Gene-expression analysis of clozapine treatment in whole blood of patients with psychosis

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Objectives **Clozapine is an atypical antipsychotic primarily prescribed for treatment-resistant schizophrenia. We tested the specific effect of clozapine versus other drug treatments on whole-blood gene expression in a sample of patients with psychosis from the UK.**

Methods **A total of 186 baseline whole-blood samples from individuals receiving treatment for established psychosis were analysed for gene expression on Illumina HumanHT-12.v4 BeadChips. After standard quality-control procedures, 152 samples remained, including 55 from individuals receiving clozapine. In a within-case study design, weighted gene correlation network analysis was used to identify modules of coexpressed genes. The influence of mood stabilizers, lithium carbonate/lithium citrate and sodium valproate was studied to identify their possible roles as confounders.**

Results **Individuals receiving clozapine as their only antipsychotic (clozapine monotherapy) had a nominal association with one gene-expression module, whereas no significant change in gene expression was found for other drugs.**

Conclusion **Overall, this study does not provide evidence that clozapine treatment induces medium to large different gene-expression patterns in human whole blood versus other antipsychotic treatments. This does not rule out the possibility of smaller effects as observed for other common antipsychotic treatments.** ***Psychiatr Genet*** **26:211–217**

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Introduction

Psychosis is a common symptom of several psychiatric disorders, including bipolar disorder and schizophrenia. Schizophrenia has a lifetime prevalence of 1%. Up to 30% of schizophrenic patients develop treatment-resistant schizophrenia that is diagnosed after unsuccessful treatment with two or more typical antipsychotics (Meltzer, 1997). Clozapine is considered a 'drug of last resort' in these patients as, despite its well-documented side effects, clozapine can lead to significant clinical improvements (Kane, 1992; Agid *et al.*, 2007; Cohen *et al.*, 2012). However, around 50% of treatment-resistant patients respond poorly to clozapine (Lieberman *et al.*, 1994).

Early identification of patients who may benefit from clozapine treatment is not currently possible. On average, there is a 4-year delay in starting clozapine because of prescribing guidelines, side effects, uncertainty of response and the need for regular blood monitoring during treatment (Howes *et al.*, 2012). Clozapine response can take up to a year to stabilize, meaning that long-term clozapine treatment is required before schizophrenia is defined as clozapine nonresponsive (Meltzer, 1989). Clozapine response biomarkers could help identify suitable candidates for clozapine treatment or be useful for early termination of unsuccessful trials. This would minimize the detrimental effects associated with persistent psychotic symptoms.

Previous evidence for clozapine-induced changes in gene expression mainly derives from animal studies. In mouse brain, Duncan *et al.* (2008) found that haloperidol, clozapine and olanzapine generally decrease gene expression, including for potassium channel subunits. In mice, clozapine has been reported to alter the expression of glutamate receptor g2 subunits and ubiquitin-conjugating

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enzyme E2R, whereas both clozapine and haloperidol modify the expression of genes associated with apoptosis, proteolysis and lipid metabolism (Thomas *et al.*, 2003). In rats, long-term clozapine exposure identified 278 downregulated genes and 73 upregulated genes in the frontal cortex relative to controls (Fatemi *et al.*, 2012). The genes identified were involved in pathways such as protein metabolism, nucleotide metabolism and signal transduction (Fatemi *et al.*, 2012). Clozapine and other atypical antipsychotics have also been shown to alter the metabolism of cholesterol and fatty acids *in vivo*. Cells treated with clozapine have significantly higher levels of SREBP (sterol-regulatory element-binding protein), HMGR (HMG-CoA reductase) and LDLR (LDL receptor) mRNA than control cells (Canfrán-Duque *et al.*, 2013). Studies have also shown that other drugs such as lithium and valproate may also influence gene expression (Phiel *et al.*, 2001; Brandish *et al.*, 2005; Sharp *et al.*, 2013). As clozapine-treated patients may receive other non-antipsychotic medications, it is important to avoid the confounding effects induced by other medications.

