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Alteration of miRNA-mRNA interactions in lymphocytes of individuals with schizophrenia

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ABSTRACT

The aetiology of schizophrenia is complex, heterogeneous, and involves interplay of many genetic and environmental influences. While significant progress has been made in the understanding the common heritable component, we are still grappling with the genomic encoding of environmental risk. One class of molecule that has tremendous potential is miRNA. These molecules are regulated by genetic and environmental factors associated with schizophrenia and have a very significant impact on temporospatial patterns of gene expression. To better understand the relationship between miRNA and gene expression in the disorder we analysed these molecules in RNA isolated from peripheral blood mononuclear cells (PBMCs) obtained from an Australian cohort of 36 individuals with schizophrenia and 15 healthy controls using next-generation RNA sequencing. Significant changes in both mRNA and miRNA expression profiles were observed implicating important interaction networks involved in immune activity and development. We also observed sexual dimorphism, particularly in relation to variation in mRNA, with males showing significantly more differentially expressed genes. Interestingly, while we explored expression in lymphocytes, the systems biology of miRNA-mRNA interactions was suggestive of significant pleiotropy with enrichment of networks related to neuronal activity.

1. Introduction

miRNA are highly enriched in the brain and have been shown to have a profound effect on the regulation of gene expression. Several studies have also implicated their involvement in schizophrenia and other psychiatric disorders through the analysis of post-mortem brain (Beveridge et al., 2010; Miller et al., 2012). While there is significant variation between studies, which highlight the heterogeneity of miRNA expression in the brain, there are some recurrent features and influences that suggest there are both genomic and epigenomic factors driving disease-associated expression. Several miRNAs have been repeatedly associated with schizophrenia at both the genetic and expression levels. One of the most significant common genetic risk factors for developing schizophrenia is a single nucleotide polymorphism (SNP) proximal to the MIR137HG locus (PGC, 2014). Haploinsufficiency of the miRNA biogenesis gene *DGCR8* is also implicated in the aetiology of some cases, as this gene is part of the DiGeorge critical region at chromosome 22q11.2, one copy of which is lost in 22q11.2 deletion syndrome; this condition predisposes individuals to a 20–30% risk of developing schizophrenia (Bassett and Chow, 2008). *DGCR8* and two other miRNA biogenesis genes – *DROSHA* and *DICER1* – have also been found overexpressed in the dorsolateral prefrontal cortex (DLPFC) and superior temporal gyrus (STG) in schizophrenia (Beveridge et al., 2010; Santarelli et al., 2011). And differential expression of individual miRNAs has been associated with schizophrenia on several occasions; the miR-132/212 cluster, for example, appears to be involved in both neurodevelopment and synaptic plasticity (Miller et al., 2012; Remenyi et al., 2013).

Peripheral blood mononuclear cells (PBMCs) are a broad class of leukocytes that are easily obtainable and have been a useful source of data in various studies of schizophrenia and other psychiatric disorders (Gardiner et al., 2013; Wang et al., 2015; Yu et al., 2015). A limitation of these cells is that they cannot provide a clear picture of brain-specific changes associated with these disorders. Nevertheless, they are a useful tool, as they can reflect other disease-associated environmental exposures, including changes in immune system activity, effects of antipsychotic drugs on gene expression, and can also reveal genetic and epigenetic perturbations that are involved in disease aetiology, and may even provide biomarkers of disease state. Importantly, their peripheral nature allows collection from living individuals; expression changes in brain tissue can only be assessed with post-mortem tissue studies, meaning only a single time-point can be studied. Compared to PBMCs,

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post-mortem tissue also suffers from additional confounding factors that can impact the results obtained, including the interval between death and tissue collection, the pH of the brain tissue, and issues with RNA integrity.

While studies of miRNA expression in schizophrenia have often investigated the expression of their target genes, this has typically been to a limited extent, looking at a handful of predicted targets using low-throughput technologies, such as qPCR. In contrast, few studies have offered an integrated investigation of miRNA-mRNA interaction networks. Using high-throughput, next-generation RNA sequencing technology, such integrated studies are now more feasible. In the present study, we analysed PBMC total RNA obtained from a small schizophrenia cohort and healthy controls. Using RNA sequencing technology, we looked for changes to both mRNA and miRNA expression, and additionally investigated these two expression datasets for significant, negative correlations between miRNAs and their predicted target mRNAs, to identify likely miRNA-mRNA interaction networks. The results show a large difference between males and females, whereby male subjects displayed much greater perturbation of mRNA expression. These results have biological implications for the regulation of development, and function of the nervous and immune systems.

2. Materials and methods

2.1. Tissue collection

Total RNA isolated from peripheral blood mononuclear cells (PBMCs) was obtained from the Australian Schizophrenia Research Bank (ASRB) (Loughland et al., 2010). A total of 51 samples consisting of 15 healthy controls (7 males and 8 females) and 36 individuals diagnosed with schizophrenia or schizoaffective disorder, as per ICD-10 criteria (18 males and 18 females) were analysed in the present study (Supplementary Table 1). The ASRB provided summary data for all individuals concerning demographics, duration of disease, medications, and other factors (Supplementary Table 2). No significant difference was observed between the mean ages of any of the groups (control males, control females, schizophrenia males, and schizophrenia females; $p > 0.9$) as determined by one-way ANOVA (Supplementary Figure 1).

2.2. RNA sequencing and preliminary analysis

RNA sequencing was performed on both mRNA and small RNA fractions separately using the Illumina HiSeq 2000 platform (BGI). The output *.fastq* files were analysed with *FastQC* for quality control analysis. Good-quality reads were deemed those that possessed a Phred quality score ≥28 for all bases and lacked any sequencing adapter content. Remnant sequencing adapter content and poor quality base calls (Phred score < 28) were trimmed with *cutadapt*. Reads were mapped to the human genome build GRCh38 using *HISAT2* and *Bowtie2* for mRNA and small RNA data, respectively. Reads aligning to annotated genes or miRNAs were counted with *htseq-count*. See Supplementary Methods for further details on software and commands used.

