Whole-blood expression of inflammasome- and glucocorticoid-related mRNAs correctly separates treatment-resistant depressed patients from drug-free and responsive patients in the BIODEP study

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Abstract

The mRNA expression signatures associated with the ‘pro-inflammatory’ phenotype of depression, and the differential signatures associated with depression subtypes and the effects of antidepressants, are still unknown. We examined 130 depressed patients (58 treatment-resistant, 36 antidepressant-responsive and 36 currently untreated) and 40 healthy controls from the BIODEP study, and used whole-blood mRNA qPCR to measure the expression of 16 candidate mRNAs, some never measured before: interleukin (IL)-1-beta, IL-6, TNF-alpha, macrophage inhibiting factor (MIF), glucocorticoid receptor (GR), SGK1, FKBP5, the purinergic receptor P2RX7, CCL2, CXCL12, c-reactive protein (CRP), alpha-2-macroglobulin (A2M), aquaporin-4 (AQP4), ISG15, STAT1 and USP-18. All genes but AQP4, ISG15 and USP-18 were differentially regulated. Treatment-resistant and drug-free depressed patients had both increased inflammasome activation (higher P2RX7 and proinflammatory cytokines/chemokines mRNAs expression) and glucocorticoid resistance (lower GR and higher FKBP5 mRNAs expression), while responsive patients had an intermediate phenotype with, additionally, lower CXCL12. Most interestingly, using binomial logistics models we found that a signature of six mRNAs (P2RX7, IL-1-beta, IL-6, TNF-alpha, CXCL12 and GR) distinguished treatment-resistant from responsive patients, even after adjusting for other variables that were different between groups, such as a trait- and state-anxiety, history of childhood maltreatment and serum CRP. Future studies should replicate these findings in larger, longitudinal cohorts, and test whether this mRNA signature can identify patients that are more likely to respond to adjuvant strategies for treatment-resistant depression, including combinations with anti-inflammatory medications.

Introduction

While there is overwhelming evidence of increased inflammation in depression1–4, the molecular signature underpinning this ‘pro-inflammatory’ phenotype is still unknown. A multitude of studies and meta-analyses show that patients with major depressive disorder (MDD) have, on average, increased serum levels of pro-inflammatory cytokines, like interleukin 1 beta (IL-1-beta), IL-6 and...
tumour necrosis factor alpha (TNF-alpha), and of the acute phase protein, C-reactive protein (CRP)\textsuperscript{1,2,4,5}. Patients with ‘treatment resistant depression’ (TRD) are more likely to have increased inflammation\textsuperscript{6,7}, as do patients with cardiovascular disorders, obesity, anxiety, and a history of childhood maltreatment\textsuperscript{3,8–13}.

Whole blood mRNA expression analyses measure mRNAs coding for inflammatory genes and for genes operating upstream and downstream of these immune mechanisms, such as the glucocorticoid receptor (GR)\textsuperscript{14}. We have been the first to demonstrate that drug-free depressed patients have increased mRNA expression of IL-1-beta, IL-6 and TNF-alpha, together with reduced expression of the GR and increased expression of the FK506 binding protein 5 (FKBP5)\textsuperscript{15}, which reduces GR function and promotes inflammation\textsuperscript{16}. Together, these results suggest that inflammation in depression is potentially caused by escape of the immune system from the anti-inflammatory effects of glucocorticoid hormones (glucocorticoid resistance) as well as the pro-inflammatory effects of FKBP5\textsuperscript{16}. Interestingly, we have also found that patients who do not respond to antidepressants have, before starting the antidepressant, higher levels of IL-1-beta, macrophage inhibiting factor (MIF) and TNF-alpha mRNAs, compared with antidepressant-responsive patients\textsuperscript{15,17}. Separately, we have found increased mRNA expression of the GR-target gene, SGK1, in the blood of depressed patients, in human hippocampal cells treated with cortisol, and in the hippocampus of rats exposed to stress, thus indicating that mRNA in the human blood can reflect changes in the brain\textsuperscript{18}.

Other blood mRNA studies on depressed patients have measured the whole genome, rather than focusing on a set of candidate genes, and have consistently found pro-inflammatory signatures. In one of the first such studies, Savitz et al.\textsuperscript{19} measured mRNA expression in peripheral blood mononuclear cells of depressed patients and identified differentially-expressed mRNAs that were linked to inflammatory pathway, such as nuclear factor kappa-B (NFkB), transforming growth factor beta (TGFb), and extracellular signal-regulated kinase (ERK). In the Netherlands Study of Depression and Anxiety (NDSA), Jansen et al.\textsuperscript{20} found an upregulation of IL-6- and natural killer cell-related related pathways. Mellon et al.\textsuperscript{21} found over-expression of genes involved in Type I interferon responses, antimicrobial responses, and cytokine and chemokine signalling, and we have recently found over-expression of genes specialised for innate immunity and myeloid cells\textsuperscript{22}. Two studies using RNAseq have found differential regulation of type I interferon-related pathways\textsuperscript{23,24}, with one study also showing enrichment for several other pathways involving immune function\textsuperscript{23}. Finally, a very recent study has used genome-wide DNA methylation and gene expression analyses in patients prospectively-defined as responders and non-responders to an 8-week trial of escitalopram treatment\textsuperscript{25}, and found two genes that exhibited increases in both DNA methylation and mRNA expression in non-responders: CHN2, which could affect hippocampal neurogenesis, and JAK2, which activates both innate and adaptive immunity.

