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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

5

Annamaria Cattaneo^{1,2}, Clarissa Ferrari³, Lorinda Turner⁴, Nicole Mariani¹, Daniela 6 Enache¹, Caitlin Hastings¹, Melisa Kose¹, Giulia Lombardo¹ Anna P. McLaughlin¹, Maria 7 A. Nettis¹, Naghmeh Nikkheslat¹, Luca Sforzini¹, Courtney Worrell¹, Zuzanna 8 Zajkowska¹, Nadia Cattane², Nicola Lopizzo², Monica Mazzelli², Linda Pointon⁵, Philip J. 9 Cowen⁶, Jonathan Cavanagh⁷, Neil A. Harrison⁸, Peter de Boer⁹, Declan Jones¹⁰, 10 Wayne C. Drevets¹¹, Valeria Mondelli¹, Edward T. Bullmore⁵, the Neuroimmunology of 11 Mood Disorders and Alzheimer's Disease (NIMA) Consortium*, and Carmine M. 12 13 Pariante¹.

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1. Stress, Psychiatry and Immunology Laboratory & Perinatal Psychiatry, King's College 15 London, Institute of Psychiatry, Psychology and Neuroscience, Department of 16 17 Psychological Medicine, Maurice Wohl Clinical Neuroscience Institute, Kings College London, SE5 9RT, UK; 2. Biological Psychiatric Unit, IRCCS Istituto Centro San 18 19 Giovanni di Dio Fatebenefratelli, 25125 Brescia, Italy; 3. Statistical Service, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, 25125 Brescia, Italy; 20 21 4. Department of Medicine, School of Clinical Medicine, University of Cambridge, 22 Cambridge, CB2 0QQ, UK; 5. Department of Psychiatry, School of Clinical Medicine, University of Cambridge, CB2 0SZ, UK; 6. University of Oxford Department 23

24 of Psychiatry, Warneford Hospital, Oxford, OX3 7JX, UK; 7. Centre for Immunobiology, 25 University of Glasgow and Sackler Institute of Psychobiological Research, Queen 26 Elizabeth University Hospital, Glasgow, G51 4TF, UK; 8. School of Medicine, School of 27 Psychology, Cardiff University Brain Research Imaging Centre, Maindy Road, Cardiff, 28 CF24 4HQ, UK; 9. Neuroscience, Janssen Research & Development, Janssen 29 Pharmaceutica NV, 2340, Beerse, Belgium; 10. Neuroscience External Innovation, 30 Janssen Pharmaceuticals, J&J Innovation Centre, London, W1G 0BG, UK; 11. Janssen 31 Research & Development, Neuroscience Therapeutic Area, 3210 Merryfield Row, San 32 Diego, CA 92121, USA. * A list of authors and their affiliations appears in the 33 Supplementary Material

- 34
- 35

36 **Correspondence to:**

37 Carmine M. Pariante, MD, FRCPsych, PhD

Stress, Psychiatry and Immunology Lab, Institute of Psychiatry, Psychology and
 Neuroscience, King's College London

40 G.32.01, The Maurice Wohl Clinical Neuroscience Institute, Cutcombe Road, London,

- 41 SE5 9RT
- 42 carmine.pariante@kcl.ac.uk
- 43
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49 ABSTRACT

50 The mRNA expression signatures associated with the 'pro-inflammatory' phenotype of 51 depression, and the differential signatures associated with depression subtypes and the 52 effects of antidepressants, are still unknown. We examined 130 depressed patients (58 53 treatment-resistant, 36 antidepressant-responsive, and 36 currently untreated) and 40 54 healthy controls from the BIODEP study, and used whole blood mRNA gPCR to 55 measure the expression of 16 candidate mRNAs, some never measured before: 56 interleukin (IL)-1-beta, IL-6, TNF-alpha, macrophage inhibiting factor (MIF), 57 glucocorticoid receptor (GR), SGK1, FKBP5, the purinergic receptor P2RX7, CCL2, 58 CXCL12, c-reactive protein (CRP), alpha-2-macroglobulin (A2M), acquaporin-4 (AQP4), 59 ISG15, STAT1 and USP-18. All genes but AQP4, ISG15 and USP-18 were differentially 60 regulated. Treatment-resistant and drug-free depressed patients had both increased 61 inflammasome activation (higher P2RX7 and proinflammatory cytokines/chemokines 62 mRNAs expression) and glucocorticoid resistance (lower GR and higher FKBP5 63 mRNAs expression), while responsive patients had an intermediate phenotype with. 64 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) 65 66 distinguished treatment-resistant from responsive patients, even after adjusting for other 67 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 68 69 findings in larger, longitudinal cohorts, and test whether this mRNA signature can 70 identify patients that are more likely to respond to adjuvant strategies for treatment-71 resistant depression, including combinations with anti-inflammatory medications.