2.3. Differential expression analysis

Differential expression analysis was performed in *edgeR* (Robinson et al., 2010) using the pairwise exact test mode. Significance was determined as all genes with a p-value < 0.05 and Benjamini-Hochberg FDR < 0.1. Initial inspection of the data was performed using multidimensional scaling (MDS) and biological coefficient of variation (BCV) plots using edgeR's inbuilt functions (Supplementary Figures 2, 3, and 4). These analyses were used to check for outlying samples and variance issues that may bias downstream analyses. A counts-per-million (CPM) threshold was applied to filter out features with consistently low counts. See Supplementary Methods for further details.

2.4. microRNA-mRNA correlation analysis

The *R* package ***miRComb*** (Vila-Casadesus et al., 2016) was employed to find significant negative (i.e. canonical) correlations between the miRNA and mRNA datasets. The correlation data was filtered for all miRNA-mRNA interactions (conserved and poorly-conserved) as predicted by the TargetScan v7.2 database with cumulative weighted Context+ + scores of less than −0.2 (Grimson et al., 2007).

2.5. Pathway and gene ontology analyses

The ToppFun functional enrichment suite (Chen et al., 2009) was used to determine pathways and gene ontologies that were significantly enriched for differentially expressed genes in the sequencing datasets.

2.6. RT-qPCR and sequencing validation

To confirm the validity of our sequencing libraries, RT-qPCR analysis was performed on a subset of sequenced samples and checked for correlation between CPM and ΔC_t values. 200–1000 ng total RNA was reverse transcribed for production of both miRNA and mRNA cDNA. See Supplementary Methods for full details. Briefly, for miRNA cDNA, total RNA polyadenylated with 1.25 units (U) *E. coli* poly(A) polymerase (NEB) and 1 mM ATP. All RNA was treated with amplification-grade 1U DNase I (Invitrogen), then blocked with 2.5 mM EDTA at 65 °C prior to reverse transcription with 200U SuperScript II reverse transcriptase (Invitrogen) as per manufacturer's instructions. 40U RNaseOUT RNase Inhibitor (Invitrogen) was included in the reaction to prevent RNA degradation. Reverse transcription was primed with either 5 ng/μL random hexamer primers (mRNA) or 2 μM miRNA reverse transcription primer, which possessed the sequence: 5' – CAGGTCCA-GTTTTTTTTTTTTTTTVN – 3' (V = either A, G, or C; N = either A, T, G, or C) (Balcells et al., 2011). For reverse transcription-negative samples, nuclease-free water was substituted for SuperScript II.

Quantitative real-time PCR (qPCR) was performed using Power SYBR Green master mix (Applied Biosystems). Briefly, for each reaction 5 μL of 1/20 cDNA dilution was mixed with 6.25 μL SYBR Green, 0.5 μL of both forward and reverse 10 μM primers, and 0.25 μL nuclease-free water. Each reaction was carried out in triplicate. A no-template control (NTC) was also run for each gene of interest by substituting nuclease-free water for cDNA. Glucuronidase beta (*GUSB*) and hydroxymethylbilane synthase (*HMBS*) were used as the reference for mRNA qPCRs; *U6* small nuclear and *U44* small nucleolar RNAs were used as references for miRNA qPCRs. The qPCR reactions were run on an Applied Biosystems 7500 Real Time PCR System with a dissociation curve to ensure specificity, as previously described (Beveridge et al., 2010). See Supplementary Methods and Supplementary Information for details on primers used for this study and their design.

Correlation analysis was performed to determine if there was a negative linear relationship between the $\log_2$-transformed miRNA CPM or mRNA RPKM (reads per kilobase per million mapped reads) values from the sequencing data and the normalised C_t values (ΔC_t) from the qPCR analysis. ΔC_t was calculated by subtracting the geomean of the two reference gene C_t values from the target gene C_t. RPKM values were calculated by dividing a gene's CPM value by the non-overlapping sum of its exons in kilobases.

3. Results

3.1. RNA sequencing analysis

Quality control analysis by *FastQC* revealed that the small RNA sequencing data contained approximately 1.18×10^7 ($\pm 3.44 \times 10^5$) reads per sample, while the mRNA data contained 3.98×10^7

(± 1.03 × 10^6) reads per sample. Both datasets had a high overall mapping rate; 94.8% (± 1.8%) of the small RNA reads and 96.9% (± 1.3%) of the mRNA reads mapped to GRCh38. The small RNA data showed a high rate of multiple-mapping; 71.3% (± 4.42%) of the reads mapped to multiple regions of the genome, while only 23.5% (± 4.2%) mapped once. This is to be expected since many mature miRNAs originate from multiple genomic loci. In contrast, only 5.8% (± 2.0%) of the mRNA reads mapped multiple times, with 88.4% (± 2.7%) mapping only once.

On average, 2.88×10^7 ($\pm 1.67 \times 10^6$) mRNA reads aligned to known exons, and 2.14×10^6 ($\pm 6.46 \times 10^5$) small RNA reads aligned to mature miRNA features (Supplementary Table 4). Analysis in edgeR using a multidimensional scaling (MDS) plot (Supplementary Figures 2 and 3) identified two outlying samples – one male control and one female case – that had a very discordant small RNA (but not mRNA) expression profile compared to the remaining members of their respective groups. Upon removing these samples from the small RNA analysis, the MDS plots showed a much tighter clustering of samples. This also slightly reduced the overall mean small RNA library size to 2.13×10^6 ($\pm 6.45 \times 10^5$) reads. Both small RNA and mRNA libraries were then filtered for lowly-expressed miRNAs or genes (detailed in the methods section), which did not have a significant impact on overall library size ($2.88 \times 10^7 \pm 1.67 \times 10^6$ reads for the mRNA data, and $2.13 \times 10^6 \pm 6.44 \times 10^5$ reads for the small RNA data), but did further tighten the MDS clustering for the small RNA dataset.