In order to understand the specific molecular signatures associated with TRD vs. responsive depression, and their interaction with antidepressant treatment, in the present study we use whole blood mRNA quantitative polymerase chain reaction (qPCR) to measure the expression of 16 candidate mRNAs in 130 depressed patients (58 TRD, 36 antidepressant-responsive and 36 currently drug-free) and 40 healthy controls. We have recently published, in an overlapping sample, that only TRD patients have increased inflammation as measured as body mass index (BMI)-adjusted CRP\textsuperscript{3}. Thus, here we hypothesise that TRD patients have the strongest mRNA-based evidence of inflammation and glucocorticoid resistance, as shown by higher expression of IL-1-beta, IL-6, TNF-alpha and MIF, together with lower GR, higher FKBP5 and higher SGK1 expression. Moreover, and examining mRNA expression of genes hitherto unmeasured in psychiatric patients, we hypothesise that this increased inflammation is associated with higher expression of the purinergic receptor, P2RX7, which mediates stress-induced activation of the inflammasome\textsuperscript{26}; higher CCL2 and lower CXCL12 expression, as in the well-established animal model of ‘repeated social defeat’ (RSD) stress, characterised by increased inflammation and glucocorticoid resistance\textsuperscript{27}; higher expression of CRP and of the other acute phase protein, alpha-2-macroglobulin (A2M)\textsuperscript{28}; and higher expression of the interferon-responsive genes, aquaporin-4 (AQP4), ISG15, STAT1 and USP-18, which we have recently shown to be elevated in the blood mRNA of patients with chronic viral hepatitis taking IFN-alpha\textsuperscript{29}, an established model of inflammation-induced depression\textsuperscript{30,31}, and to mediate the IFN-alpha-induced increase in neuronal apoptosis and decrease in neurogenesis\textsuperscript{32}. Finally, to explore the clinical implications of these findings, we examined which genes would best classify depressed subjects in either TRD or antidepressant-responsive, even after adjusting for the effects of other clinical and immune variables, including serum CRP and white blood cells counts.

**Methods**

**Study design and clinical measures**

In total, 190 cases of MDD, meeting SCID-based DSM-5 criteria for a diagnosis for MDD\textsuperscript{33}, and 54 healthy controls, were recruited in the non-interventional, case-control, Biomarkers of Depression (BIODEP) study\textsuperscript{3}; 130 depressed patients and 40 healthy controls with available gene expression data are included in the present
study. The cases were divided into three sub-groups based on current depressive symptom scores at the Hamilton Rating Scale for Depression (HAM-D), and current and previous drug treatment: (1) responsive patients had no depressive symptoms (HAM-D < 7) while currently on an antidepressant at standard therapeutic dose for at least 6 weeks; (2) drug-free had depressive symptoms (HAM-D > 17) and had not been medicated with any antidepressants for at least 6 weeks and (3) TRD patients had depressive symptoms (HAM-D > 13) while currently on an antidepressant at standard therapeutic dose for at least 6 weeks, plus they had at least one historical failure to a different antidepressant. Lifetime antidepressants use was measured using the antidepressant treatment response questionnaire (ATRQ)34, anxiety using the Spielberger State-Trait Anxiety Rating scale35 and exposure of stressors in childhood using the childhood trauma questionnaire (CTQ)36.

The study was part of the Wellcome Trust Consortium for Neuroimmunology of Mood Disorder and Alzheimer’s disease (NIMA), approved by the National Research Ethics Service East of England, Cambridge Central, UK (15/EE/0092). The study was conducted according to the Declaration of Helsinki, and all participants provided informed consent in writing.

Clinical and sociodemographic features of the sample
Inclusion and exclusion criteria are presented in the Supplementary Material. The demographic and clinical characteristics of each group are summarised in Table 1. We had n = 58 TRD patients, n = 36 responsive patients, n = 36 drug-free patients and n = 40 healthy controls. Briefly, all the main within-group comparisons were similar to those already published in the larger sample3, and the groups did not differ significantly in age, gender distribution, educational level and BMI. As expected by design, each group differed significantly from the others on HAM-D total score (ANOVA, F = 683.6; df = 3, 166; P < 0.001), with drug-free (HAM-D around 20) being more depressed than TRD (HAM-D around 18), and both being more depressed than responsive (HAM-D around 3) and controls (HAM-D less than 1). Moreover, both TRD and drug-free patients had higher state and trait anxiety compared with responsive and controls (ANOVA, F = 51.2 and 114.5, respectively; df = 3, 166; P < 0.001). Finally, all patient groups had higher CTQ scores than controls, and both TRD and untreated patients had higher CTQ scores than responsive (generalised linear model (GLM), Wald chi-square = 106.6; df = 1, 3; P < 0.001).

Similar to the published larger sample3, the majority of TRD patients were currently taking selective serotonin reuptake inhibitors (72%), with smaller numbers exposed to noradrenergic and specific serotonergic reuptake inhibitors (14%), mirtazapine (9%), tricyclic antidepressants (4%) or bupropion (1%). Treatment-responsive patients were also predominantly taking selective serotonin reuptake inhibitors (69%), followed by noradrenergic and specific serotonergic reuptake inhibitors (22%) and mirtazapine (9%). Drug-free patients were all currently not on antidepressants for at least 6 weeks; however, n = 20 (55% of the group) had been on an antidepressant in the past, mostly (17 out of 20) on a selective serotonin reuptake inhibitor. As expected, the TRD group had more failed treatments than the other depressed groups (average of 1.7 vs. 0.8 in responders and 0.9 in drug free, ANOVA, df = 3, 166; P < 0.001; see Table 1).

