Schizophrenia is a severe mental illness affecting approximately 1% of the population worldwide. Previous studies have inferred a strong genetic component in schizophrenia. However, the genetic variants involved in the susceptibility to schizophrenia remain unclear.

Aims
To detect potential gene pathways and networks associated with schizophrenia, and to explore the relationship between common and rare variants in these pathways and abnormal white matter integrity in schizophrenia.

Method
The analysis included 100 first-episode treatment-naïve patients with schizophrenia and 140 healthy controls. A network-based analysis was carried out on the data collected from the Psychiatric Genomics Consortium Phase I (PGC-I). Based on our genome-wide association study and whole-exome sequencing data-sets, we performed a gene-set analysis to detect associations between the combining effects of common and rare genetic variants and abnormal white matter integrity in schizophrenia.

Results
Patients had significantly reduced functional anisotropy in the left and right anterior cingulate cortex, left and right precuneus and extra-nuclear (τ = 4.61–5.10, P_{FDR} < 0.01), compared with controls. Generated from co-expression network analysis of the PGC-1 summary statistics of schizophrenia, a subnetwork of 207 genes associated with schizophrenia was identified (P < 0.01), and 176 genes were co-expressed in four gene modules. Functional enrichment analysis for genes in each module revealed that the yellow module was enriched with highly co-expressed, innate immune response genes. Furthermore, rare variants of enriched genes in the yellow module were associated with reduced functional anisotropy in the left anterior cingulate cortex (P = 0.006; P_{FDR} = 0.024) in patients only.

Conclusions
The pathogenesis of schizophrenia may be substantially influenced by genes involved in the immune system, via both pathway and network.

Declaration of interests
None.

Keywords
Schizophrenia; GWAS; network analysis; immune system; imaging genetics.

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The neuroimaging of schizophrenia
The inconsistent replications among many GWAS of schizophrenia so far might be because of the limited statistical power arising from the small sample size and the clinical heterogeneity among recruited patients with schizophrenia. One feasible strategy to overcome these barriers is to use endophenotypes or intermediate phenotypes in molecular genetic studies. Endophenotypes are considered to be more proximal to the biological aetiology of a disorder, and might provide an alternative strategy in the identification of the pathogenesis of schizophrenia. For instance, previous magnetic resonance imaging (MRI) studies reported significant abnormalities in the white matter integrity of patients with schizophrenia when compared with healthy controls, and this finding has been supported by a meta-analysis study. Furthermore, fractional anisotropy, a measure reflecting the fibre bundle connectivity of white matter, has been found to be significantly low in patients with schizophrenia compared with healthy controls, especially in brain regions such as the genu of the corpus callosum, posterior cingulum fibres, superior and inferior fronto-occipital fasciculus and the posterior corona radiata. In fact, some other studies have indicated that fractional anisotropy is a highly heritable trait and could be used as a potential quantitative endophenotype in understanding the aetiology of schizophrenia.

In the present study, instead of targeting the individual gene, we hypothesised that the abnormal endophenotypes of schizophrenia might be mainly associated with the dysfunctional gene pathways
or networks, the identification of which could provide pivotal information on the biological mechanism for schizophrenia.

Building on our hypothesis, we aim to (a) examine fibre bundle connectivity of white matter indexed by fractional anisotropy values in schizophrenia and (b) explore the association between rare variants in gene pathways or networks and abnormal white matter integrity in schizophrenia.

In the current study, we first performed voxel-wise comparisons in a sample set of first-episode and treatment-naive patients with schizophrenia and matched controls by diffusion tensor imaging (DTI), and identified the brain regions with abnormal fractional anisotropy values. Each differential fractional anisotropy value and a composite index of overall fractional anisotropy value computed by principal component analysis (PCA) were then used as one of endophenotypes in the subsequent genetic analysis. Furthermore, by leveraging the summary statistics generated from the GWAS of schizophrenia in PGC-1,4 we carried out a gene-wise subnetwork analysis to identify the pathways/networks associated with schizophrenia, followed by a weighted gene correlation network analysis, finding modules in 15 brain regions that are highly correlated with genes in the associated pathway/network. Finally, in each identified module, association analysis was carried out to identify the genes associated with the value of each differential and overall fractional anisotropy value in an attempt to explore the potential role of genetic variants in schizophrenia, specifically, its fibre bundle disconnectivity.

