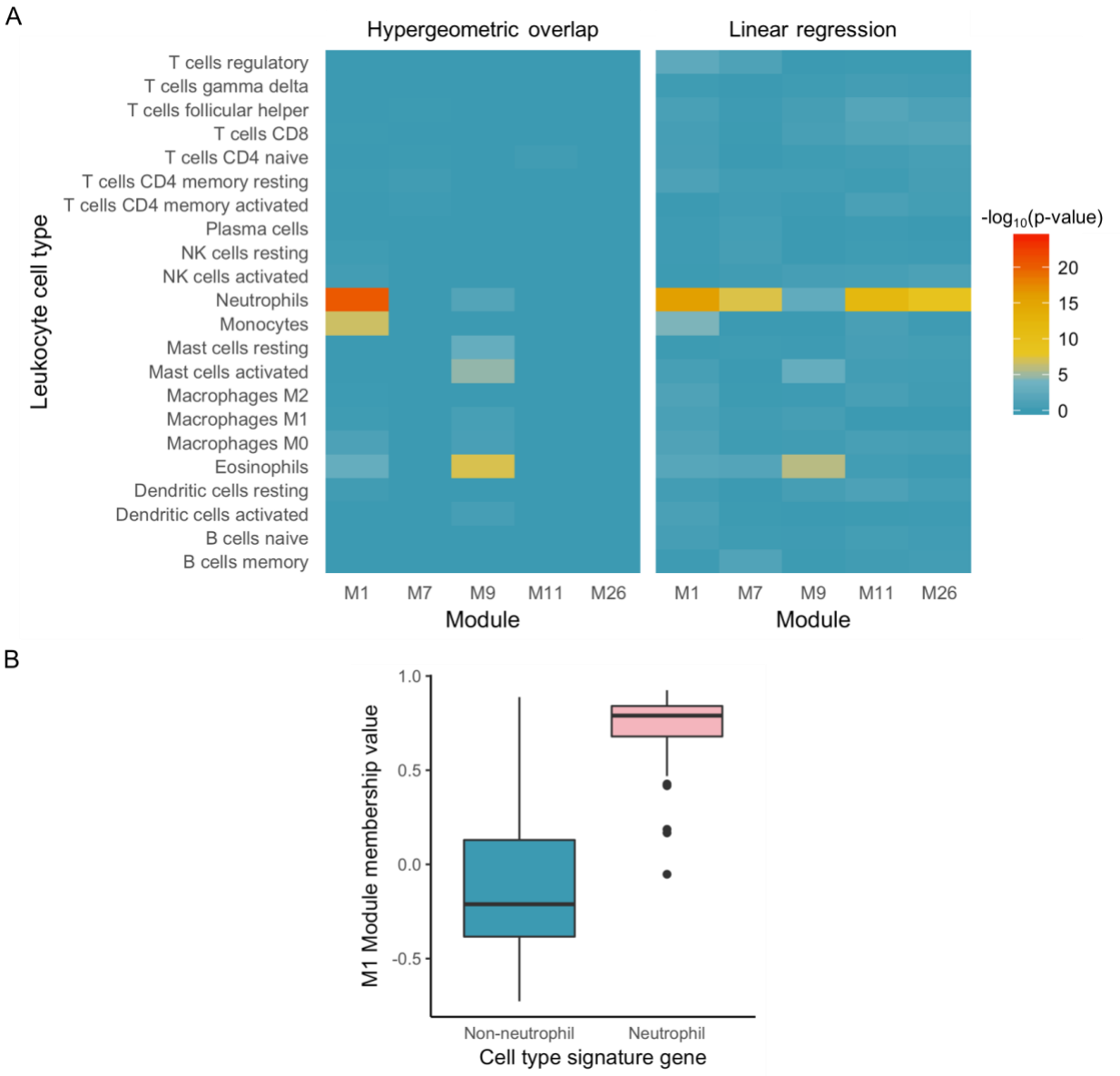


Figure 7. Lithium-associated co-expression module M1 enrichment for neutrophil gene expression signatures. (A) Lithium-associated module enrichment for leukocyte cell types. Left, Hypergeometric overlap between leukocyte cell type signature genes and genes in each module. Right, Linear regression of leukocyte cell type signature genes to predict module membership values. (B) Neutrophil signature genes have higher module membership values for M1 than other leukocyte signature genes (regression $P < 2.20 \times 10^{-16}$).