Biomarkers
Venous blood was sampled from an antecubital vein between 08:00 and 10:00 h on the day of clinical assessment. Participants had fasted for 8 h, refrained from exercise for 72 h, and had been lying supine for 0.5 h prior to venepuncture. Whole blood (2.5 mL) was collected in PaxGene tubes at each recruitment site, and all PaxGene tubes were then kept at −80°C and later transferred to a central site (Brescia) for RNA isolation and gene expression analyses. Isolation of total RNA was performed using the PAXgene blood miRNA kit according to the manufacturer’s protocol (PreAnalytiX, Hombrechtikon, CHE). RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrophotometer (NanoDrop Technologies, Delaware, USA) and by Agilent BioAnalyzer (Agilent Technologies); the RNA integrity number was above 8 for all sample. Samples were stored at −80°C until processing.

Candidate gene expression analyses was performed using real-time PCR. For quality control, all samples were assayed in duplicate, and were randomised in different plates, also adding a calibrator, in order to control for possible differences in the efficiency of the Real Time reaction. Each target gene was normalised to the expression of three reference genes (glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and beta-2-microglobulin). We used commercially-available Taqman primer and probes by using Taqman assays that are all available at the Thermofisher website (https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/taqman-gene-expression.html) on a 384-wells Real Time PCR System (Biorad); the assays had been already tested for efficiency by Thermo Fisher Scientific; catalogue numbers are available on request. The expression levels of each target gene were normalised to the geometric mean of all three reference genes, and the Pfaffl method was used to determine relative target gene expression of each gene in the patients’ groups compared
Table 1  Demographic, clinical and immune data.

| Category | N | Mean [95% confidence interval]/N (%) in category | Drug-free (Free) N = 36 | Treatment-resistant (TRD) N = 58 | Group test |
|----------|---|-----------------------------------------------|-------------------------|-----------------------------------|------------|
| Healthy controls (Con) | 40 | Age, years [95% CI] 35.1 (32.7–37.5) | 36.0 (33.2–38.7) | 34.3 (31.8–36.9) | F = 0.43 |
| Treatment-responders (Resp) | 36 | Gender, female, N [%] 26 (65.0%) | 24 (66.7%) | 23 (63.9%) | Chi² = 0.59 |
| Drug-free (Free) | 30 | Education level [below university yes/no %] 9/31 (22.5%/77.5%) | 9/27 (25.0%/75.0%) | 15/21 (41.7%/58.3%) | Chi² = 14.6 |
| Treatment-resistant (TRD) | 58 | Relationship status [divorced, separated or single yes/no] 8/32 (20.0%/80.0%) | 13/23 (36.1%/63.9%) | 18/18 (50.0%/50.0%) | Chi² = 21.6 |
| | | HAM-D total score [95% CI] 0.7 (0.3–1.0) | 3.1 (2.5–3.8) | 19.9 (19.0–20.9) | F = 683.6 |
| | | State anxiety [95% CI] 26.7 (24.7–28.7) | 36.8 (33.2–40.4) | 52.8 (40.0–56.6) | F = 51.19 |
| | | Trait anxiety [95% CI] 27.8 (26.2–29.5) | 44.1 (40.4–47.8) | 60.2 (56.8–63.9) | F = 11.45 |
| | | Number of failed antidepressants (lifetime) [95% CI] 0.0 | 0.83 (0.47–1.20) | 0.89 (0.45–1.33) | F = 15.7 |
| | | Duration of exposure to antidepressants (lifetime) [95% CI] 0.0 | 20.7 (15.8–25.6) | 18.9 (12.2–25.6) | F = 20.1 (three groups) |
| | | Total Score CTQ 40.1 (38.2–42.1) | 47.6 (45.4–49.9) | 54.1 (51.7–56.6) | Wald Chi² = 1066 |
| | | Smoking % current/past/never 128/256/461 | 147/176/467 | 114/200/686 | Chi² = 2.8 |
| | | Alcohol use % current/past/never 590/0/410 | 543/143/314 | 555/139/306 | Chi² = 9.9 |
| | | BMI, kg/m² 25.7 (23.8–27.0) | 27.6 (25.6–29.7) | 260 (24-273) | F = 2.35 |
| | | CRP, mg/L 1.1 (0.8–1.6) | 2.2 (1.5–3.2) | 2.9 (2.0–4.2) | Wald Chi² = 40.49 |
| | | Total white cells 5.9 (5.5–6.4) | 6.2 (5.5–6.9) | 6.6 (6.1–7.2) | Wald Chi² = 9.82 |
| | | Lymphocytes absolute 1.9 (1.7–2.0) | 1.9 (1.7–2.1) | 1.9 (1.8–2.1) | Wald Chi² = 6.22 |
| | | Monocytes absolute 0.4 (0.35–0.44) | 0.43 (0.37–0.49) | 0.42 (0.38–0.47) | Wald Chi² = 0.611 |
| | | Neutrophils absolute 3.1 (3.14–3.89) | 3.64 (3.15–4.41) | 4.09 (3.60–4.57) | Wald Chi² = 14.6 |
| | | Basophils absolute 0.02 (0.002–0.03) | 0.03 (0.002–0.03) | 0.03 (0.02–0.03) | Wald Chi² = 0.26 |
| | | Eosinophils absolute 0.15 (0.12–0.19) | 0.19 (014–0.24) | 0.18 (0.14–0.24) | Wald Chi² = 0.051 |

*Post hoc ‘specific group category vs. others’ means that the specific group has mean score statistically different (larger or smaller) than the scores of others group categories; one group > /< one group’ means that the first category group has score statistically larger/smaller than the second group.
with controls. The analyses were conducted by researchers who were blind to group allocation.