3.2. *Differential expression*

Both the small RNA and mRNA sequencing data were analysed for pairwise differences in RNA expression using *edgeR*. Initially the data was analysed with all cases compared against all controls. Using an FDR threshold of 0.1, 35 miRNAs and 97 mRNA were observed to be differentially expressed. Among the dysregulated miRNAs, 21 were downregulated in contrast to the 80 altered mRNAs, which were upregulated compared with controls (Fig. 1a and b, Supplementary Tables 6 and 7).

To explore whether sex was a significant confounding factor in the data, males and females were analysed for differential expression separately. Since performing separate analyses for both sexes resulted in a drop in statistical power for each pairwise analysis, fewer significant results were expected. Indeed, fewer miRNAs reached significance: in males, 14 miRNAs were altered – 12 downregulated – and in females, only 7 miRNAs were differentially expressed, all downregulated (Fig. 1d, f; Fig. 2b; Supplementary Table 6). Some overlap was observed between all three analyses of the miRNA data; 6 of the 7 miRNAs downregulated in females were also significantly altered in the whole cohort; 5 of these were also differentially expressed in the males (Fig. 1h). Nine of the miRNAs significant in the male-only analysis, were also common to the whole cohort.

There was a larger difference in mRNA dysregulation in males compared to females; when considering only the male subjects, 365 genes were differentially expressed, with 318 being upregulated (Figs. 1e and 2a), while among the female individuals, only 1 gene was differentially expressed – *NRCAM* (neuronal cell adhesion molecule), which was downregulated (Fig. 1c,e, Supplementary Table 7). *NRCAM* was not significantly altered in the male-only analysis ($p = 0.50$, $q = 0.72$), and did not pass multiple test correction in the whole cohort analysis ($p = 0.0046$, $q = 0.13$), suggesting this dysregulation was specific to females. Furthermore, there were only 32 mRNAs common between the male-only and the whole cohort analyses; an additional 333 mRNAs reached significance once the female subjects were removed, despite a drop in statistical power.

3.3. *RT-qPCR validation*

To validate the sequencing results, 17 samples were analysed *via* RT-qPCR for the expression of 5 mRNA including: *NRCAM*, *DGCR2*, *ACTN1*, *KREMEN1*, *RXRA*; and 3 miRNAs including: miR-1271-5p, miR-221-5p, and let-7f-5p, all of which were differentially expressed between groups. A significant correlation between the ΔC_t values for each gene and the corresponding $\log_2$-transformed RPKM and CPM values for genes and miRNAs, respectively (Fig. 2c and d).

3.4. *Pathway and gene set enrichment*

Enrichment of cellular pathways and gene ontologies was determined *via* the online ToppFun functional enrichment tool. First, the whole-cohort mRNA dataset was analysed (Supplementary Table 8). The top gene ontology observed was *extracellular space*, with 22 genes in the dataset. *Defense response*, *cytokine production*, and *immune response* were also among the top ontologies observed. Furthermore, three immune-related REACTOME pathways were also significantly enriched for differentially expressed genes: *neutrophil degranulation*, *innate immune system*, and *immunoregulatory interactions between a lymphoid and a non-lymphoid cell*. This suggests a substantial dysregulation of immunity-related genes in PBMCs in schizophrenia. The male-only dataset was similarly enriched for immune-relevant terms, including *immune response* and *inflammatory response* (Fig. 3a, b, c and Supplementary Table 9). Additionally, numerous genes related to cell motility were differentially expressed; *chemotaxis*, *taxis*, and *locomotion* were among the top gene ontologies observed in males. Again, REACTOME pathways *innate immune system* and *neutrophil degranulation*, as well as *ROS, RNS production in phagocytes* and *interleukin-10 signalling* were significantly perturbed. Among males, there also appeared to be a substantial over-representation of genes originating from chromosome 19 (Fig. 4 and Supplementary Table 9). In total, 46 differentially expressed genes were located on chromosome 19, representing approximately 1.5% of its known annotated genes. Functional enrichment analysis revealed five genomic loci significantly enriched for differentially expressed genes, with 20 genes originating from three loci on chromosome 19, including four leukocyte immunoglobulin-like receptor genes: *LILRA2*, *LILRA4*, *LILRA5*, and *LILRB3*. Several genes relating to Wnt signalling were also differentially expressed in the male dataset (Supplementary Table 10), including Wnt signalling antagonist *KREMEN1* and Wnt receptor subunit *FZD3*, which were up- and downregulated, respectively. Functional enrichment analysis additionally revealed genes whose differential expression have been associated with stimulation of the Wnt signalling pathway, *via* comparison with the Broad Institute's curated gene sets in their molecular signatures database.

3.5. *miRNA-mRNA expression correlation*

The expression data from the male-only cohort was analysed with *miRComb* (Vila-Casadesus et al., 2016) to determine if any miRNA-mRNA pairs predicted by TargetScan v7.2 were significantly negatively correlated with one another, suggesting potential direct regulation of gene expression by the miRNAs. At an FDR threshold of 0.05, nine miRNAs were found to be significantly correlated with 54 mRNAs (Fig. 4, Supplementary Figure 4; Supplementary Table 11). The 7 most significant and strongest correlations were with miR-1271-5p, which was one of the 5 miRNAs significantly altered in both males and females. Furthermore, 10 genes were significantly correlated with two or three miRNAs. Gene ontology analysis again revealed an enrichment of immunity- and cell motility-related genes (Fig. 3d and Supplementary Table 12). Additionally, several brain- and schizophrenia-relevant genes were among this subset, including schizophrenia-associated *DGCR2* (DiGeroge Critical Region 2), *BASP1* (brain acid-soluble protein 1), and *RARA* (retinoic acid receptor alpha).