73 **INTRODUCTION**

74

While there is overwhelming evidence of increased inflammation in depression $^{1-4}$, the 75 76 molecular signature underpinning this 'pro-inflammatory' phenotype is still unknown. A 77 multitude of studies and meta-analyses show that patients with major depressive 78 disorder (MDD) have, on average, increased serum levels of pro-inflammatory 79 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) ^{1,2,4,5}. Patients 80 81 with 'treatment resistant depression' (TRD) are more likely to have increased inflammation ^{6,7}, as do patients with cardiovascular disorders, obesity, anxiety, and a 82 history of childhood maltreatment ^{3,8–13}. 83

84

85 Whole blood mRNA expression analyses measure mRNAs coding for inflammatory genes and for genes operating upstream and downstream of these immune 86 mechanisms, such as the glucocorticoid receptor $(GR)^{14}$. We have been the first to 87 demonstrate that drug-free depressed patients have increased mRNA expression of IL-88 89 1-beta, IL-6 and TNF-alpha, together with reduced expression of the GR and increased expression of the FK506 binding protein 5 (*FKBP5*)¹⁵, which reduces GR function and 90 promotes inflammation ¹⁶. Together, these results suggest that inflammation in 91 depression is potentially caused by escape of the immune system from the anti-92 93 inflammatory effects of glucocorticoid hormones (glucocorticoid resistance) as well as the pro-inflammatory effects of FKBP5¹⁶. Interestingly, we have also found that patients 94 who do not respond to antidepressants have, before starting the antidepressant, higher 95 levels of *IL-1-beta*, macrophage inhibiting factor (*MIF*) and *TNF-alpha* mRNAs, 96

97 compared with antidepressant-responsive patients 15,17 . Separately, we have found 98 increased mRNA expression of the GR-target gene, *SGK1*, in the blood of depressed 99 patients, in human hippocampal cells treated with cortisol, and in the hippocampus of 100 rats exposed to stress, thus indicating that mRNA in the human blood can reflect 101 changes in the brain ¹⁸.

102

103 Other blood mRNA studies on depressed patients have measured the whole genome, 104 rather than focusing on a set of candidate genes, and have consistently found proinflammatory signatures. In one of the first such studies, Savitz et al.¹⁹ measured 105 106 mRNA expression in peripheral blood mononuclear cells of depressed patients and 107 identified differentially-expressed mRNAs that were linked to inflammatory pathway, 108 such as nuclear factor kappa-B (NFkb), transforming growth factor beta (TGFb), and 109 extracellular signal-regulated kinase (ERK). In the Netherlands Study of Depression and 110 Anxiety (NESDA), Jansen et al. found an upregulation of IL-6- and natural killer cellrelated related pathways²⁰. Mellon et al. found over-expression of genes involved in 111 112 Type I interferon responses, antimicrobial responses, and cytokine and chemokine signalling ²¹, and we have recently found over-expression of genes specialised for 113 innate immunity and myeloid cells ²². Two studies using RNAseg have found differential 114 regulation of type I interferon-related pathways ^{23,24}, with one study also showing 115 enrichment for several other pathways involving immune function²³. Finally, a very 116 117 recent study has used genome-wide DNA methylation and gene expression analyses in 118 patients prospectively-defined as responders and non-responders to an 8-week trial of escitalopram treatment ²⁵, and found two genes that exhibited increases in both DNA 119

methylation and mRNA expression in non-responders: CHN2, which could affect
 hippocampal neurogenesis, and JAK2, which activates both innate and adaptive
 immunity.