3.3.8 *Lithium-induced gene expression differences are not enriched for genes with common variants associated with BD and other psychiatric disorders*

To evaluate if BD and lithium-use DEG sets were associated with a higher burden of psychiatric risk alleles, we performed gene-set analyses using MAGMA[109]. Analyses were performed across three psychiatric GWAS traits: BD[42], SCZ[65], and self-reported depression[110]. SCZ and depression were used because of their high degree of overlap in SNP-based heritability with BD[43]. The self-reported depression GWAS was used instead of MDD GWAS because of the large sample size and successful findings of this study. The lithium-response traits were not used because the SNP-based heritability estimates for these traits were zero (Figure 8A). Because the set of BD DEGs at FDR < 0.05 was too small to test, instead we tested the set of BD DEGs at FDR < 0.2. None of the comparisons demonstrated an association with genetic risk across the genes implicated in the current study (except for the Positive control gene set), even after stratifying by up- and down-regulated genes (Figure 8B).

3.4 Discussion

We present the largest BD case-control gene expression study conducted to date. After carefully controlling for technical and clinical factors, there were minimal transcriptomic differences between BD cases and controls (Figure 1, Table 5). One of the top differentially expressed genes, *COG4*, encodes a part of a multiprotein complex that is a key determinant of Golgi apparatus structure and capacity for intracellular transport and glycoprotein modification[112]. *COG4* mRNA is expressed widely across body tissues including the brain[113]. It has been reported as having alternative splicing in subjects with BD[114], and concordant with our results, was reported as down-regulated in three of the ten Stanley Genomics BD brain datasets[115]. Further work will be needed to determine the role of *COG4* in BD, but perhaps

neuronal hyperexcitability in BD[89] destabilizes internal cellular processes including Golgi function[116].