Global expression changes in blood have been observed to correlate with those in the brain. The correlation between transcripts present in both central nervous system and whole blood was ~0.5 (Sullivan *et al.*, 2006). Of the candidate schizophrenia genes investigated, half were expressed in whole blood and the prefrontal cortex (Sullivan *et al.*, 2006). Using a methodology similar to that of the current study, De Jong *et al.* (2012) found two gene coexpression modules enriched for brain-expressed genes in whole blood in schizophrenic patients. A recent study examining DNA methylation in response to antipsychotics and mood stabilizers (including lithium and valproic acid) found that these medications influence cell-type composition and that psychotropic medications investigated influenced DNA methylation at both the gene and the network level (Houtepen *et al.*, 2016).

Given the previous evidence that clozapine may influence gene expression, we hypothesize that antipsychotic medications could have a detectable effect on blood expression in whole blood. In this study, we aimed to clarify the specific effects of clozapine treatment on gene expression in whole blood of psychosis patients using both a single gene and a network-driven analysis approach (Zhang and Horvath, 2005; Langfelder and Horvath, 2008).

Methods

The data presented here were collected previously as part of the IMPACT randomized controlled trial. This aimed to improve physical health by addressing issues such as substance use, poor diet and lack of exercise through cognitive behaviour therapy (Gaughran *et al.*, 2013).

Ethical approval

Ethical approval was obtained from The Joint South London and Maudsley and The Institute of Psychiatry NHS Research Ethics Committee (REC ref no. 09/H080/41).

Cohort description

The patients recruited to IMPACT were between 18 and 65 years of age, with the following ICD 10 psychiatric diagnoses: F20–F29 (schizophrenia, psychotic disorders and schizoaffective disorder), ICD 10 F31.2 (bipolar) and ICD 10 F32.3, F33.3 (depressive episode with psychotic symptoms). Exclusion criteria were as follows: learning disability, a physical health problem that would influence metabolic measures or substance use habit, pregnancy or less than 6 months postpartum or under intensive care [for further details, see Gaughran *et al.* (2013)]. A total of 186 patients provided consent for prerandomisation bloods for gene expression. In all, 152 remained following quality control and removal of outliers. Outliers were defined as those with insufficient clinical data, inadequate quality information or technical outliers. The main attributes of this sample are shown in Table 1. The clinical diagnoses, stratified by clozapine medication status, are shown in Table 2. Out of 152 individuals, 104 had a diagnosis of schizophrenia, 19 had a diagnosis of bipolar disorder, 18 had a schizoaffective disorder and six had a depressive disorder with psychosis. The less common diagnoses included one with schizotypal disorder, one with delusional disorder and three with 'Other non-organic psychosis'. Only 148 individuals had scores on the positive and negative symptom score (Leucht *et al.*, 2005). The mean positive and negative symptom score was 50 ± 12. The distribution of drugs shown in Table 3 is not mutually exclusive as some individuals were receiving several antipsychotics or other medications.

Gene-expression data preprocessing

RNA samples were extracted from postfasting samples using Tempus Blood RNA tubes according to the

Table 1 Attributes of the 152 individuals drawn from the IMPACT sample divided into nonclozapine ($n = 97$) and clozapine treatment groups ($n = 55$)

Attribute	Total sample	Nonclozapine group	Clozapine group
Sample size	152	97	55
Age (mean ± SD) (years)	45 ± 9.34	45 ± 9.55	43 ± 8.95
Male	90	50	40
Female	62	47	15
Caucasian	77	45	32
Black	57	39	18
Asian	7	5	2
Mixed	7	4	3
Other	4	4	0
RIN (mean ± SD)	8.64 ± 0.77	8.56 ± 0.85	8.72 ± 0.79
RNA concentration (mean ± SD)	83.52 ± 36.21	83.29 ± 35.85	83.94 ± 37.08

RIN, RNA integrity number.