123

124 In order to understand the specific molecular signatures associated with TRD vs. 125 responsive depression, and their interaction with antidepressant treatment, in the 126 present study we use whole blood mRNA gPCR to measure the expression of 16 127 candidate mRNAs in 130 depressed patients (58 TRD, 36 antidepressant-responsive, 128 and 36 currently drug-free) and 40 healthy controls. We have recently published, in an 129 overlapping sample, that only TRD patients have increased inflammation as measured as body mass index (BMI)-adjusted CRP³. Thus, here we hypothesise that TRD 130 131 patients have the strongest mRNA-based evidence of inflammation and glucocorticoid 132 resistance, as shown by higher expression of IL-1-beta, IL-6, TNF-alpha and MIF, 133 together with lower GR, higher FKBP5 and higher SGK1 expression. Moreover, and 134 examining mRNA expression of genes hitherto unmeasured in psychiatric patients, we 135 hypothesise that this increased inflammation is associated with: higher expression of 136 the purinergic receptor, P2RX7, which mediates stress-induced activation of the inflammasome ²⁶; higher CCL2 and lower CXCL12 expression, as in the well-137 138 established animal model of 'repeated social defeat' (RSD) stress, characterised by increased inflammation and glucocorticoid resistance ²⁷; higher expression of *CRP* and 139 140 of the other acute phase protein, alpha-2-macroglobulin (A2M) ^{4,28}; and higher 141 expression of the interferon-responsive genes, acquaporin-4 (AQP4), ISG15, STAT1 142 and USP-18, which we have recently shown to be elevated in the blood mRNA of

patients with chronic viral hepatitis taking IFN-alpha ²⁹, an established model of inflammation-induced depression ^{30,31}, and to mediate the IFN-alpha-induced increase in neuronal apoptosis and decrease in neurogenesis 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.

- 150
- 151
- 152 **METHODS**
- 153
- 154 Study design and clinical measures
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156 In total, 190 cases of major depressive disorder, meeting SCID-based DSM-5 criteria for a diagnosis for major depressive disorder ³³, and 54 healthy controls, were recruited in 157 the non-interventional, case-control, Biomarkers of Depression (BIODEP) study ³; 130 158 159 depressed patients and 40 healthy controls with available gene expression data are 160 included in the present study. The cases were divided into 3 sub-groups based on 161 current depressive symptom scores at the Hamilton Rating Scale for Depression (HAM-162 D), and current and previous drug treatment: 1) responsive patients had no depressive 163 symptoms (HAM-D < 7) while currently on an antidepressant at standard therapeutic 164 dose for at least 6 weeks; 2) drug-free had depressive symptoms (HAM-D > 17) and 165 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 scale ³⁵, and exposure of stressors in childhood using the Childhood Trauma Questionnaire (CTQ) ³⁶.

172

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.

178

179 Clinical and sociodemographic features of the sample

180

181 Inclusion and exclusion criteria are presented in the Supplementary Material. The 182 demographic and clinical characteristics of each group are summarized in Table 1. We 183 had n=58 TRD patients, n=36 responsive patients, n=36 drug-free patients and n=40 184 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 185 186 age, gender distribution, educational level and BMI. As expected by design, each group 187 differed significantly from the others on HAM-D total score (ANOVA, F=683.6; df=3, 188 166; 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 (GLM, Wald chi-square=106.6; df=1, 3; P<0.001).

195

Similar to the published larger sample³, the majority of TRD patients were currently 196 197 taking selective serotonin reuptake inhibitors (72%), with smaller numbers exposed to 198 noradrenergic and specific serotonergic reuptake inhibitors (14%), mirtazapine (9%), 199 tricyclic antidepressants (4%) or bupropion (1%). Treatment-responsive patients were 200 also predominantly taking selective serotonin reuptake inhibitors (69%), followed by 201 noradrenergic and specific serotonergic reuptake inhibitors (22%) and mirtazapine (9%). 202 Drug-free patients were all currently not on antidepressants for at least 6 weeks; 203 however, n=20 (55% of the group) had been on an antidepressant in the past, mostly 204 (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 205 206 responders and 0.9 in drug free, ANOVA, df=3, 166; P<0.001; see Table 1).

207

208 Biomarkers

209

Venous blood was sampled from an antecubital vein between 08:00-10:00 h on the day
of clinical assessment. Participants had fasted for 8 h, refrained from exercise for 72 h,

212 and had been lying supine for 0.5 h prior to venepuncture. Whole blood (2.5 mL) was 213 collected in PaxGene tubes at each recruitment site, and all PaxGene tubes were then 214 kept at -80 °C and later transferred to a central site (Brescia) for RNA isolation and gene 215 expression analyses. Isolation of total RNA was performed using the PAXgene blood 216 miRNA kit according to the manufacturer's protocol (PreAnalytiX, Hombrechtikon, CHE). 217 RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 218 ratios using a Nanodrop spectrophotometer (NanoDrop Technologies, Delaware, USA) 219 and by Agilent BioAnalyzer (Agilent Technologies); the RNA integrity number (RIN) was 220 above 8 for all sample. Samples were stored at -80 °C until processing.