Methods for the immune assessments are described in the Supplementary Material.

Statistical analyses

Socio demographic, clinical and immune measurements were compared among the four study groups by ANOVA, chi-square or GLM according to the statistical distribution of the variables (respectively, Gaussian, categorical and non-Gaussian). Group mean comparisons of the 16 genes were evaluated by ANOVA test followed by post hoc comparisons with Bonferroni correction. Correlations among the genes, as well as between genes and immune measures, were evaluated by Spearman’s rho coefficient. Binomial and multinomial logistic regression models were performed to detect the best predictors of the ‘study group’ outcome variable while adjusting for the effects of the other variables that were significantly different among the study groups in previous analyses. A stepwise-forward selection procedure was applied for the selection of the best (in terms of goodness of fit) predictors of the categorical ‘study group’ outcome, and predictive performances were evaluated by the Negelkerke pseudo-Rsquare goodness of fit index. Partial least square-discriminant analysis (PLS-DA) was conducted to define which genes contributed to discriminate between each study groups; the contribution of each variable (gene) which genes contributed to discriminate between each

Results

TRD patients and drug-free depressed patients have the strongest signatures of inflammation and glucocorticoid resistance

TRD and drug-free depressed patients had increased levels of circulating serum CRP (see Table 1), as previously reported in the overlapping sample. Specifically, CRP was higher in TRD patients compared with responsive and controls, and in drug-free patients compared with controls (GLM, Wald chisq = 40.5; P < 0.001). Numerically, CRP was higher in TRD patients (average of 5 mg/L), followed by drug-free (2.9 mg/L), followed by responsive (2.2 mg/L), with controls averaging at around 1.1 mg/L. There were also significant differences in total white cell count (ANOVA, F1,166 = 4.09; P = 0.008) and absolute number of neutrophils (ANOVA, F3,166 = 3.3; P = 0.022): both were significantly higher in TRD patients compared with controls, and the gradient present for CRP (TRD > drug-free > responsive > controls) was present also for these measures. Thirteen of the 16 genes were significantly different among the four groups (see Table 2, ANOVAs and post hoc comparisons with Bonferroni correction). In general, TRD and drug-free patients had similarly increased levels of inflammation-related genes: this applied to both the genes that had been measured before in depression (IL1-beta, IL-6, TNF-alpha and MIF) and those never measured before (A2M, CRP, P2RX7, CCL2 and STAT1). Moreover, TRD and drug-free patients also showed similar evidence of glucocorticoid resistance (lower GR and higher FKBPs expression). Responsive patients had an intermediate phenotype with only some of these genes (IL-6, MIF, TNF-alpha and A2M as well as FKBPS) different from controls.

Contrary to our primary hypothesis that TRD patients would have the strongest evidence of inflammation and glucocorticoid resistance, none of the above genes were significantly higher in TRD compared with drug-free patients; indeed, CCL2 was significantly higher in drug-free than in TRD patients (see Table 2). This suggests that TRD and drug-free patients came, at least in part, from phenotypically similar groups (see Discussion).

Interestingly, SGK1 was significantly higher only in the drug-free group, while TRD and responsive patients had levels similar to controls. Thus, albeit elevated in depression as we hypothesised, SGK1 levels were not linked with glucocorticoid resistance, since they were normal in TRD patients even if they had low GR mRNAs (see also correlation analyses below).

It is also of note that both P2RX7 and CXCL12 were lower in the responsive group compared with controls. For CXCL12, this confirms our hypothesis, based on the RSD animal model, that this gene would be reduced in (at least some) patients with depression.

The three genes that were not differentially regulated were three of the four interferon-responsive genes, AQP4, ISG15 and IUSP-18.

The correlation matrix (Spearman’s rho) for 13 differentially expressed genes together with serum CRP and immune cell counts is presented in Fig. 1. There were significant, positive correlations between P2RX7, pro-inflammatory cytokines and FKBPs mRNAs, and significant negative correlations between all of these genes and GR mRNA. Moreover, white cell and neutrophil counts were (not-significantly) positively correlated with FKBPs (rho = 0.20/0.21) and negatively correlated with GR mRNA (rho = −0.21/−0.22). Together, these correlations indicate that, as hypothesised, the inflammasome/inflammatory gene over-expression and resulting immune activation are associated with glucocorticoid resistance and with FKBPs-mediated pro-inflammatory signalling. Interestingly, GR was negatively correlated with FKBPs, but neither was correlated with SGK1, confirming that SGK1 is not a marker of GR resistance. It is also of note
Table 2  Candidate gene expression data.