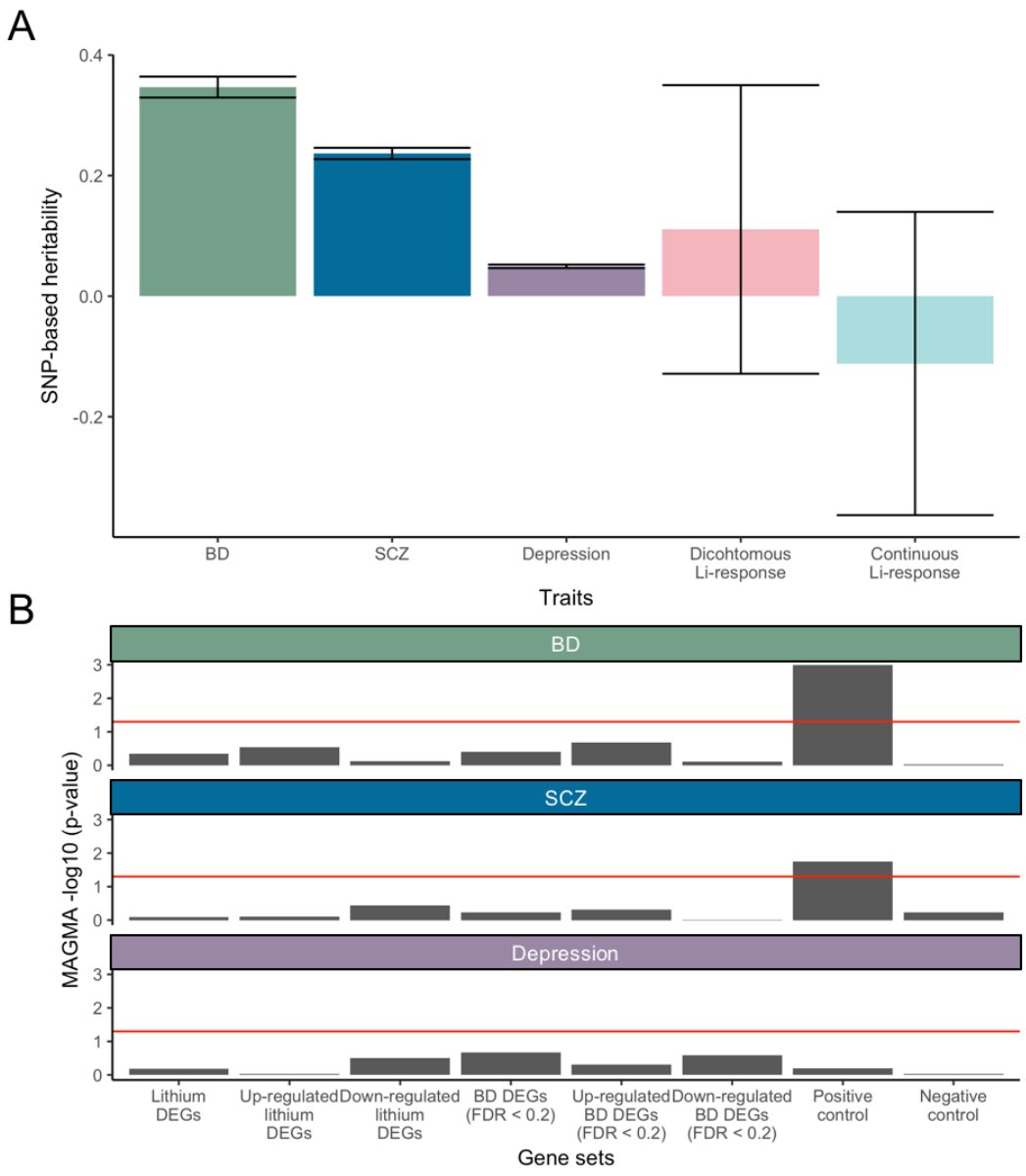


Figure 8. Lithium-induced gene expression differences are not enriched for genes with common variants associated with BD. (A) SNP-based heritabilities of psychiatric GWAS traits. The SNP-based heritability estimates for both the dichotomous and continuous lithium-response traits were not significantly different from zero and were therefore not used in the subsequent analysis. (B) Gene-set enrichment of DEG sets with genes in psychiatric trait-associated loci. DEG sets stratified by up- and down-regulated genes. The BD DEG set was extended to include genes with FDR-corrected $P < 0.2$. The red line represents the significance threshold of $-\log_{10}(0.05)$.

The therapeutic mood-stabilizing mechanisms of lithium are not well understood despite it being the single most extensively studied psychopharmacological agent[18]. One likely reason for this is the magnitude of lithium's physiological interactions[117]. In pharmacological terms, lithium is a *small molecule* (the third smallest element in fact) without a defined target[103]. This lack of specificity makes it difficult to discern therapeutic mechanisms from off-target effects, which likely lead to many of the undesirable side effects and even the toxicity of lithium at doses that are too high. Lithium ions (Li^+) have a single positive charge and are hypothesized to mimic and disrupt the actions and targets of more ubiquitous metal ions such as magnesium (Mg^{2+})[103]. Theorized therapeutic mechanisms of lithium include its inhibition of the protein GS3Kβ, and its effect on intracellular signaling cascades such as those involving protein kinases and phosphatidylinositol[20]. It is not clear how these mechanisms relate to higher order properties thought to be involved in BD etiology like neuronal function, chronobiology, and brain structure. Examining lithium mechanisms at high biological resolution is therefore not only crucial for understanding the high rates of non-response and non-adherence to prophylactic lithium treatment in BD patients but also for understanding BD etiology itself.

The investigation of gene expression differences in lithium users in our large BD cohort makes this the largest naturalistic study of the transcriptional effects of lithium treatment to date. Our analysis revealed widespread but subtle changes in gene expression in response to lithium treatment (Figure 2). The large number of genes altered each with small effect is in line with lithium's broad scope of physiological effects[117] and with the complex genetic architecture of BD[93]. These genes were enriched for functional annotations related to transmembrane, cell signaling, protein kinase, and immunity. These pathways have been implicated in previous BD transcriptome studies[86,87,89,96,99] and are known targets of lithium[21,118]. Although a direct replication was unavailable, the lithium-use DEGs presented herein significantly overlapped with DEGs from two previous studies, which we present as high-confidence lithium-associated genes

(Table 6, Figure 3). There were two genes shared among all three of these lists, *RFX2* and *SLC29A1. RFX2* was among the top DEGs in the current study (FC = .581, FDR adjusted-*P* = 1.40 x 10^{-11}) and encodes regulatory factor X2, a transcriptional activator that regulates human leukocyte antigen (HLA) class II expression[119]. HLA proteins play a key function in the immune system and are encoded in the major histocompatibility complex (MHC) locus containing a number of genetic associations to autoimmune diseases[120]. The MHC locus also contains the strongest associated signal in SCZ GWAS (rs115329265 on chromosome 6, combined *P* = 3.48 x 10^{-31})[65]. *SLC29A1* encodes a member of the solute carrier family of transmembrane glycoproteins that mediate the essential transport of substrates across cell membranes[121] and is a reasonable putative target of lithium[122]. While this gene has not been implicated in BD GWAS, other solute carrier family genes were present in genome-wide significant loci[42].