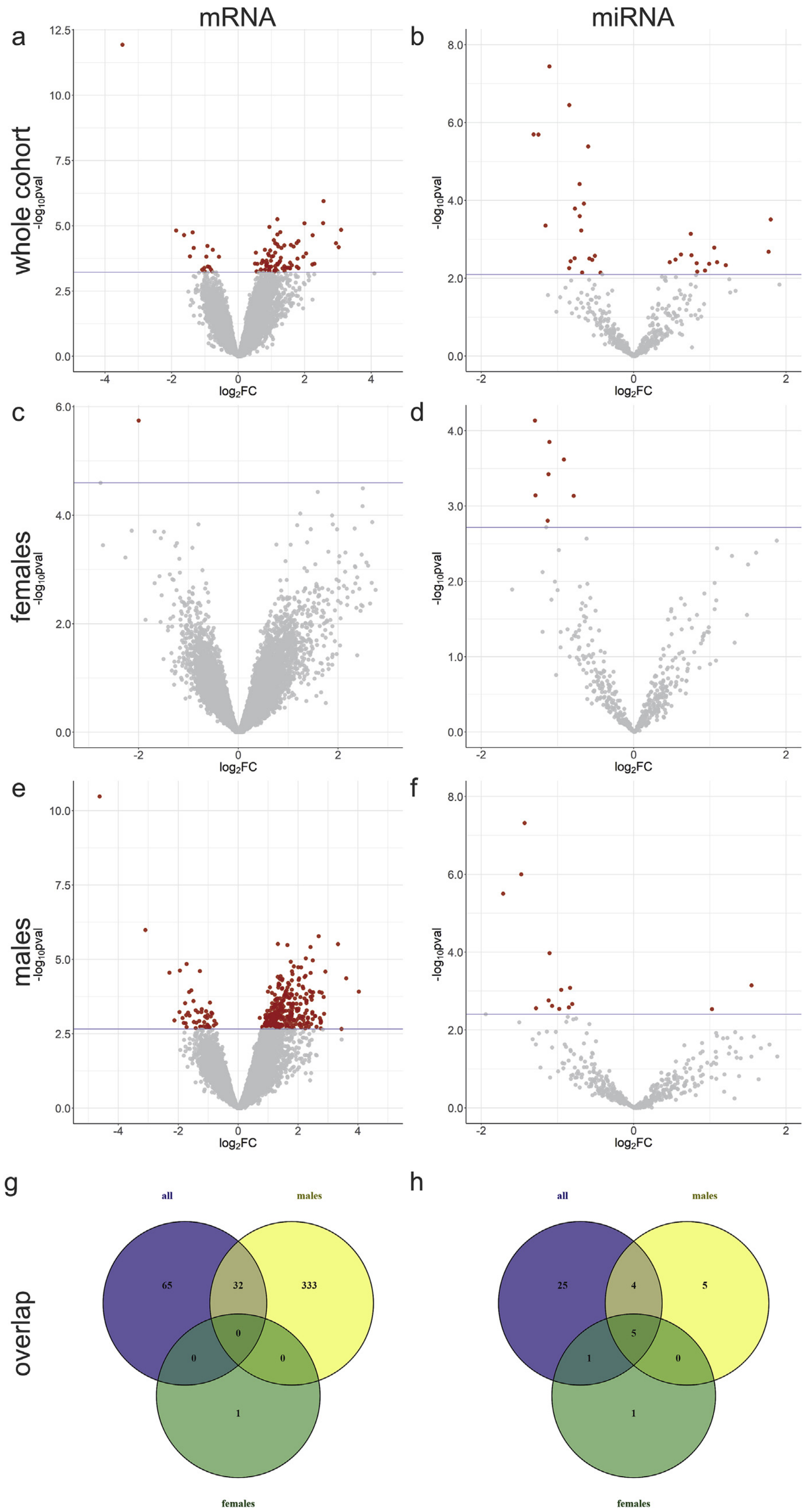

Fig. 1. (a–f) Volcano plots showing $\log_2$ fold-change vs $\log_{10}$ p-value for differentially expressed genes (a, c, e) and miRNAs (b, d, f) in the whole cohort (a, b), as well as in the female- (c, d) and male-only (e, f) groups. Dark red points represent genes passing the FDR cut-off of 0.1 (blue line). (g, h) Venn diagrams displaying the overlap between the mRNA (g) and miRNA (h) datasets.