| Genes   | Mean expression levels [95% confidence interval] | Group test                                                                 |
|---------|--------------------------------------------------|-----------------------------------------------------------------------------|
|         | Healthy controls (Con) \[40\]                    |                                                                              |
|         | Treatment-responders (Resp) \[36\]               |                                                                              |
|         | Drug-free (Free) \[36\]                          |                                                                              |
|         | Treatment-resistant (TRD) \[58\]                 |                                                                              |
| A2M     | 1.02 [0.95–1.09]                                 | F = 14.11 <0.001 TRD > Con Free > Con Resp > Con                            |
| CRP     | 1.03 [0.96–1.09]                                 | F = 4.54 0.004 TRD > Con Free > Con                                        |
| IL-1beta| 1.07 [1.04–1.10]                                 | F = 12.24 <0.001 TRD > Con TRD > Resp Free > Con                           |
| IL-6    | 1.06 [1.03–1.08]                                 | F = 19.675 <0.001 TRD > Con TRD > Resp TRD > Resp > Con                    |
| MIF     | 1.00 [0.96–1.05]                                 | F = 29.62 <0.001 TRD > Con TRD > Resp Free > Con Free > Resp Con            |
| TNF-alpha| 1.06 [1.00–1.11]                                | F = 35.09 <0.001 TRD > Con TRD > Resp Free > Con Resp Free > Con            |
| P2RX7   | 1.03 [0.95–1.12]                                 | F = 29.69 <0.001 TRD > Con TRD > Resp Free > Con Free > Resp Con            |
| CCL2    | 1.03 [0.99–1.06]                                 | F = 27.485 <0.001 TRD > Con TRD > Resp Free > Con Free > Resp Free > TRD    |
| CXCL12  | 1.06 [0.98–1.14]                                 | F = 4.49 0.005 TRD > Resp Con > Resp                                       |
| AQP4    | 1.03 [0.97–1.09]                                 | F = 0.62 0.065                                                             |
| ISG15   | 0.99 [0.91–1.06]                                 | F = 0.64 0.59                                                              |
| STAT1   | 1.06 [1.00–1.11]                                 | F = 9.67 <0.001 TRD > Con TRD > Resp Free > Con Free > Resp                |
| USP18   | 0.99 [0.91–1.07]                                 | F = 0.245 0.865                                                            |
| FKBPS   | 1.04 [0.97–1.10]                                 | F = 30.31 <0.001 TRD > Con TRD > Resp Free > Con Free > Resp               |
| GR      | 1.05 [1.02–1.08]                                 | F = 40.28 <0.001 TRD < Con TRD < Resp Free < Con Free < Resp               |
| SGK1    | 1.06 [1.03–1.09]                                 | F = 32.34 <0.001 Free > Con Free > Resp Free > TRD                         |

*Post hoc: ‘one group > < one group’ means that the first category group has score statistically larger/smaller than the second group.
that serum CRP (largely produced by the liver) was significantly, positively correlated with CRP mRNA (from the whole blood).

**Binomial logistic models show that a signature comprising** P2RX7, IL-1-beta, IL-6, TNF-alpha, CXCL12 and GR, **discriminates between TRD and responder patients over and above standard clinical and blood immune assessments**

Binomial logistic models were performed applying the step-forward procedure, in order to examine the predicting performance of mRNA gene expression, clinical data and blood immune variables, in classifying depressed patients in the TRD or responders study group, while addressing the co-variance between the immune genes and adjusting for all the other clinical and immune variables (see Table 3).

The first model included the six clinical and immune variables significantly different between the study groups (see Table 1): state anxiety, trait anxiety, total score CTQ, CRP, total white cells and neutrophils numbers. HAM-D and number of failed antidepressants were excluded as these were part of the decisional process leading to group allocation. Trait anxiety and neutrophils numbers were the only significant predictors, with a Nagelkerke’ pseudo-R-squared equal to 0.53.

The second model included the 13 significant genes from the univariate analyses (see ANOVA in Table 2). Ten genes were significant predictors (P2RX7, IL-1b, IL-6, MIF, TNF-alpha, CCL2, CXCL12, GR, FKBP5 and STAT1), with a Nagelkerke’ pseudo-R-squared = 0.89.

Finally, the third model included the two significant variables from model 1 (trait anxiety and neutrophils number) and the ten significant genes from model 2. It resulted in six genes (P2RX7, IL-1-beta, IL-6, TNF-alpha, CXCL12 and GR) remaining the only significant predictors, with a Nagelkerke’ pseudo-R-squared = 0.90. Thus, the expressions of these six genes remain significant predictors of the allocation of depressed patients to the TRD or responders group even after adjusting for the

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**Fig. 1** Correlations (Spearman’s rho) between significantly-different genes and immune measures. Coloured coefficients are statistically different from zero at level $P < 0.05$; red = negative correlations, blue = positive correlations.
other clinical and immune variables, whose variability was fully captured by trait anxiety and neutrophils number, and with a larger predictive ability than the standard clinical and immune variables in Model 1 (Nagelkerke’s pseudo-$R^2$ = 0.90 vs. 0.53).

A second series of multinomial logistic models were performed to examine the predicting performance of gene expression, clinical data and blood immune variables, in classifying all study subjects in the four study groups, including drug–depressed patients and controls (see Supplementary Results and Supplementary Table 1). We found that a signature of five mRNAs (P2RX7, IL-6, GR, SGK1 and TNF-alpha) together with trait anxiety significantly predicted the allocation of subjects to their study group.

**PLSDA show that P2RX7 best discriminates TRD patients vs. all other patients, while GR best discriminates responsive vs. all other depressed patients**

The PLSDA is presented in Fig. 2. This was conducted to define which genes mainly contribute to discriminate between each of the four groups or between the three patient groups. Panel A (on the three depressed groups only) shows that: P2RX7, and, less, CXCL12 and IL-1-beta (all in red), best discriminate TRD vs. the other depressed groups; CCL2, and, less, FKBP5 and MIF (all in green), best discriminate drug-free vs. the other depressed groups; and GR and, less, IL-6 and A2M (all in blue), best discriminate responsive vs. the other depressed groups. Panel B (on the four groups) shows GR (in black) as the gene that best discriminates controls from all the other depressed groups. It is worth noting that the discriminant performance of some genes overlaps on more than one patient group, as also indicated by the PCA of the 13 differentially expressed genes presented in Supplementary Material (Fig. S1).