Similar to the individual gene expression analysis, the gene co-expression analysis revealed lithium-associated changes in expression but not BD-associated ones (Figure 4). The lithium-associated modules of co-expressed genes were enriched for many of the same functional annotations as the lithium-use DEGs, including transmembrane and immunity, which is expected considering the extensive overlap between the genes in M1, M9, and M11 and the lithium-use DEGs. In addition to lithium-use, M1 was also associated with estimated neutrophil proportions (Figure 7). The M1 hub gene was *MXD1* (k_{IM} = 1), which encodes max dimerization protein 1, a member of the Myc/Max/Mad network of transcription factors that mediate cellular proliferation, differentiation and apoptosis[123]. That a transcription factor is a hub gene is not surprising given the extensive interactions of transcription factors with target genes. Myc proteins are regulated by glycogen synthase kinase-3 (GSK3)[124], which is known to be inhibited by lithium and therefore hypothesized to play a role in mood disturbances[125]. Lithium-associated inhibition of GSK3 and the hypothesized subsequent effect on Myc proteins may also affect Myc's dimerization partner, Max.

The lithium-use effect on gene expression we observed was partially due to differences in cell types, although not entirely as evidenced by the remainder of 233 genes with differential expression after correcting for estimated cell type composition (Figure 6). The cell type effect seems to be driven primarily by neutrophils, which displayed higher estimated proportions in individuals undergoing prophylactic lithium treatment (Figure 5). Indeed, lithium-induced leukocytosis and more specifically neutrophilia has been described since the medication's early use in psychiatry[118]. Lithium induces neutrophilia through a complex pathway involving GSK3 and immune-related transcription factors and genes[126]. Increased levels of neutrophils are typically associated with anti-inflammatory or an infection-fighting immune response[127]. Whether these immunity-related mechanisms play a role in the mood stabilizing effects of lithium remains to be determined. Immune components of psychiatric illness including BD[128] have long been recognized, but it remains unclear if they represents a causal pathway, a property of the disease state, or a consequence of environmental factors like body mass index or smoking.

Finally, we hypothesized that genes whose expression is altered by lithium treatment may be involved in BD etiology and therefore enriched for genes implicated from GWAS. To test this, we performed a gene-set analysis of DEG sets with multiple psychiatric GWAS, which revealed a lack of association with common risk alleles (Figure 8). This indicates that genes displaying a transcriptional response to lithium treatment in blood do not harbor genetic risk for psychiatric disorders. This can be interpreted several ways. First, it could indicate that psychiatric genetic studies are thus-far underpowered and therefore our DEGs do not show enrichment for genetic risk even though it may actually be there. This may be the case given the small amount of SNP-based heritability that can be explained by known loci. This is certainly the case for lithium-response, which has a SNP-based heritability of zero and was therefore left out of the subsequent analysis. Second, it could indicate that our study is underpowered to demonstrate enrichment of genetic risk, and that with larger sample sizes we may expect to see

genes with altered expression in blood the blood of lithium users to be enriched for psychiatric genetic risk. Third, it could indicate that the transcriptomic mechanisms of genetic risk for BD are not present in whole blood. Both of these scenarios are possible, but given the substantial overlap of cis-eQTLs between brain and blood[129], we suspect there to be genetic regulatory mechanisms associated with BD and lithium detectable in blood that will be revealed with larger sample sizes of both genetic and transcriptomic studies.

These results contribute to the understanding of the genomics of lithium action, which is essential for the future of personalized psychiatric medicine for patients with BD. They suggest that lithium causes widespread gene expression changes in whole blood, converging on biological pathways related to cell signaling and immunity, and partially as a result of increased neutrophils. Future studies with larger sample sizes and independent replication datasets will be needed to confirm our findings. Whether these genes and pathways play a role in the mood-stabilizing mechanisms of lithium remains to be determined. Lithium use, as a trait only present in BD subjects and therefore confounded with BD diagnosis, likely eliminated most of the observable BD effects via confounding by indication. We caution investigators regarding the importance of correcting for cell type composition and medication use, and suggest a lithium-naive study design to optimize BD transcriptomic signal that is independent of lithium use. Nevertheless, we argue that investigating the BD transcriptome in whole blood remains valuable due to the accessibility of this tissue, its potential for biomarker discovery, and its potential for use in longitudinal study designs, which are appealing due to the episodic nature of BD. The suggested immune component of BD etiology and lithium therapy makes blood a clear choice of tissue as well. In addition, peripheral tissues such as blood partially recapitulate gene expression signatures of the brain[130], and compared to post-mortem tissues are less subject to poor quality due to rapid degradation upon death[131]. Studies involving post-mortem tissue, *in*

vitro neuronal cells, or animal models will nevertheless be needed to determine the therapeutic effect of lithium on BD-associated brain-related function.