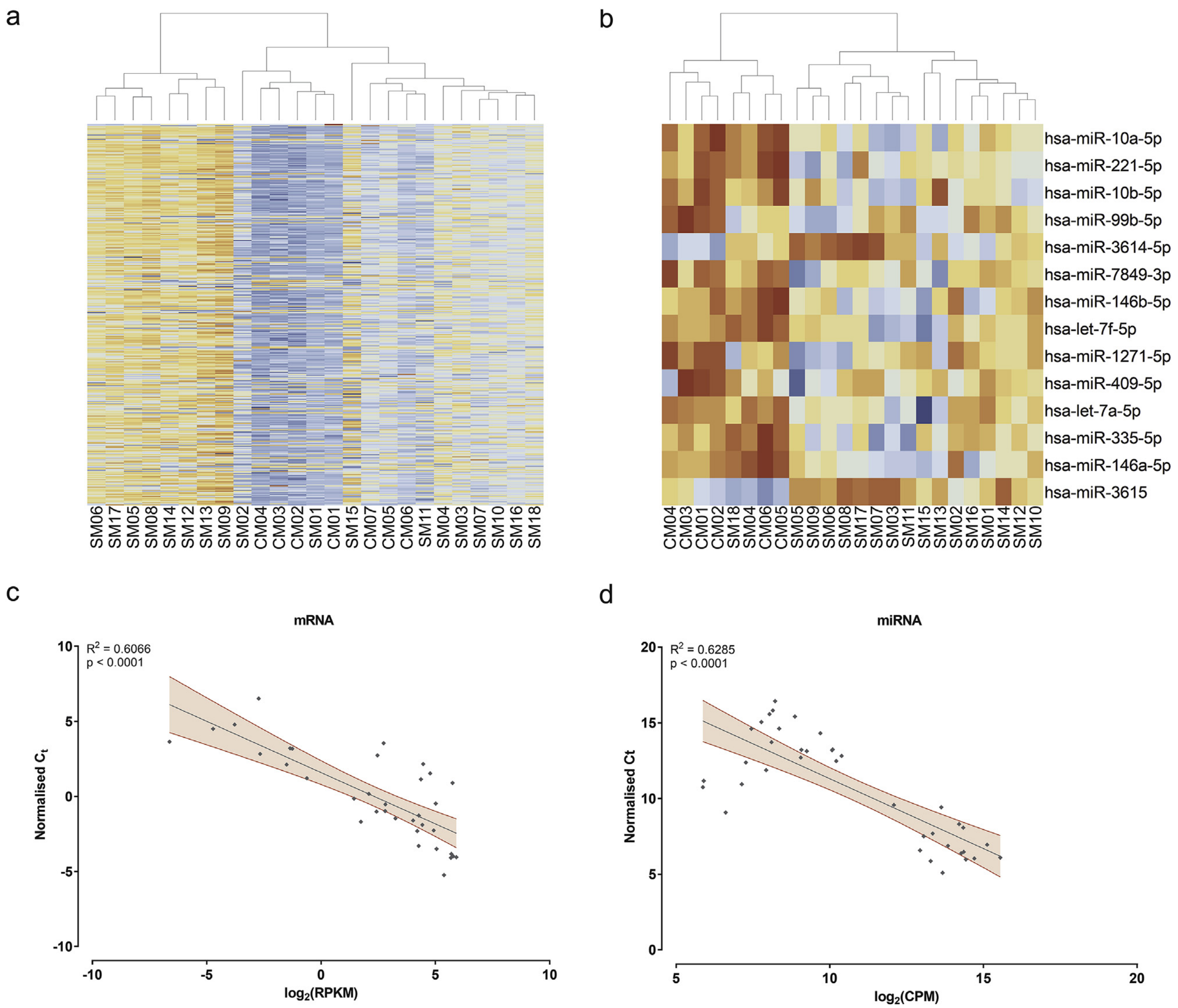

Fig. 2. (a,b) Heat maps of the significantly (FDR < 0.1) differentially expressed genes in the male-only mRNA (a) and miRNA (b) analyses. Each square represents the expression of a single gene in a single sample. Red = high expression, blue = low expression. Labels for the genes in the mRNA heatmap are omitted due to the high number of differentially expressed genes (see Supplementary Table 7 for complete list). (c,d) qPCR validation of the sequencing data. Five genes and three miRNAs were selected for validation in 17 samples. C_t values were normalised to the C_t values of housekeeping genes; *GUSB* and *HMBS* were selected for the mRNA reactions, and *U6* and *U44* were both used for miRNA analyses; in each case the geomean of the two C_t values was used as the reference. Normalised C_t values were correlated with $\log_2$RPKM and $\log_2$CPM values from the mRNA (a) and miRNA (b) sequencing data, respectively. A significant correlation was observed in both cases ($p < 0.0001$).

3.6. Role of antipsychotic medications

The ability for antipsychotics to impact gene and miRNA expression is a confounding factor in expression studies like this. The schizophrenia cases in the present cohort were mostly being treated with antipsychotics, and further separating the groups by treatment was infeasible due to the limiting sample size. However, a previous study published by our laboratory investigated the effects of three antipsychotics – chlorpromazine, clozapine, and haloperidol – on gene and miRNA expression in T-lymphocytes (Gardiner et al., 2014). No miRNAs, and only a handful of genes were common across the two studies; furthermore, of those common genes, only two – *ANXA11* and *HPSE* – were altered in the same direction, while the remaining 18 genes - *STK40*, *MBOAT7*, *TBC1D10B*, *LPPR2*, *GMIP*, *WAS*, *WBP2*, *RARA*, *ZDHHC18*, *SBNO2*, *LRP10*, *MAP3K11*, *EHD1*, *MYD88*, *PFKFB3*, *PTGFRN*, *HK1*, and *SLC39A7* – were dysregulated in opposite directions (Supplementary Table 4). This might suggest that the changes observed in the oppositely-regulated genes might be associated to the disease state, rather than medication.

4. Discussion

Complex patterns of posttranscriptional gene expression are regulated by networks of small non-coding miRNA that are prone to dysregulation in schizophrenia. In the current study we used RNA sequencing to explore their composition and identify key miRNA-mRNA interactions in PBMCs from individuals with the disorder. Our analyses revealed a number of interaction networks with miRNA hubs and associated biological pathways with relevance to the pathophysiology of schizophrenia. A surprising feature of this data was the very significant sexual dimorphism between gene expression profiles observed in schizophrenia, with over three hundred mRNA differentially expressed in males, compared to only one in females. This gene, the neuronal cell adhesion molecule (*NRCAM*), is involved in dendritic spine remodelling (Demyanenko et al., 2014) and axon growth and guidance (Sakurai, 2012). Interestingly, female mice with *Nrcam* double knockout show