Method

Participants

A total of 240 participants comprising 100 first-episode, treatment-naive patients with schizophrenia and 140 healthy controls were recruited at the Mental Health Centre of the West China Hospital, Sichuan University, China. The Structured Clinical Interview for DSM-IV, Patient Version15 was used by a trained psychiatrist to interview and diagnose patients with schizophrenia. Patients initially diagnosed with the schizophreniform disorder were followed for at least 6 months to confirm the diagnosis of schizophrenia. All patients were evaluated with the Positive and Negative Syndrome Scale for their disease severity.17 Healthy controls were unable to be sequenced because of poor DNA quantity. A total of 234 out of 240 participants (97 patients and 137 controls) were sequenced with the TruSeqExome Enrichment Kit, optimised for Illumina HiSeq2000 sequencing. Three patients and three controls were unable to be sequenced because of poor DNA quantity.

Whole-exome sequencing and variants calling

A total of 234 out of 240 participants (97 patients and 137 controls) were sequenced with the TruSeqExome Enrichment Kit, optimised for Illumina HiSeq2000 sequencing. Three patients and three controls were unable to be sequenced because of poor DNA quantity.
Data preprocessing included (a) the Burrows–Wheeler Alignment tool\textsuperscript{28} to reference against the human genome (hg19); (b) Picard tools (http://picard.sourceforge.net/) to collect quality statistics and fix read group issues; (c) Samtools (http://samtools.sourceforge.net/) to filter out low-quality reads, and GATK\textsuperscript{22} for indel alignment, and SNP and indel calling. Quality control of all variants and samples is presented in the Supplementary Material.

### Identifying significant gene-wise subnetworks associated with schizophrenia

The summary statistics from PGC-1, generated from a GWAS of 9394 patients with schizophrenia and 12 462 controls,\textsuperscript{14} were downloaded (https://www.med.unc.edu/pgc) and used to identify significant gene-wise subnetworks associated with schizophrenia. A gene-based analysis of the summary statistics was conducted, using the VEGAS program with default parameters,\textsuperscript{23} the output of which was then integrated with a human interactome network,\textsuperscript{24} using protein interaction network-based pathway analysis\textsuperscript{25} to identify the enriched subnetworks. The statistical threshold was set at <0.1 (fewer than 10\% of random networks result in a significant subnetwork) after permutation testing (shuffling the data 1000 times).\textsuperscript{26,27}

### Constructing gene co-expression networks in brain regions

The gene co-expression networks in brain regions were constructed with the whole-genome transcriptomic data from BrainSpan (http://www.brainspan.org/); the data was collected by RNA-sequencing, and the gene expression was defined by a normalised reads per kilobase million value of 1 in at least one region at one time point for 80\% of the available samples. In the current study, the expression data of 15 brain regions, including 11 neocortical regions, the striatum, the hippocampus, the thalamus and the amygdaloid, were chosen to construct the gene co-expression networks, using the R package ‘WGCNA’.\textsuperscript{15} (Supplementary Table 1).

### Gene ontology enrichment analysis

WebGestalt\textsuperscript{26} was used to perform gene ontology enrichment analysis of genes in each module. A hypergeometric test implemented in WebGestalt computed the enrichment P-value, followed by a Benjamini–Hochberg correction for multiple testing. Enriched gene ontology terms are reported at a Benjamini–Hochberg-corrected P < 0.05.