3.5 Supplementary Methods

3.5.1 *Sequencing metrics*

Transcriptome alignments were analyzed for quality control using CollectRnaSeqMetrics in Picard Tools. Eighteen sequencing metrics were obtained: number of paired reads, percent duplication, percent GC content, number of bases passing Illumina's filter, number of bases passing Illumina's filter that were aligned, number of coding bases, number of UTR bases, number of intronic bases, number of intergenic bases, number of correct strand reads, number of incorrect strand reads, percent mRNA bases, percent usable bases, median coverage, median 5' bias, median 3' bias, and median 5' to 3' bias. Upon examination of these metrics, no samples were removed for low quality. They were then processed for PCA dimension reduction using the prcomp function in R. Principal components one through three, which explain 75.9%, 16.9%, and 6.4% of the variance in sequencing metrics respectively, were used as covariates in subsequent analyses.

3.5.2 *Gene expression principal component analysis*

Principal component analysis of gene expression quantification was performed for visualization and quality control purposes. To do this, first the read count matrix obtained from HTseq was transformed using the variance stabilizing transformation function in DESeq2[132] and then filtered for the 500 most variable genes. PCA was then performed using the prcomp function in R. Principal components were then examined for their relationship to technical and biological variables. PC1 distinctly separates samples by sex except for four samples, which we removed from further analyses due to apparent mix-up or contamination.

3.5.3 *Genotyping and polygenic risk scores*

Genotyping was performed on a subset of samples (N_{cases} = 234, $N_{controls}$ = 187) using the Illumina Infinium Human OmniExpressExome. Standard genotyping quality control was performed with PLINK[133] to remove outliers and low quality SNPs and samples [36]. Imputation was performed on the Michigan Imputation Server (imputationserver.sph.umich.edu)[134] with the 1000 Genomes Phase 3 version 5 reference panel[111], Eagle phasing[135], and European ancestry. Following imputation, variants were excluded if they had mismatching alleles, if they were duplicates or indels, or if they had a SNP call rate < 90%. After imputation each sample had 46,625,935 SNPs. The imputed data were further filtered for $r^2 > 0.3$ and MAF > 0.05, yielding a final set of 6,828,668 SNPs per sample.

A polygenic risk score (PRS) for a given individual represents the cumulative genetic load of disease risk alleles and is defined as the sum of trait-associated alleles across many genetic loci, weighted by effect sizes estimated from a genome-wide association study. To calculate PRS we used the largest BD GWAS[42] with our samples removed, and used a P-value cut-off of $P < 0.05$. We compared PRS scores between BD cases and controls and between lithium using cases and non-lithium using cases by computing the Student's t-test.

3.5.4 *Curation of DEG lists from previous studies and enrichment analyses*

To compare our results with the results of previous studies, PubMed was searched for transcriptome-wide gene expression studies with BD case-control or lithium treatment designs. Four schizophrenia case control studies and one major depressive disorder study were also included because of their large sample sizes and therefore increased power to detect differentially expressed genes. Both microarray and RNA sequencing technologies were considered. A variety of tissues were considered including peripheral whole blood and post-

mortem brain from different regions, as well as several *in vitro* cell culture designs. Criteria for studies included in the comparison analysis were having > 10 samples, > 50 DEGs at FDR < 0.1, and differential expression analysis statistics available for download. The set of genes from Breen, et al.[92] was limited to genes with FDR < 0.005 because those were the only genes available for download.

Hypergeometric overlap tests were performed between the lithium-use DEGs and each of the gene lists from these previous using the GeneOverlap library in R. The full set of 12,344 genes expressed with > 10 counts in 90% of samples was considered as background gene expression. To account for multiple tests, Bonferroni correction was applied. For gene sets with significant hypergeometric *P*-values, the concordance rate and correlation statistics were calculated.

We inspected whether DEGs were differentially expressed in studies in The Stanley Medical Research Institute Online Genomics Database (www.stanleygenomics.org)[115]. We also inspected whether DEGs were expressed in brain tissues and cell types. A gene was considered to be expressed in frontal cortex tissue if it had a median gene TPM > 1 in GTEx (www.gtexportal.org). A gene was considered to be expressed in one of the six brain cell types[136] (neurons, fetal astrocytes, mature astrocytes, oligodendrocytes, microglia/ macrophages, and endothelia) if it had a mean FPKM > 1.

3.5.5 *Weighted gene co-expression network module preservation analysis*

Using the same pipeline as described in Methods, we constructed WGCNA[106] networks in four groups separately: BD cases (β = 4.2), controls (β = 5.5), lithium users (β = 7), and non-lithium users (β = 7). These networks were then assessed for module preservation using the WGCNA package in R. This module preservation analysis considers the structure of co-expression modules constructed in one group, the reference network, then tests the density and

connectivity of these same module structures in another group, the test network. Four analyses were run: with the BD case network as the reference and the control network as the test, vice versa, with the lithium use network as the reference and the non-lithium use as the test, and vice versa.