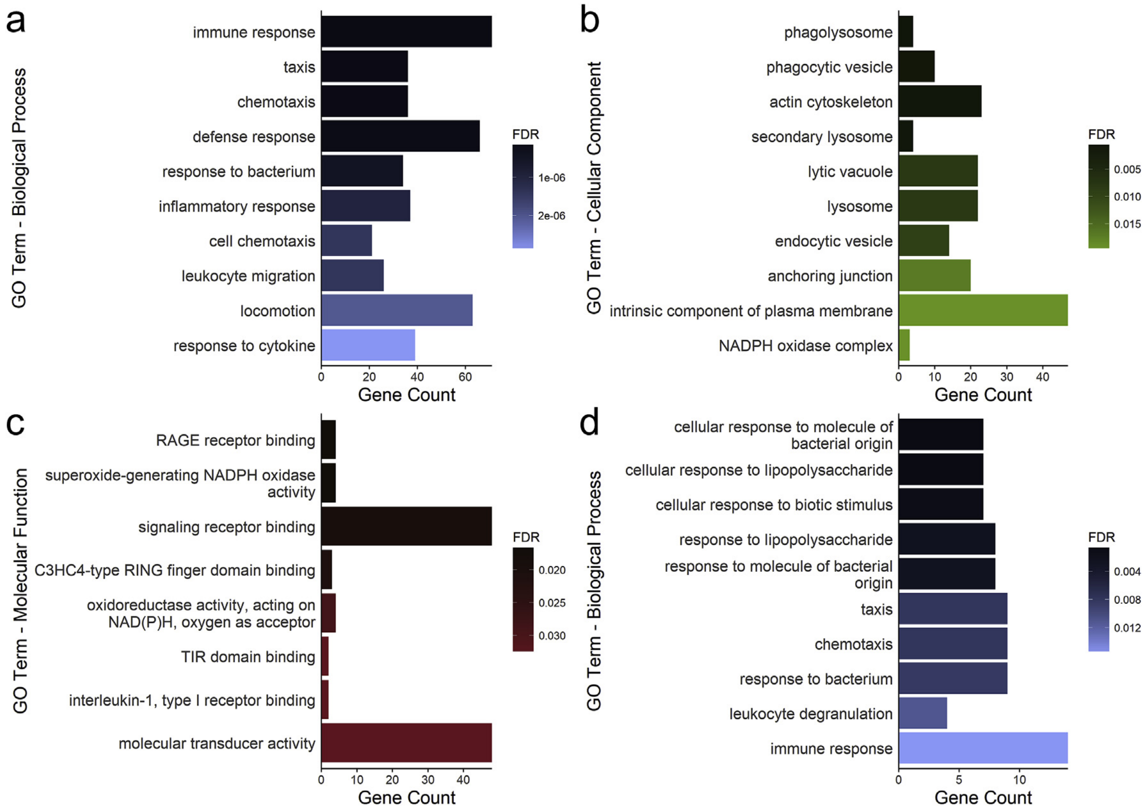

Fig. 3. | Top gene ontologies from the male-only mRNA sequencing data. (a) Top 10 biological process gene ontologies. (b) All cellular component ontologies. (c) All molecular function ontologies. (d) Top 10 gene ontologies (all biological process terms) from the male mRNA-miRNA correlation analysis.

motor and spatial learning deficits (Moy et al., 2009), whereas males show reduced sociability. It has previously been identified as a candidate gene for schizophrenia in a Korean population (Kim et al., 2009; Zhang et al., 2015), and has been associated autism spectrum disorders (Bonora et al., 2005) and addiction (Ishiguro et al., 2006). The *NRCAM* promoter region contains a putative estrogen response element (ERE) and is more highly expressed in estrogen receptor (ER)-positive cells compared to ER-negative cells (Yu et al., 2011). Estrogen is thought to play a significant role in the gender differences observed in schizophrenia, especially in a neuroprotective manner (da Silva and Ravindran, 2015), and current evidence suggests that women with schizophrenia tend to have lower estradiol levels compared to healthy controls (Huber et al., 2004). Therefore, the expression of *NRCAM* in females with schizophrenia could potentially be influenced by differences in estradiol.

Our analyses also revealed an enrichment of genes involved in inflammatory signalling pathways in schizophrenia, particularly among males. This included *IFNG* (interferon gamma) and *IFI27* (interferon-induced 27), which were downregulated, *IFITM2* (interferon-induced trans-membrane protein 2), which was upregulated, and several interleukin pathway genes. While many of these did not reach significance in the entire cohort, the gene ontology analysis still revealed an over-representation of immune response and cytokine production genes, suggesting there is a strong immune signal in the blood associated with schizophrenia, which supports previous findings (Horvath and Mirnics, 2014). Indeed, the large genome-wide association study (GWAS) published in 2014 revealed significant enrichment of SNPs in enhancers specific to immune cells in addition to cells of neuronal lineage, and identified the major histocompatibility (MHC) region to be the most significantly associated locus in schizophrenia (PGC, 2014). Taxis and locomotion genes were also differentially expressed in PBMCs in males. These processes are important for lymphocyte localisation to sites of infection and are vital for inflammatory responses. While this is consistent with inflammatory mechanisms believed to be altered in schizophrenia, the specific details of modified peripheral immune activity in the pathophysiology of schizophrenia is still unknown. Communication between the peripheral immune system and the brain has been investigated, with several pathways for peripheral proinflammatory cytokines to either directly or indirectly affect the brain (Khandaker and Dantzer, 2016). These pathways are thought to be involved in many psychiatric disorders, such as depression and schizophrenia.

We also observed dysregulation of genes relating to the Wnt/β-catenin signalling pathway in schizophrenia. This included the upregulation of *KREMEN1*, which is a negative regulator of Wnt/β-catenin signalling (Mao et al., 2002), and the downregulation of *FZD3* – frizzled 3, a Wnt receptor involved in planar cell polarity (Tissir and Goffinet, 2013). The changes both support a general downregulation of Wnt/β-catenin signalling in schizophrenia, which concurs with previous findings (Mulligan and Cheyette, 2017). There were also several other dysregulated genes previously observed co-expressed with perturbation of the Wnt-signalling pathway (Hoshida et al., 2009; Labbe et al., 2007; Ziegler et al., 2005). This pathway is not only important to neurodevelopment, it is also important in inflammatory responses; Wnt signalling in microglia can result in microglia activation and release of cytokines IL-6, IL-12, and TNF-α (Halleskog et al., 2011), which are heavily associated with immune responses in schizophrenia (Miller et al., 2011). Thus the Wnt pathway may play a role in regulating the increased inflammatory state associated with schizophrenia.

The analysis of small RNA and mRNA provided the capacity to explore miRNA-mediated posttranscriptional regulatory networks in schizophrenia. This was achieved by integrative analysis of negative correlations between the miRNA and mRNA read counts. In general accordance with expectation, we observed downregulation of miRNAs and upregulation of target genes, in support of the canonical regulatory function of miRNAs. Several significant co-expression networks were