Table 2 Clinical diagnoses of 152 individuals stratified by clozapine medication status

Diagnosis	Total sample	Nonclozapine group	Clozapine group
Schizophrenia	59	35	24
Paranoid schizophrenia	43	21	22
Residual schizophrenia	1	0	1
Simple schizophrenia	1	1	0
Schizoaffective disorders	9	7	2
Schizoaffective disorder, manic type	4	2	2
Schizoaffective disorder, depressive type	2	1	1
Schizoaffective disorder, mixed type	2	2	0
Schizoaffective disorder, unspecified	1	0	1
Schizotypal disorder	1	1	0
Delusional disorder	1	1	0
Other nonorganic psychotic disorders	1	1	0
Unspecified nonorganic psychosis	2	2	0
Severe depressive episode with psychotic symptoms	4	3	1
Recurrent depressive disorder, current episode severe with psychotic symptoms	2	2	0
Bipolar affective disorder, current episode manic with psychotic symptoms	19	18	1

Table 3 Distribution of antipsychotic treatments and other medications among the clozapine ($n = 55$) and nonclozapine-treated cohort ($n = 97$)

Medication	Total sample	Nonclozapine cohort	Clozapine cohort
Quietapine	10	9	1
Depixol	12	12	0
Amisulpiride sulpiride	18	8	10
Aripiprazole	20	12	8
Risperidone	16	16	0
Olanzapine	14	14	0
Clozapine	55	0	55
Clozapine monotherapy	39		39
Clozapine polytherapy	16		16
Other antipsychotics	17	11	6
Antidepressants	82	60	22
Benzodiazepines	19	5	14
Valproate	17	8	9
Lithium	18	9	9

These numbers are not mutually exclusive as many individuals received several antipsychotics or other medications.

manufacturer's protocol (Applied Biosystems, Foster City, California, USA). Whole-genome gene-expression data were generated using Illumina HumanHT-12.v4 BeadChips according to the manufacturer's protocol at the in-house BRC BioResource Illumina Core laboratory. Quality control and preprocessing used a standard pipeline (BRC Bioinformatics pipeline, URL 1: *https://github.com/snewhouse/BRC_MH_Bioinformatics*) that excluded sample and probe outliers and applied robust spline normalization and log2 transformation (Du *et al.*, 2008). In all, 154 individuals passed quality control and after removing additional sample outliers using the weighted gene coexpression network analysis (WGCNA) package (Zhang and Horvath, 2005; Langfelder and Horvath, 2008), 152 patients and 6357 probes remained for subsequent analysis. We applied principal component analysis (Stacklies *et al.*, 2007) to identify the correlation with possible covariates (see Supplementary digital content, Table 1S, Supplemental digital content 1, *http://links.lww.com/PG/A158*). The raw expression data were corrected using age, sex, RNA integrity number, RNA concentration and ethnicity as covariates. This was done using the lmFit function of the Limma *R* package in a linear model (Smyth, 2004, 2005). This generated residuals, which were used in subsequent modelling of the association of drug treatment with gene-expression data. In total, six models were tested. These included a test for the association of clozapine, lithium, valproate, other antipsychotics, clozapine monotherapy and clozapine polytherapy with changes in gene expression.

Identification of significant genes

Individual gene-level analysis tested for an association of drug treatment (clozapine, valproate, lithium and other antipsychotics) with expression changes in individual genes. The association was tested by a linear model in *R*, with the untested drug treatments as covariates. The significance threshold for individual probes was below a Holm–Sidak-corrected *P*-value of 0.05. The Holm–Sidak method is a more conservative measure of multiple testing easily applied to module eigengene analyses.

Power calculation

Given a sample size of 55 for the clozapine-treated group and 6357 probes, we had 80% power to detect 1.3-fold expression changes and 99% power to detect 1.5-fold changes (false discovery rate $= 0.05$, SD $= 0.7$) (Bioinformatics M.D. Anderson microarray power calculator, URL 2: *http://bioinformatics.mdanderson.org/MicroarraySampleSize/*).

Weighted gene coexpression network analysis

WGCNA (Zhang and Horvath, 2005; Langfelder and Horvath, 2008) is a systems biology method used to analyse microarray expression data in a network context. First, the pairwise Pearson correlations calculated between all genes produce a correlation matrix. When raised to a power, this correlation matrix yields an adjacency matrix of the pairwise connection of each gene. As the power increases, the fit of this network to the scale-free topology model is improved (Supplementary digital content, Figure 1S, Supplemental digital content 2,

http://links.lww.com/PG/A159). Here, we chose a power of 11 as this exceeded the 0.9 R^2 value, thus ensuring that highly connected genes were given priority. Our unsigned network accounted for the absolute correlations of genes in either direction.