Evaluating module preservation requires the module assignment of each gene in the reference network, as well as adjacency matrices for both the reference network and the test network. Using these inputs for each of the four analyses, various module preservation statistics were calculated using modulePreservation in the WGCNA package with 200 permutations. Two main types of preservation statistics were calculated: density based preservation statistics, which determine whether module nodes (genes) remain highly connected in the test network, and connectivity based preservation statistics, which determine whether the connectivity pattern between nodes in the reference network is similar to that in the test network. Significance levels (permutation test *P*-values) were calculated by using a permutation test procedure that randomly permutes the module assignment in the test data. To evaluate module preservation between networks, the composite $Z_{summary}$ statistic was considered, which is the average of the density and connectivity based preservation statistics. Lower *Z* scores correspond to reference modules that are lesser preserved in the test network. Modules with *Z* scores less than 2 were considered not to be preserved. Modules with *Z* scores greater than 2 but less than 10 were considered to be moderately preserved. Modules with *Z* scores greater than 10 were considered to be well-preserved. Because *Z* statistics and permutation test *P*-values depend on the module size, the composite module preservation statistic *medianRank*, which is less dependent on module size, was also used to compare relative preservation among the modules. The *medianRank* statistic summarizes the ranks of the observed density and connectivity preservation statistics. Results of the module preservation analyses can be seen in Supplementary Figure 2.

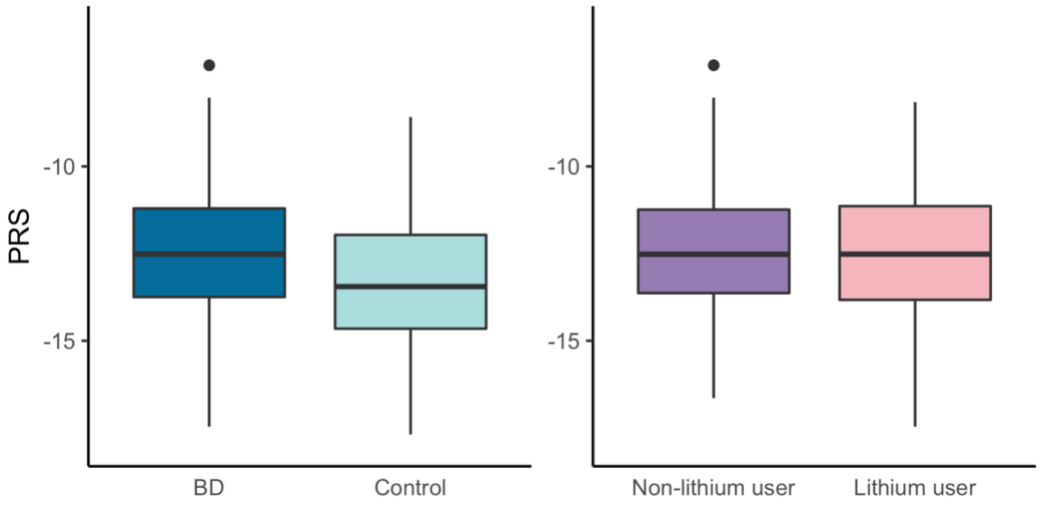
3.6 Supplementary Tables and Figures

	Case	*Control*		*Lithium user*	*Non-lithium user*	
	N (%)		*P*	*N* (%)		*P*
Diagnosis	240	204	-	152 (100%)	88 (30.1%)	<2.20E-16
Female sex	131 (54.6%)	119 (58.3%)	0.444	90 (59.2%)	160 (54.8%)	0.420
Lithium use	152 (63.3%)	0 (0%)	<2.20E-16	152	292	-
Tobacco use	74 (30.8%)	39 (19.1%)	6.14E-03	48 (31.5%)	65 (22.3%)	0.0387
Assessment group	240 (100%)	111 (53.4%)	<2.20E-16	152 (100%)	199 (68.2%)	<2.20E-16
Sequencing plate 1	48 (20.0%)	38 (18.6%)	0.810	58 (38.2%)	28 (9.6%)	0.800
Sequencing plate 2	48 (20.0%)	41 (20.1%)	1.000	60 (39.5%)	29 (11.5%)	0.803
Sequencing plate 3	48 (20.0%)	41 (20.1%)	1.000	59 (38.8%)	30 (10.3%)	1.000
Sequencing plate 4	48 (20.0%)	42 (20.6%)	0.906	57 (37.5%)	33 (11.3%)	0.619
Sequencing plate 5	48 (20.0%)	42 (20.6%)	0.906	58 (38.2%)	32 (11.0%)	0.804
	Mean (SD)			Mean (SD)		
Age	50.3 (12.4)	43.4 (14.8)	1.95E-07	48.0 (13.1)	46.7 (14.4)	0.309
RIN	7.50 (0.764)	7.70 (0.599)	1.92E-03	7.48 (0.633)	7.65 (0.726)	0.00774
Sequencing metric PC1	5.48E-4 (0.0458)	6.21E-4 (0.0462)	0.987	-7.35-4 (0.0457)	9.22E-4 (0.0461)	0.828
Sequencing metric PC2	4.55E-3 (0.0563)	-4.34E-3 (0.0324)	0.0385	6.16E-3 (0.0591)	-2.50E-3 (0.0391)	0.105
Sequencing metric PC3	6.92E-3 (0.0421)	-6.44E-3 (0.0491)	2.44E-03	6.43E-3 (0.0410)	-2.16E-3 (0.0480)	0.0495