### Cell-specific expression analysis

To detect the cell type overrepresented by the genes from each network and by taking advantage of an online tool (CSEA) for cell-specific expression analysis (http://genetics.wustl.edu/jdlab/csea-tool-2/),\textsuperscript{27} we conducted a cell-specific enrichment analysis for the genes in each network module. In brief, a large survey of central nervous system cell-specific microarray data was used to identify those genes that are significantly enriched in each population data and provide a simple perusable archive of plots of this measure across all cell types. The algorithms are available online (www.bactrap.org).\textsuperscript{27}

### Association analysis of fractional anisotropy and overall fractional anisotropy

We performed PCA with oblimin rotation for differential fractional anisotropy values, which showed a significant difference between patients with schizophrenia and controls, and the first component was extracted as overall fractional anisotropy. Taking into account the fact that these differential fractional anisotropy values are correlated with each other and with overall fractional anisotropy, the correlation analyses among the five original fractional anisotropy variables, and between the overall fractional anisotropy and each differential fractional anisotropy variable were conducted. The SNP-set (Sequence) Kernel Association Test (SKAT) program,\textsuperscript{28} which accounts for both linear and nonlinear interactions, was used to test the association of common and rare variants of genes in each module with the value of each differential fractional anisotropy and the overall fractional anisotropy, respectively, with age, gender, years of education and the top three principal components served as covariates. In an attempt to control the type I error in the identification of the rare variants in the genes constituting each module associated with each differential fractional anisotropy and the overall fractional anisotropy, a permutation test was used to obtain empirical P-values. In our analysis, the rare variants were defined as those with an MAF <1\% from the whole-exome sequencing data, and the common variants as those with an MAF ≥5\% from the GWAS data. Genetic loci (single nucleotide variations) mapped to genes were extracted based on whole-exome sequencing and GWAS data in each module. Phenotypes and genetic loci were then randomly shuffled, leaving the correlation of phenotype with genotype unchanged. This process was repeated 10 000 times and obtained 10 000 results (Zns).\textsuperscript{29} The empirical P-value for the association of genes in each module with the overall fractional anisotropy was evaluated by calculating the proportion of the Zns less than or equal to the overall observed Zns.

### Results

**Demographic data and clinical characteristics**

The demographic and clinical characteristics of all participants who completed the clinical assessments and the whole-brain diffusion-weighted images in the study are shown in Table 1. There appeared no significant differences in mean age ($t = -0.285$, $P = 0.776$), gender distribution ($\chi^2 = 0.011$, $P = 0.918$) and years of...
education ($t = -0.831, P = 0.407$) between patients with schizophrenia and healthy controls.

### Voxel-wise comparisons of fractional anisotropy

Fractional anisotropy was significantly reduced in the left and right anterior cingulate cortex, the left and right precuneus and the extra-nuclear in patients with schizophrenia compared with controls (false discovery rate-corrected $P < 0.01$, cluster size $\geq 100$; Supplementary Figure 2, Table 2). The overall fractional anisotropy was obtained by extracting the first component of the PCA from the fractional anisotropy values of the above five regions. There was a significant correlation among the five original fractional anisotropy variables (Supplementary Table 2), and between the overall fractional anisotropy and all of the five original fractional anisotropy variables (Supplementary Table 3).

### Gene-wise subnetworks and co-expressed gene modules

A subnetwork including 207 genes was identified by the network-based analysis of the summary statistics from PGC-1 (Supplementary Figure 3 and Table 4). The co-expression network analysis found that 176 out of the 207 genes passed the quality control and were highly co-expressed in four gene modules (named yellow, blue, brown and turquoise; Fig. 1, Supplementary Table 5). Supplementary Table 6 displays the gene ontology enrichment of genes in each module. The yellow module contained co-expressed innate immune response genes (10 out of 24, 41.6%), major histocompatibility complex (MHC) class I receptor activity genes (4 out of 24, 16.6%) and MHC class I protein complex genes (5 out of 24, 20.8%). It is noteworthy that the average expression of genes in the yellow module is increased mainly during the early prenatal development (post-conception weeks 13–37; Supplementary Figure 4). The blue and brown modules predominantly contain genes functioning in histone methyltransferase activity and histone-lysine $\text{N}^\text{methyl}$-transferase activity. The turquoise module contains genes related to phosphotransferase activity, alcohol group as acceptor and DNA binding. In addition, genes in the yellow module indicated a significantly increased cell type-specific enrichment in astrocytes ($P = 0.002$, $P_{\text{adjusted}} = 0.04$).