Genes are subsequently clustered using the topological overlap matrix (TOM) in a gene dissimilarity measure (1 − TOM). This considers each gene pair relative to all other genes taking into account 'shared neighbours'. Branches of the dendrogram are cut using the DynamicTreeCut algorithm (D'haeseleer, 2005), assigning each gene to a module represented by a colour. A module eigengene for each module is defined by taking the first principal component of the expression values per module. Therefore, the module eigengene represents a summary of the expression profile of all genes in a module for each sample. The module eigengenes are tested for association with drug treatment using a linear model in *R* (Smyth, 2005). We tested the association of each module with clozapine, valproate, lithium and 'other antipsychotic' treatment. As for the individual gene-level analysis, the covariates used were the drug treatments that were not being tested. Clozapine monotherapy and polytherapy was also tested in this way. We define clozapine monotherapy as individuals receiving clozapine as their only antipsychotic ($n = 39$). However, all individuals on clozapine were receiving additional medications, including antidepressants, benzodiazepines or mood stabilizers. Clozapine polytherapy was defined as being on clozapine and other antipsychotics ($n = 16$).

Significance thresholds

WGCNA alleviates the multiple-testing problem by relating relatively few modules to traits rather than thousands of probes. To determine significance thresholds, the matSPD spectral decomposition approach by Dale Nyholt was used (URL 3: *http://neurogenetics.qimrberghofer.edu.au/matSpD/*). The *P*-values generated from the association of lithium, valproate, clozapine and other antipsychotics were used to create a correlation matrix, from which the number of independent variables was measured (Nyholt, 2004; Li and Ji, 2005) This yielded a total of seven independent tests from 11 correlated tests (the total number of modules). For the full cohort of 152 individuals, four further tests were carried out. Therefore, the independence threshold was defined as $0.05/(7 \times 4) = 0.0018$.

Significant genes and pathways were characterized using Entrez IDs in WebGestalt (Zhang *et al.*, 2005; Wang *et al.*, 2013). Pathway analysis utilized Gene Ontology (version 1.2) and KEGG pathways, referenced against all probes passing quality control, a hypergeometric statistical model and Holm–Sidak correction for multiple testing ($P < 0.05$). Each gene category was required to contain at least two genes.

Results

Single-gene analysis

After correcting for age, sex, ethnicity, RNA concentration and RNA integrity number, the residuals of each gene were tested for association with clozapine, valproate, lithium and other antipsychotic treatment. The covariates were the above drug treatments, excluding the drug that was being tested. At the single gene level, no individual gene reached significance for association with clozapine, lithium, valproate or other antipsychotics according to the Holm–Sidak threshold of 8.068×10^{-6}.

Network construction

Figure 1 shows the dendrogram representing the unsigned network from 152 individuals and 6357 probes. The network contains 11 modules, with sizes ranging from turquoise (1118 probes) to purple (39 probes). The grey module (2781 probes) represents genes not belonging to any other module (background noise).