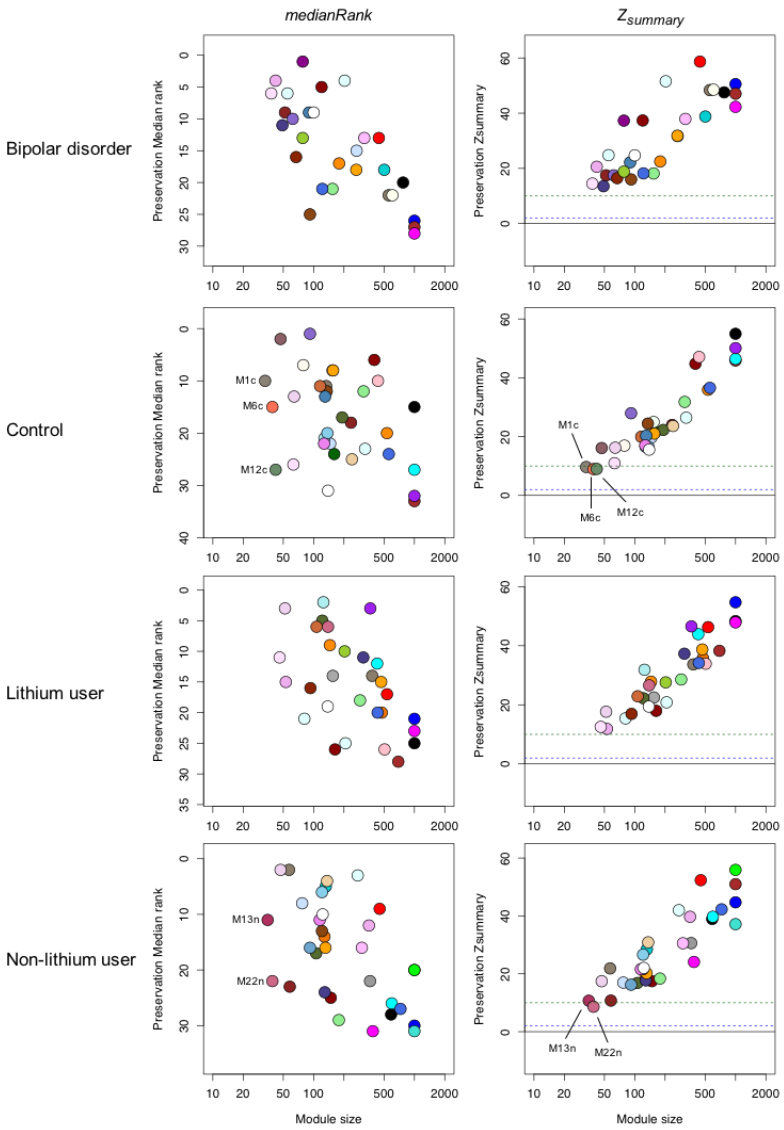
Supplementary Table 1. Covariate relationships with BD diagnosis and lithium use. P-values computed by Fisher's exact test (binary variable) or t-test (continuous variable). SD, standard deviation.

Module	Correlation P		Hyper-geometric P
	Lithium use	*BD diagnosis*	
M1	9.40E-04*	0.208	2.03E-97*
M2	0.363	0.989	0.902
M3	0.356	0.172	1.000
M4	0.634	0.505	0.979
M5	0.615	5.31E-02	0.966
M6	5.49E-02	0.105	0.998
M7	4.50E-04*	3.10E-03	1.000
M8	0.508	0.733	1.000
M9	1.15E-03*	0.165	6.15E-07*
M10	3.01E-03	1.92E-02	0.390
M11	3.12E-04*	1.76E-02	4.93E-13*
M12	3.05E-02	1.93E-02	1.000
M13	0.378	0.413	1.000
M14	0.585	0.134	0.564
M15	0.176	0.176	1.000
M16	0.821	0.227	1.000
M17	0.342	4.37E-02	1.000
M18	1.87E-02	0.163	0.830
M19	0.131	0.865	0.975
M20	5.50E-03	1.02E-02	1.000
M21	0.687	0.498	0.998
M22	0.349	0.238	1.000
M23	0.637	0.978	1.000
M24	0.584	0.451	1.000
M25	1.16E-02	0.377	3.83E-02
M26	2.00E-04*	2.68E-03	1.000
M27	0.309	8.46E-02	1.000