**Discussion**

In a study examining whole-blood mRNA expression of candidate genes in depressed patients characterised for their depressive symptoms and response to antidepressants, and testing both established and hitherto unmeasured mRNAs, we find evidence of inflammation some activation and glucocorticoid resistance in both drug-free depressed patients and antidepressant-treated TRD patients (less so in antidepressant-treated responsive patients). Moreover, a mRNAs signature of six genes (P2RX7 and CXCL-12, both measured for the first time in psychiatric patients, as well as IL-1-beta, IL-6, TNF-alpha and GR) is a significant predictor of allocation of depressed patients to the TRD or responder group in binominal logistics models, even after adjusting for other clinical variables that are different between groups, such as a history of childhood maltreatment and serum CRP.

Our data confirm our previous findings showing increased whole blood mRNA expression of IL-6, MIF and TNF-alpha in depressed patients vs. controls\(^ {15} \), with higher levels of IL-1-beta and MIF predicting TRD when measured in drug free-depressed patients before starting an antidepressant treatment\(^ {15,17} \). This consistency is particularly noticeable since the above-mentioned studies are clinical trials with a pre–post assessment\(^ {15,17} \), and thus the biomarkers were measured before starting the antidepressants (at a time where patients were all drug-free and their response status was still unknown) and the response was measured prospectively. Admittedly, this was a much better design than the present study, which instead compares patients allocated to different groups based on a combination of current symptomatology and medication use as well historical treatment response. As shown in Table 1, these leads to groups that are different in a number of biological and clinical risk factors. All things considered, it is thus reassuring that we replicate both the increased IL-6, MIF and TNF-alpha in all our depressed groups vs. controls, as well as the increased IL-1-beta, TNF-alpha and MIF in TRD vs. responsive.

Meta-analyses of longitudinal studies have shown that antidepressant treatment (on average, for 6–12 weeks) is associated with decreases in serum or plasma cytokines, such as IL-6 and TNF-alpha, both in general\(^ {40} \) and for SSRIs in particular\(^ {41} \), with the most recent meta-analyses showing that TNF-alpha, but not IL-6, is differentially affected in responders only\(^ {42} \). Data on longitudinal changes in mRNA expression are much more limited; for example, we published\(^ {15} \) that 8-weeks of antidepressants (escitalopram or nortriptyline) decrease IL-6 mRNA, but this is driven by responders only, while TNF-alpha mRNA levels do not change. In the present study we find that levels of IL-6 and TNF-alpha mRNAs are higher in responders than controls, although with slightly different patterns, that is, responders have the highest IL-6 (higher even than TRD) while TNF-alpha is lower than in TRD patients. However, it is important to emphasise that it is difficult to compare the present study with all the others, because of the cross-sectional, rather than longitudinal, nature of our study: we simply do not know what the cytokines levels in these patients were before they started the antidepressants.

P2RX7 is a purinergic receptor that activates the NLR family pyrin domain containing 3 (NLRP3), a pattern-recognition receptor that precipitates the pro-inflammatory cascade\(^ {26,43} \). P2RX7 is ubiquitously expressed in cells of the immune system\(^ {44} \), but recent research has identified its expression also in neuronal cells, where it regulates the function of neurotransmitters relevant to depression\(^ {45} \). In our study, P2RX7 is not only associated with other markers of inflammation and with GR expression, as hypothesised, but it is also the strongest predictors of TRD in the PLSDA,
and one of the predictive genes in the signature originated by the binomial and multinomial models. While one previous study found increased levels of NLRP3 in the monocytes of depressed patients\textsuperscript{46}, the only evidence so far of a direct involvement of P2RX7 in depression comes from genetic studies associating a polymorphism in the gene with severity of depressive symptoms\textsuperscript{45,47}.

We replicate here our previous findings showing reduced GR mRNA and higher FKBP5 mRNA in depressed patients\textsuperscript{15}. While increased FKBP5 expression

| Logistic models | Explanatory variables | Likelihood ratio test | Negelkerke's pseudo-$R^2$ |
|-----------------|-----------------------|-----------------------|---------------------------|
|                 |                       | Chi$^2$ (degree of freedom) | $P$ value |
| Mod. (i)        | Trait-anxiety         | 23.9 (1)               | <0.001 | 0.53 |
|                 | State-anxiety         | 0.4 (1)                | 0.533 |
|                 | CRP                   | 0.2 (1)                | 0.961 |
|                 | Neutrophils absolute  | 5.9 (1)                | 0.015 |
|                 | Total white cells     | 0.3 (1)                | 0.601 |
|                 | Total score CTQ       | 0.2 (1)                | 0.727 |
| Mod. (ii)       | CXCL12                | 4.0 (1)                | 0.038 | 0.89 |
|                 | CCL2                  | 4.9 (1)                | 0.023 |
|                 | IL-1beta              | 3.8 (1)                | 0.048 |
|                 | IL-6                  | 3.6 (1)                | 0.037 |
|                 | GR                    | 18.4 (1)               | <0.001 |
|                 | P2RX7                 | 11.5 (1)               | 0.003 |
|                 | SGK1                  | 2.2 (1)                | 0.125 |
|                 | TNF-alpha             | 3.7 (1)                | 0.042 |
|                 | FKBP5                 | 4.3 (1)                | 0.004 |
|                 | A2M                   | 2.1 (1)                | 0.076 |
|                 | MIF                   | 6.1 (1)                | 0.018 |
|                 | STAT1                 | 5.6 (1)                | 0.009 |
|                 | CRP                   | 2.8 (1)                | 0.086 |
| Mod. (iii)      | GR                    | 5.7 (1)                | 0.017 | 0.90 |
|                 | P2RX7                 | 14.0 (1)               | <0.001 |
|                 | TNF-alpha             | 4.1 (1)                | 0.040 |
|                 | Trait-anxiety         | 3.9 (1)                | 0.051 |
|                 | IL-6                  | 4.2 (1)                | 0.042 |
|                 | CCL2                  | 3.8 (1)                | 0.053 |
|                 | IL-1beta              | 6.6 (1)                | 0.010 |
|                 | CXCL12                | 5.7 (1)                | 0.031 |
|                 | Neutrophils absolute  | 1.2 (1)                | 0.277 |
|                 | FKBP5                 | 2.4 (1)                | 0.124 |
|                 | MIF                   | 2.5 (1)                | 0.113 |
|                 | STAT1                 | 1.4 (1)                | 0.235 |