### Gene-set association test in each module

The cumulative evidence of rare or common variants for all genes in each module was evaluated in patients with schizophrenia and controls. Using the whole-exome sequencing data, we found that the combining effect rare variants of genes in the yellow module were significantly associated with schizophrenia ($P = 0.002$, $P_{\text{adjusted}} = 0.008$). Furthermore, we found that the same combining effect in the yellow module was also associated with reduced fractional anisotropy in the left anterior cingulate cortex ($P = 0.006$, $P_{\text{adjusted}} = 0.024$) and with reduced overall fractional anisotropy ($P = 0.041$, uncorrected) only in patients with schizophrenia, not in the controls (Table 3). However, no significant association between common variants of genes in each module and the overall fractional anisotropy was detected.

| Table 2 | Fractional anisotropy differences between patients with schizophrenia and controls |
|---|---|---|---|---|
| Voxel | $P$-value (FDR corrected) | $T$ | Peak (MNI: X Y Z) |
| Precuneus (right) | 263 | 0.001 | 5.10 | 12 – 30 32 |
| Precuneus (left) | 292 | 0.001 | 5.03 | -16 – 52 30 |
| Extra-nuclear (right) | 158 | 0.001 | 4.88 | 34 – 56 28 |
| Anterior cingulate cortex (right) | 104 | 0.002 | 4.66 | 30 22 26 |
| Anterior cingulate cortex (left) | 108 | 0.002 | 4.61 | -26 30 8 |

Threshold at $P < 0.01$ (FDR corrected) and cluster size $\geq 100$. FDR, false discovery rate; MNI, Montreal Neurological Institute.

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**Fig. 1** Co-expression module containing genes in protein–protein interaction.
In the present study, we found that the first-episode, treatment-naive patients with schizophrenia had significantly reduced fractional anisotropy in the left and right anterior cingulate cortex, the left and right precuneus and the extra-nuclear compared with healthy controls. By integrating the results of the PGC-1 GWAS for schizophrenia, interactome data and brain co-expression networks, a subnetwork with 207 genes were found to be associated with schizophrenia; of these, 176 genes were highly co-expressed in four gene modules. Furthermore, we found that the rare variants with schizophrenia; of these, 176 genes were highly co-expressed in four gene modules. Furthermore, we found that the rare variants in the genes constituting the yellow module, mainly the genes related to the immune pathways, were associated with reduced fractional anisotropy in the anterior cingulate cortex only in patients with schizophrenia. Our findings thus further support the hypothesis that gene pathways or networks might substantially contribute with schizophrenia. Our findings thus further support the hypotheses that gene pathways or networks might substantially contribute to the abnormal endophenotypes in schizophrenia.

The abnormal fractional anisotropy of white matter in patients with schizophrenia identified in this study reflects a disruption of neural circuitry. Our findings are consistent with previous studies, including our own, suggesting that the impaired fibre bundle connectivity between the brain regions plays a critical role in the neuropathology of schizophrenia. Further identification of genes or pathways involved in neural connectivity circuits could deepen our understanding of the complex causes of schizophrenia.

The same approach has been taken previously to identify the genetic subnetworks associated with other complex diseases; for example, Han et al integrated the GWAS and human protein interaction networks to identify a subnetwork of 39 genes that was not only enriched for genes associated with alcohol dependence, but also collectively associated with alcohol dependence in three independent samples. In the current study, following the identification of a subnetwork with 207 genes associated with schizophrenia, we found that 176 of those genes were highly co-expressed in four gene modules. Each module represents particular gene ontology biological processes, molecular functions and cellular components. Of note, the yellow module was found in our data to be associated with reduced fractional anisotropy in the left anterior cingulate cortex, and it is also enriched by genes related to MHC class I (HLA-A, HLA-B and HLA-C) and genes (DGK1, IRF3, MICA, Pias2 and TAP1) previously reported to be associated with schizophrenia.