Supplementary Table 2. Co-expression module association with BD diagnosis and lithium use. Correlation *P*-values were calculated by correlating gene module membership values with gene significance values for the traits shown. Hypergeometric *P*-values were calculated by testing for overlap between the list of lithium-use DEGs and the list of genes within each module. *Significant at $P < \alpha = 0.05/27$.



Supplementary Figure 1. Polygenic risk scores across groups. PRS calculated from the largest BD GWAS[42] was significantly different between cases and controls (Left, Student's t-test P = 3.85 x 10^{-6}) but not between cases using lithium and cases not using lithium (Right, Student's t-test P = 0.690).



Supplementary Figure 2. Module preservation analysis. The blue dashed line represents a $Z_{summary}$ = 2, below which a module is considered not to be preserved. The green dashed line represents a $Z_{summary}$ = 10. If a module falls between the green and blue dashed lines it is considered to be weakly to moderately preserved. If a module falls above the green dashed line, it is considered to be strongly preserved. The 28 modules in the BD case network were significantly preserved in controls. The 33 modules in the control network were significantly preserved in BD cases. Three modules in the control network displayed moderate preservation in cases, M6c, M12c, and M1c ($Z_{summary}$ = 8.9, 9, and 9.7, respectively), but their median ranks were not among the top indicating that their low summary preservation statistics were due to small module size. The 29 modules in the lithium-use network were all significantly preserved in non-lithium users. The 32 modules in the non-lithium use network were all significantly preserved in lithium users. Two modules in the non-lithium-treated network displayed moderate preservation, M22n and M13n ($Z_{summary}$ = 8.6 and 9.5, respectively), but their median ranks were not among the top indicating that their low summary preservation statistics were due to small module size.

CHAPTER 4

Conclusion

We know from family studies that a large portion of risk for BD comes from genetic variation, and have confirmed this via genome-wide examinations of common genetic variation[42]. However, much of the genetic variation underlying risk for BD remains unknown, and even in known loci, which variant is causal and how variants mechanistically lead to disease are a mystery. Several studies have been successful in defining disease mechanisms in SCZ and MDD via gene expression analyses[83,93], but the results of similar studies in BD have been inconclusive due to small sample sizes and varying study designs (see Table 1). In our own attempt to characterize BD mechanisms, we explored gene expression signatures in whole blood in a large Dutch cohort. We found that almost all of the variation in gene expression observed in BD was due to lithium use.

Lithium is the first-line treatment for BD[23], but it is associated with high non-adherence rates due to adverse side effects[22], and there is a high degree of interindividual variability in response[69]. Lithium responsive-BD tends to run in families[25], but larger GWAS samples are needed to determine if it is a heritable subtype of BD[27]. Lithium is hypothesized to act by competing with Mg^{2+} and Ca^{2+} to alter internal cellular signaling mechanisms like those related to GSK3, inositol monophosphatase protein kinase A (PKA) and G-protein-coupled receptor signaling, ultimately leading to higher order effects on neuroprotection, chronobiology, and the immune system[21]. Our findings provided high resolution evidence for lithium targeting cellular processes related to signaling, calmodulin, protein kinase, and immunity. These effects were partially mediated by increased neutrophil content, adding to mounting evidence that lithium affects immune cells[118]. It remains to be determined if these effects contribute to lithium's

therapeutic mood-stabilizing mechanisms. A lack of psychiatric genetic signal within the lithium effects we observed suggests that our findings exist outside the context of underlying genetic risk. But larger sample sizes in both transcriptomic and genetic studies are needed to confirm this.

The future of BD and lithium genomics will undoubtedly see larger studies with higher resolution. Large-scale genomic studies should emphasize phenotyping to homogenize samples and improve detection power. Ascertaining lithium-responsive BD subjects may be one way to accomplish this. Gene expression studies should carefully assess and correct for medication use and cell type composition, or design around these issues (e.g. by collecting medication-naïve subjects and by performing single-cell RNA sequencing). As we delve deeper into the underlying biology of BD, biomarkers will emerge, and we may one day be able to diagnose and determine the best course of treatment from a simple blood draw. Outside of the lab, investigators should promote psychiatric genetic literacy to help dismantle the undue stigma attached to mental illness. Only through a cultural shift in care-seeking and care-giving will we ever see a world unburdened by mental illness.

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