\textsuperscript{a}Explanatory variables of the model (iii) were standardised in order to take into account the different variable ranges.

Mod. (i) considering only (significantly different between group) clinical and blood immune variables; mod. (ii) considering only (significantly different between group) genes variables and mod. (iii) considering both genes and clinical-blood immune variables resulted remained significant in mod. (i) and (ii).
is well known to induce glucocorticoid resistance\(^{48,49}\), new evidence indicates that FKBP5 can also directly promote inflammation by strengthening the interactions of NF-κB regulatory kinases\(^{16}\), and our findings showing that pro-inflammatory genes are positively correlated with FKBP5 expression confirm these functional links. Indeed, the ultimate role of the reduced GR mRNA in our findings is difficult to define, as most clearly exemplified by the fact that responsive patients have GR levels indistinguishable from controls yet have increased IL-6, MIF, TNF-alpha and A2M levels. Moreover, recent data from the larger BIODEP sample show that only drug-free patients have increased cortisol levels\(^{50}\), but we show here that both drug-free and TRD have reduced GR mRNA. While the concept of reduced GR function and expression leading to ‘glucocorticoid resistance’ in depression has been extensively discussed before\(^{51–55}\), including for TRD patients\(^{56–58}\), the present study shows that reduced GR mRNA expression alone cannot fully explain the increased inflammation. Indeed the aforementioned study by Mellon et al.\(^{21}\) found upregulation of immune pathways in mononuclear cells from depressed patients in the absence of changes in GR function, and our own clinical meta-analysis on this topic has found only limited evidence linking ‘glucocorticoid resistance’ to inflammation\(^{59}\). Furthermore, it is important to emphasise here the additional confounding effects of antidepressant treatment. Previous studies have shown that antidepressants increase the expression and the function of the GR in experimental and clinical models\(^{51,53,60,61}\), and we have also found that GR mRNA levels are increased by antidepressants in the aforementioned longitudinal mRNA gene expression study, irrespective of response\(^{15}\). In the present study, we find that GR mRNA levels are ‘normal’ in responsive patients but lower in TRD, even if both groups have similar profiles of antidepressant treatment.
In contrast, we find increased levels of the GR-target gene, SGK1, in drug-free depressed patients but not in antidepressant-treated (TRD and responsive) patients, and Frodl et al. also measured SGK1 mRNA in depressed patients who were mostly on antidepressants and found no differences compared with controls. As mentioned above, the lack of longitudinal data in the present study makes it difficult to dissect the differential effects of antidepressant treatment vs. clinical improvement on mRNAs expression.

CCL2 and CXCL12 are chemokines involved in the RSD model of depression, characterised by increased inflammation and glucocorticoid resistance27. These mice show increased CCL2 in circulation and increased levels of the receptor for CCL2, C-C chemokine receptor type 2 (CCR2), in the brain, leading to monocyte recruitment to the brain and increased microglia activation. Consistently, we find increased CCL2 mRNA expression in TRD and drug-free patients, and other studies found elevated serum CCL2 (also known as Monocyte chemoattractant protein 1, MCP-1) in depressed patients63. Interestingly, in the present study we find lower levels of CCL2 in TRD patients than in drug-free patients (even if both are higher than in controls), and we have previously found, in a different sample, lower levels of serum CCL2 (MCP-1) in TRD vs. responsive patients64. Thus, it is possible that lower CCL2 in depression identifies a more severe, TRD group. Differently from CCL2, CXCL12 inhibits the trafficking of monocytes to the circulation, and in fact CXCL12 levels are reduced in the RSD model65. A recent meta-analysis did not find any studies measuring CXCL12 in depression66, but it is interesting that we find reduced CXCL12 in responsive depressed patients in our study (and normal levels in the other depressed groups), showing some consistency with the RSD model.

Both CRP and A2M mRNAs are elevated in TRD and drug-free depressed patients in our study. There is an extensive literature showing elevated levels of serum (protein) CRP in depression, with more than 13,000 patients included in recent meta-analyses34 and evidence of increased CRP also in the cerebrospinal fluid42. Interestingly, while the liver is considered the most important source of CRP, CRP mRNA has been detected in macrophages from the lung66 and from atherosclerotic plaques67. Our study not only finds that CRP mRNA is expressed in circulating blood cells, but also that the whole-blood CRP mRNA is highly correlated with the levels of (liver-produced) serum CRP protein. A2M is another acute phase protein, like CRP, but there are only three studies looking at A2M serum levels in depression, with conflicting findings68–70. We have recently described higher A2M mRNA in both whole blood mRNA of adult humans exposed to early life trauma and the hippocampus of adult rats exposed to prenatal stress, and identified seven polymorphisms in the A2M gene that show significant gene × environment interactions with childhood stress in predicting depressive symptoms in adulthood28. Together, this evidence supports a role of A2M in depression, but further studies are needed.