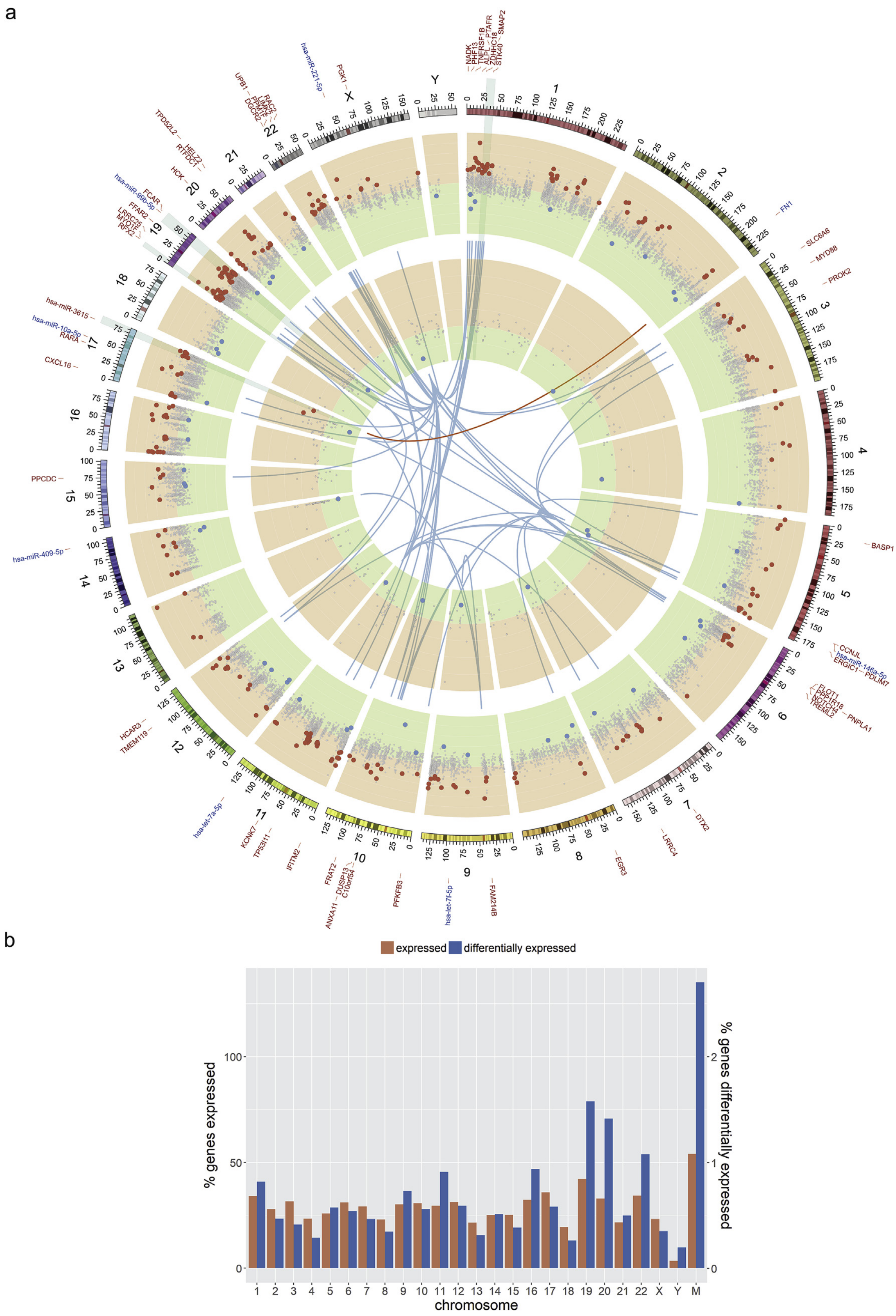

Fig. 4. (a) Circos plot showing all differentially expressed genes (outer scatter plot) and miRNAs (inner scatter plot), with links representing significant negative correlations between miRNAs and mRNAs. Labels and points in blue = downregulated genes and miRNAs; red = upregulated. Labels shown only for significantly correlated genes and miRNAs. Light blue links = correlations between downregulated miRNAs and upregulated genes; orange link = upregulated miRNA and downregulated gene. (b) Percentage of all genes from each chromosome which were expressed (orange) vs significantly differentially expressed (blue).

observed in biologically relevant systems, including enrichment of inflammation and locomotion ontologies. This suggests that these miRNAs, in particular, represent a core subset involved in regulating immune system activity in schizophrenia. This is supported by previous studies which suggest miR-221 is an activator of the immune response (Chen et al., 2015; Peng et al., 2018; Zhao et al., 2016). Several of these miRNAs and their families have also been related to brain function and schizophrenia in previous studies. Two let-7 family members – let-7a-5p and let-7f-5p – were downregulated in our analyses. Members of this miRNA family have been identified across a number of psychiatric disorders, including schizophrenia, bipolar disorder, and major depressive disorder (Beveridge et al., 2010; Miller et al., 2012; Shi et al., 2012; Shih et al., 2012; Smalheiser et al., 2011). Furthermore, both let-7 and miR-10 family miRNAs appear to be involved in neurodevelopment (Parsons et al., 2012), and miR-10b-5p (which differs from miR-10a-5p by only a single base outside the seed sequence, and thus is predicted to mostly target the same mRNAs) has been demonstrated to regulate BDNF expression, which is a vital signalling molecule in neurogenesis and synaptic plasticity (Varendi et al., 2014). Therefore, the downregulation of these molecules in schizophrenia may be reflective of both immune system dysregulation and neurodevelopmental deficiencies.