No association of module eigengenes with clozapine treatment

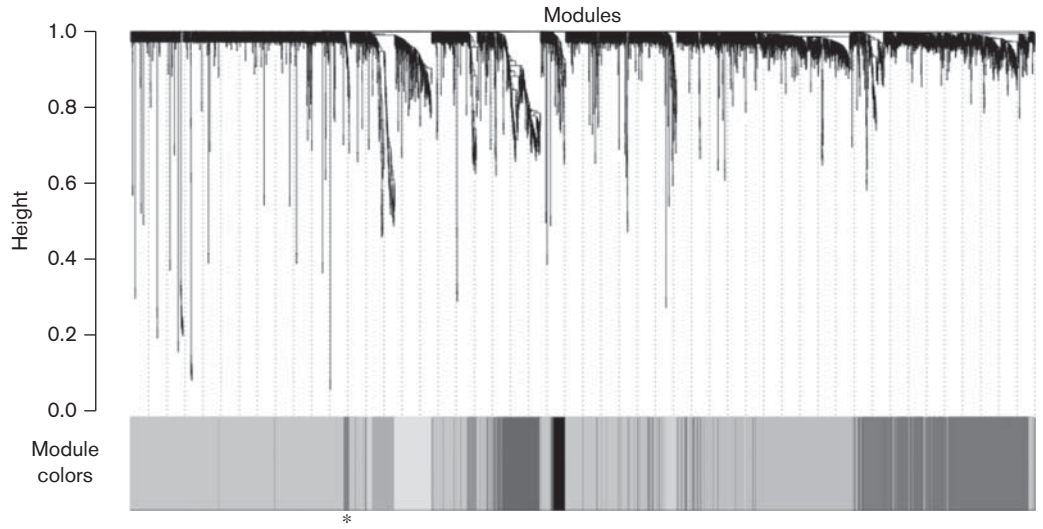
A linear model was used to test associations of module eigengenes with clozapine, valproate, lithium and other antipsychotics (all antipsychotics except clozapine) treatment with covariates as described in the Methods section, excluding the tested treatment. No modules were significantly associated with clozapine treatment, lithium, valproate or other antipsychotics (Supplementary digital content Tables 2S–5S, Supplemental digital content 3, *http://links.lww.com/PG/A160*; Supplemental digital content 4, *http://links.lww.com/PG/A161*; Supplemental digital content 5, *http://links.lww.com/PG/A162*; Supplemental digital content 6, *http://links.lww.com/PG/A163*, which present the statistics of each module association with drug treatment).

Clozapine antipsychotic monotherapy

A possible confound was polypharmacy within the clozapine group. We defined a clozapine monotherapy group of individuals receiving clozapine as their only antipsychotic. All individuals on clozapine were receiving additional medications, including antidepressants, benzodiazepines or mood stabilizers.

We tested for association between module eigengenes and clozapine antipsychotic monotherapy ($n = 39$) versus polytherapy ($n = 16$) with lithium and valproate as covariates (Table 4). The strongest association was between clozapine monotherapy and the purple module ($P = 0.002$), just above our significance threshold ($P < 0.0018$). This was a downregulation of expression. This purple module contained 36 genes (represented by 39 probes), the functions of which included cell junctions and adhesion, platelet degranulation ($P = 0.0297$), blood

Fig. 1



The network was constructed using gene-expression data of 6357 probes of 152 individuals with psychosis, of whom 55 were medicated with clozapine. The dendrogram was produced using hierarchical clustering and modules were grouped and assigned colours by cutting the tree using the DynamicTreeCut procedure. The asterisk indicates the purple module, associated with clozapine monotherapy treatment.

Table 4 ***P*-value association of each module with clozapine monotherapy ($n = 39$) and polytherapy ($n = 16$) in 152 individuals**

Module	*P*-value for association with clozapine polytherapy	*P*-value for association with clozapine monotherapy
Pink	0.317 (−0.999)	0.204 (−1.270)
Blue	0.005 (−2.832)	0.236 (1.187)
Red	0.222 (−1.221)	0.990 (0.013)
Brown	0.185 (1.328)	0.374 (−0.889)
Purple	0.110 (1.597)	0.002* (−3.065)
Green	0.0293 (2.181)	0.547 (−0.603)
Yellow	0.004 (2.914)	0.530 (0.628)
Magenta	0.660 (0.440)	0.122 (1.548)
Black	0.164 (1.391)	0.786 (0.272)
Turquoise	0.212 (1.249)	0.906 (−0.118)
Grey	0.0288 (−2.189)	0.365 (0.906)

t-values are shown in brackets. Each module was generated from corrected residuals. Module eigengenes summarizing the modules were tested for association with clozapine therapy, corrected for lithium and valproate in a linear model. **P*-values near the significance threshold ($P = 0.0018$).

coagulation ($P = 0.0009$), wound healing ($P = 0.0033$) and muscle contraction ($P = 0.0093$).