Finally, we measure here the four interferon-responsive genes, aquaporin-4, ISG15, STAT1 and USP-18, which are elevated in the whole blood29 and in human neurones following IFN-alpha32. Only STAT1 is increased in the present study, in both drug-free and TRD patients, suggesting that the upregulation of the other three genes is only visible after pharmacological inflammation induced by IFN-alpha, or in brain tissue. Although this is the first study measuring STAT1 in the blood of depressed patients, the above-mentioned studies in the NESDA cohort20 and in non-responders to citalopram25 found an upregulation of, respectively, STAT3 and JAK2 mRNAs, and another study found STAT3 cell signalling alterations in depression71.

The study has two main limitations that must be discussed. Firstly, as mentioned above, this is not a clinical trial with pre–post measures of gene expression or longitudinal ascertainment of antidepressant resistance, and thus cross-sectional comparisons between groups are likely to be influenced by other clinical and sociodemographic variables that differ between groups. Of course, our analyses attempt to adjust for such group differences in the binomial/multinomial logistic regression models. Moreover, we had already measured the mRNA levels of seven of the 16 genes (IL-1-beta, IL-6, TNF-alpha, MIF, GR, FKBP5 and SGK1) in drug-free depressed patients15 and in ‘prospectively-defined’ TRD patients15,17,18, and in the present paper we replicate all of these findings. Nevertheless, the cross-sectional design of the present study implies that, especially for the genes never measured before, the findings need to be replicated. The second important limitation is that the measurement of mRNA gene expression is in the whole blood rather than sorted immune cells. Of course, the ‘whole-blood’ approach has the advantages of speed and simplicity of blood collection and handling ‘at the bedside’, which is essential for the development of clinically useful biomarkers. However, we do not know which cells predominantly contributes to the mRNA findings, and furthermore we lack functional cellular data, for example, to measure inflammasome activation or glucocorticoid resistance. Thus, future studies should include an in-depth characterisation of immune cells-specific mRNA profiles as well as functional methodologies.

Notwithstanding these limitations, we believe that our paper is relevant to novel approaches for personalised psychiatry and novel targets for immune-related antidepressants therapies. We find that a combination of six
genes (P2RX7, IL-1-beta, IL-6, TNF-alpha, CXCL-12 and GR) performs better than the routine clinical and immunological variables in identifying patients who are TRD or responsive to antidepressants. If replicated in larger, longitudinal samples, this signature might be helpful in identifying patients that should be fast-tracked into augmentation regimes—potentially a step toward overcoming the classic ‘trial and error’ approach in treating depression. In terms of novel targets, antagonists of P2RX7, JAK, CCR2 and FKBP5 are all novel antidepressant tools supported by our findings. Future studies will need to examine if these new treatments work, and whether responses to such new treatments can be improved by selecting patients with abnormal levels of relevant mRNAs.

Acknowledgements
This work was supported by the Wellcome Trust strategy award to the Neuroimmunology of Mood Disorders and Alzheimer’s Disease (NIMA) Consortium (104025), which is also funded by Janssen, GlaxoSmithKline, Lundbeck and Pfizer; the full list of consortium members is presented in the Supplementary Material. As part of the Wellcome Trust strategy award to the Neuroimmunology of Mood Disorders and Alzheimer’s Disease (NIMA) Consortium, the authors will be conducting a clinical trial testing a CNS-penetrant P2RX7 antagonist by Johnson & Johnson, JNJ-54175446, in TRD patients (EudraCT Number 2018-001884-21). C.M.P. and E.T.B. are each supported by the NIHR Biomedical Research Centre (BRC) at the South London and Maudsley NHS Foundation Trust and King’s College London, London, the NIHR Cambridge Biomedical Research Centre (Mental Health) and the Cambridge NIHR BRC Cell Phenotyping Hub. C.M.P. and V.M. are in receipt of research funding from J&J. E.T.B. consults for Sosei Heptares. L.T. is supported by the NIHR Cambridge BRC. N.H. consults for GlaxoSmithKline and is in receipt of research funding from J&J. P.B., D.J. and W.D. are employees of Janssen Research & Development, LLC of Johnson & Johnson, and hold stock in Johnson & Johnson. All the other authors have no disclosures to declare. Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Cambridge. For the members of the NIMA Consortium (as detailed in the Supplementary Material): Khan, Murphy, Parker, Patel and Richardson are employees of GSK; Acton, Austin, Bhattacharya, Carruthers, Isaac, Kemp, Kolb, Nye (deceased) and Wittenberg are employees of Janssen; Campbell, Egbebjerg, Eriksson, Gastambide, Adams, Jeggo, Moeller, Nelson, Plath, Thomsen, Pederson and Zorn are employees of Lundbeck; Balice-Gordon, Binnuen, Duerr, Fullerton, Golli, Hughes, Pio, Samad and Sporn are employees of Pfizer; all other consortium members have no disclosures to declare. The sharing of data used in this study is restricted by the informed consent process. Our data cannot be made available on public repositories but will be shared with other scientifically accredited research groups on request.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41398-020-00874-7).

Received: 9 February 2020 Revised: 28 May 2020 Accepted: 28 May 2020
Published online: 23 July 2020

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