Two mature miRNAs – miR-1271-5p and miR-221-5p – were correlated with their common predicted target *DGCR2* (DiGeorge Critical Region 2). Notably, *DGCR2* is one of the many genes within the "minimal DiGeorge critical region" (MDGCR) (Gong et al., 1996) affected by 22q11.2 deletion syndrome (22q11.2 DS), which conveys an approximately 20–30% risk of developing schizophrenia (Bassett and Chow, 2008). *DGCR2* is a transmembrane protein, with potential ligand-binding potential at its extracellular domain (Demczuk et al., 1995). It has been hypothesised to be involved in neuronal crest cell migration, and plays a role in migration of mouse cortical projection neurons at post-natal day 2.5 (Molinard-Chenu and Dayer, 2018). This appears to be partially due to interactions with the secreted protein Reelin, another schizophrenia-associated gene with important roles in regulating neuronal migration and neurodevelopment. SNP variants in the *DGCR2* gene have been associated with schizophrenia in males from an Ashkenazi Jewish population; in particular, the C risk allele for the coding SNP rs2072123 appears to have a negative impact on allele expression, concurring with the schizophrenia risk for haploinsufficiency of the 22q11.2 region (Shifman et al., 2006). Furthermore, elevated *DGCR2* expression has been reported in the DLPFC in schizophrenia (Shifman et al., 2006), which may be related to antipsychotic treatment, as rats treated with olanzapine, haloperidol, or clozapine displayed higher *DGCR2* mRNA expression in the frontal cortex compared to controls. The present study also supports previous observations of miR-1271-5p downregulation in PBMCs in schizophrenia (Yu et al., 2015). That same study also demonstrated that this miRNA appears to be positively regulated by the antipsychotic drug risperidone. Therefore miR-1271-5p and *DGCR2* may be involved in both schizophrenia pathophysiology and antipsychotic drug function, and miR-1271-5p may regulate *DGCR2* expression and may modulate its role in neurodevelopment and neuronal function. Further investigation of this miRNA and its interaction with *DGCR2* and other schizophrenia-associated genes will be the focus of future study.

A limitation to the general applicability of the study findings was sample size which may contribute to the disparity between males and females. To mitigate this possibility, the sample sizes of males and females in cases and controls were all evenly matched. We also applied identical lower-bound CPM thresholds were also applied (0.2 for gene expression and 5 for miRNAs), and the numbers of genes and miRNAs tested in each group were similar (16,700 genes and 479 miRNAs for males; 16,478 genes and 439 miRNAs for females). While few studies perform separate analyses for males and females due to the effect on sample size, we suspect this may masks discrete differences in males and females and reduce the power to identify important differences in disorders known to display significant sexual dimorphism. To further test this assertion, it will be important to investigate larger cohorts in future with the power to more confidently resolve differences associated with gender.

As with most expression studies in psychiatric cohorts it is challenging to resolve the influence of antipsychotic medication, which are a present factor most of the time. Antipsychotic drugs have been shown previously to alter gene and miRNA expression in model systems and have the potential to confound studies from tissues that have been exposed to medication in human subjects. As it was infeasible to further dissect the data to examine the role of antipsychotics., some caution should be exercised in interpreting these results. In particular, the immune system-related gene expression changes observed in this study may be confounded, since several studies have previously investigated the effects of antipsychotic medications on the immune system, with varying results. For example, one study identified three cytokine molecules – IL-1RA (interleukin 1 receptor antagonist), IL-10 and IL15 – to be elevated in serum of first-onset, antipsychotic-naïve schizophrenia patients, of which IL-1RA and IL10 were downregulated after 6 weeks' treatment with either olanzapine or risperidone. Furthermore, the changes in IL-10 levels correlated with negative, general, and total PANSS (positive and negative syndrome scale) symptom improvement (de Witte et al., 2014). Clozapine has been found to inhibit interferon gamma production, while haloperidol slightly enhances its production (Chen et al., 2012). Antipsychotics may also negatively affect macrophage adhesion and phagocytic uptake, and risperidone and clozapine appear to have an inhibitory effect on macrophage IL-6, IL-8, and TNF-α production in response to lipopolysaccharide (Chen et al., 2013). Yet other studies appear to support an increase in the activity of the T helper-1 system and B cells (Muller et al., 2000). Thus, a complex relationship between schizophrenia, antipsychotic medication, and the immune system exists, and confounds these results. Nevertheless, the minimal overlap of genes with a previous study from our lab (Gardiner et al., 2014) investigating antipsychotic medication effects on gene expression in T-lymphocytes suggests that many of the results may be genuinely associated with the disease state rather than treatment. Where there was correspondence in gene expression, most were in opposite directions in schizophrenia compared to the antipsychotic drug study, and may even be therapeutic targets for antipsychotic drug action.

Finally, several of the miRNAs identified in this study have been previously observed in schizophrenia and other psychiatric disorders. Three of the miRNAs downregulated in the male data – miR-10a-5p, miR-146a-5p, and miR-335-5p – were downregulated in prefrontal cortex from major depressive disorder patients (Smalheiser et al., 2012). miR-10a-5p has also been found differentially expressed in lymphoblastoid cells in autism spectrum disorder (Ghahramani Seno et al., 2011), as has miR-146a-5p (Talebizadeh et al., 2008). Interestingly, while miR-409-5p was downregulated in this study, the accompanying 3′ miRNA was found downregulated in a previous study from our laboratory (Gardiner et al., 2012), and upregulated in PBMCs from major depressive disorder patients (Belzeaux et al., 2012). It is likely that the 5′ form was only observed in the current study using deep sequencing as it is expressed at lower levels compared to the 3' form and may have been below the detection threshold previously. Variation between miRNA expression studies is an ongoing concern, especially in complex disorders, and likely results from various sources, including sample size, technology platform and normalization, as well as the heterogeneity of transcription in a population related to genetic background and environmental exposures. Nevertheless, the repeated detection of particular miRNAs and miRNA family members – e.g. miR-10, let-7, and miR-1271 – in schizophrenia and across other psychiatric disorders gives weight to their involvement in regulating pathways important to the aetiology of neuropsychiatric disease.

The present study adds to growing research linking dysregulation of miRNAs to schizophrenia. It demonstrates several significant negative correlations between peripheral miRNA and mRNA expression profiles, suggesting miRNAs including miR-1271-5p, miR-221-5p, and let-7 family members are key molecules in regulating peripheral immune cell gene expression. This strengthens the importance of these particular miRNAs for the pathophysiology of neuropsychiatric disorders like schizophrenia, and suggests they may represent useful targets for future treatments. The present data also demonstrates a large difference in the peripheral mRNAs differentially expressed between males and females, highlighting the importance of accounting for sex in analyses of such complex disorders.

Declarations of interest

None.

Human ethics and consent

Participants in the Australian Schizophrenia Research Bank (ASRB) were recruited through a national media campaign and consented to data and sample collection genomic analyses as outlined in PMID:21034186. The use of these data was approved by the University of Newcastle Human Ethics Research Committee.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.02.023.

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