Enrichment analyses using WGCNA gene lists (UserListEnrichment) also indicated that the purple module was significantly enriched for platelet-expressed genes. The purple module was enriched for the top 50 marker genes for platelets ($P = 0.86 \times 10^{-6}$) and for platelet-specific genes from a custom microarray ($P = 4.85 \times 10^{-16}$) according to Gnatenko *et al.* (2009, 2010). Using KEGG (URL 4: *http://www.genome.jp/kegg/pathway.html*) pathway terms, this module is enriched for ECM receptor interaction ($P = 0.0143$). To our knowledge, none of these genes have been implicated previously in clozapine response. Of the genes implicated in cell junctions, one of potential interest is YWHAH (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide). This is an adapter protein of the 14-3-3 family, which has been implicated previously in schizophrenia (Toyooka *et al.*, 1999). None of the other genes within this module appear to have been reported in schizophrenia studies.

Discussion

This study used a naturalistic, cross-sectional, within-case design with the aim of identifying specific effects of clozapine on gene expression relative to other antipsychotics. We applied WCGNA to whole-blood gene-expression data of 152 individuals with psychosis to examine gene–trait associations at a network level. None of the 11 gene coexpression modules showed a significant association with clozapine treatment. Clozapine monotherapy (clozapine as the only antipsychotic) may induce small differences in gene expression.

The distribution of the diagnoses between the clozapine and the nonclozapine groups was similar, with the exception of bipolar disorder. We investigated the effect of bipolar diagnosis on the association of the module eigengenes to clozapine monotherapy treatment in a linear model (Supplementary digital content, Table 6S, Supplemental digital content 7, *http://links.lww.com/PG/A164*). This did not have a major effect on the final associations, and was therefore not included as a covariate in primary analyses, given that this was a study on the effects of clozapine medication and the distribution of mood stabilizers was equal between the two groups.

The ability to find clozapine-specific effects may be confounded by other medications causing global effects in gene expression (De Jong *et al.*, 2012), although this was not evident here. No individual antipsychotic had significant effects on gene expression (data not shown) and other antipsychotics were corrected for as a single group. Correction for mood stabilizers was justified to avoid lithium or valproate treatment confounding gene expression in clozapine-treated individuals. It was also not possible in our study to distinguish between expression changes because of refractory schizophrenia and those because of the effects of medication. Given that clozapine is known to induce agranulocytosis in 0.8% of patients on clozapine, we considered whether this could be an explanation for the enrichment of blood cell-type markers here (Alvir *et al.*, 1993). However, in this sample, the likely prevalence would be less than one individual, which is not likely to have a significant effect on the results shown here.

Our study had some limitations because of the heterogeneity of the cohort used (Table 1). We corrected for this using age, sex and principal components, and are confident that this has controlled for any major influences on gene expression. Another potential limitation was the heterogeneous medication received by individuals as only 39 received antipsychotic monotherapy of clozapine. However, this sample represents the reality of a sample derived from a retrospective clinical trial. This means that any findings are more likely to be applicable to clinical practice but, equally, our study has more potential confounding factors.

Searching for expression changes directly related to pathology in whole blood is more difficult than in brain tissue, although it has been shown that whole-blood expression profiles correlate with brain expression abnormalities in schizophrenic patients (Davies *et al.*, 2012; Liu *et al.*, 2014). Also, given that clozapine exerts effects on blood cells, it is reasonable to expect these changes to be reflected in a gene-expression profile. Finally, a blood biomarker may be more useful in predicting treatment response than a brain biomarker because of ease of access to tissue, and could improve diagnostics and treatment of patients.

Concluding remarks and further work

The current study found no gene-expression changes related to clozapine, lithium or valproate use on a gene-by-gene or network level. However, a potentially interesting nominal association was found for clozapine monotherapy. The current study is limited by the relatively small sample size used and our within-case design, which resulted in insufficient power to detect very small changes in gene expression. Further work could use a larger sample size and take into account the duration of clozapine treatment and other factors, such as the likely epigenetic effects of longer term clozapine treatment.

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Conflicts of interest

G.B. has acted as a consultant in preclinical genomics and has received grants from Eli Lilly. R.M.M. has received honoraria for lectures from Janssen, Lundbeck and Otsuka. FG has received honoraria for advisory work and lectures from Roche, BMS, Lundbeck and Sunovion and has a family member with professional links to Lilly and GSK. For the remaining authors there are no conflicts of interest.

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