It is worth highlighting here that innate immune response genes, such as MHC class I receptor activity genes and MHC class I protein complex genes, were significantly enriched in the yellow module. MHC class I belongs to the MHC gene family, which is involved in the immune system through the response to viral infection. Melnik et al reported that IRF3 might be critical in regulating the development of neuronal progenitor cells. The analysis of protein–protein interaction networks showed that IRF3 interacts with other schizophrenia susceptibility genes, such as CREB1, AKT1 and ESR1. Furthermore, several studies identified that AKT1 and CREB1 were associated with synaptic plasticity in the hippocampus. Liu et al identified that Pias1 has an important role in neuronal plasticity, learning and memory function. Also, CDK2AP1 in the yellow module was recently identified as a susceptibility gene for intellectual disability. Alsayegh et al have also shown that CDK2AP1 gene was associated with the differentiation of human embryonic stem cells and the percentage of cells in the S phase. On the other hand, Manolio et al reported that the cumulative effect of rare variants, especially genes regulating immune activation, may influence brain structure and neurodevelopment deficit in patients with schizophrenia as well as account for the part of ‘missing heritability’ in schizophrenia. A recent next-generation sequencing study, including 4877 patients with schizophrenia and 45 376 controls from Sweden, confirmed the effects of rare variant burden in patients and contributed to the understanding of the genetic architecture and biological patterns of schizophrenia.

As shown in this study, the expression of genes in the yellow module increased during early prenatal development, with a significant cell type-specific enrichment in astrocytes. The present study, in line with previous studies, provides another piece of evidence that brain development and immune dysregulation in the prenatal environment affect the pathology of schizophrenia in a temporal- and cell-specific interaction. Further, animal studies also provide supportive evidence. For example, the previous study by Bauman et al reported that prenatal exposure to maternal immune activation might alter brain structure and behaviour related to schizophrenia in offspring in rhesus monkeys. Another study showed that prenatal exposure to maternal inflammation influences the levels of N-acetylaspartate/creatine and myo-inositol/creatine in the brain.
cingulate cortex of mice. In fact, abnormal white matter microstructure in the left anterior cingulate cortex has been found in mice exposed to an immune challenge in early or late prenatal development, which is consistent with our findings in the current study.

Using data from the PGC-I, we found that the blue and brown modules harboured the genes predominantly related to the histone methyltransferase activity and the histone-lysine H3K4 methyltransferase activity. It has previously been reported that multiple immune system-related genes and genes related to histone H3K4 methylation are associated with schizophrenia and bipolar disorder. In agreement with previous studies, the current findings provide further evidence for the role of histone methylation in schizophrenia. Furthermore, previous studies have shown that the histone methylation pathway may coordinate complex cognitive processes involved in long-term memory in schizophrenia, depression, autism and neurodegenerative disease, and could play an important role in the interaction between environmental factors and genetics on susceptibility to psychiatric manifestations throughout life.

By studying first-episode and treatment-naive patients with schizophrenia, we minimised the effects of confounding factors such as chronicity of the illness and antipsychotic treatment. The main limitation of this study is the moderate sample size, which limited statistical power, especially for common variants with small or medium effects. Further replication in a larger and independent sample is required.

In summary, through an integrated analysis of genetic data from the PGC-I for schizophrenia, interactome data and brain co-expression network, the current results indicate that dysregulation of genes involved in the immune system play an important role in the pathogenesis of schizophrenia. The combining effect of the rare variants in the genes making up the yellow module, especially genes related to the immune system, was associated with abnormal fractional anisotropy of white matter in patients with schizophrenia. These findings provide some important clues as to the aetiology of schizophrenia. Moreover, the present study also highlights the feasibility of using endophenotypes in exploring the pathogenesis of neuropsychiatric diseases such as schizophrenia.

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