221

233

222 Candidate gene expression analyses was performed using real-time PCR. For guality 223 control, all samples were assayed in duplicate, and were randomized in different plates, 224 also adding a calibrator, in order to control for possible differences in the efficiency of 225 the Real Time reaction. Each target gene was normalized to the expression of three 226 reference genes (glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and beta-2-227 microglobulin). We used commercially-available Tagman primer and probes by using 228 Tagman assays that are all available at the Thermofisher website 229 (https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-230 assays/tagman-gene-expression.html) on a 384 wells Real Time PCR System (Biorad); 231 the assays had been already tested for efficiency by Thermo Fisher Scientific; catalogue 232 numbers are available on request. The expression levels of each target gene were

was used to determine relative target gene expression of each gene in the patients'

11

normalized to the geometric mean of all three reference genes, and the Pfaffl method

groups compared with controls. The analyses were conducted by researchers who wereblind to group allocation.

237

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

- 239 Statistical analyses
- 240

241 Socio demographic, clinical and immune measurements were compared among the four 242 study groups by ANOVA, Chi-Square or Generalized linear model (GLM) according to 243 the statistical distribution of the variables (respectively, Gaussian, categorical, and non-244 Gaussian). Group mean comparisons of the 16 genes were evaluated by ANOVA test 245 followed by post-hoc comparisons with Bonferroni correction. Correlations among the 246 genes, as well as between genes and immune measures, were evaluated by 247 Spearman's rho coefficient. Binomial and multinomial logistic regression models were 248 performed to detect the best predictors of the 'study group' outcome variable while 249 adjusting for the effects of the other variables that were significantly different among the 250 study groups in previous analyses. A stepwise-forward selection procedure was applied 251 for the selection of the best (in terms of goodness of fit) predictors of the categorical 252 'study group' outcome, and predictive performances were evaluated by the Negelkerke 253 pseudo-Rsquare goodness of fit index. Partial Least Square-Discriminant Analysis 254 (PLS-DA) was conducted to define which genes contributed to discriminate between 255 each study groups ^{37,38}; the contribution of each variable (gene) in the group discrimination was displayed by the loadings plots ³⁹. The data-reduction technique, 256 257 Principal Component Analysis (PCA), was used to derive, through the biplot, a graphical

representation of the association between genes and subjects, labelled by study group(see Supplementary Material).

- 260
- 261
- 262 **RESULTS**
- 263

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

266

TRD and drug-free depressed patients had increased levels of circulating serum CRP 267 (see Table 1), as previously reported in the overlapping sample ³. Specifically, CRP was 268 269 higher in TRD patients compared with responsive and controls, and in drug-free patients compared with controls (GLM, Wald Chi²=40.5; P<0.001). Numerically, CRP was higher 270 271 in TRD patients (average of 5 mg/L), followed by drug-free (2.9 mg/L), followed by 272 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, F_{3.164}=4.09; P=0.008) and 273 274 absolute number of neutrophils (ANOVA, $F_{3.164}$ =3.3; P=0.022): both were significantly 275 higher in TRD patients compared with controls, and the gradient present for CRP 276 (TRD>drug-free>responsive>controls) was present also for these measures.

277

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*, *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 *FKBP5* expression). Responsive patients had an intermediate phenotype with only some of these genes (*IL-6*, *MIF*, *TNFalpha* and *A2M*, as well as *FKBP5*) different from controls.

287

288 Contrary to our primary hypothesis that TRD patients would have the strongest 289 evidence of inflammation and glucocorticoid resistance, none of the above genes were 290 significantly higher in TRD compared with drug-free patients; indeed, *CCL2* was 291 significantly higher in drug-free than in TRD patients (see Table 2). This suggests that 292 TRD and drug-free patients came, at least in part, from phenotypically similar groups 293 (see Discussion).

294

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).

300

301 It is also of note that both *P2RX7* and *CXCL12* were *lower* in the responsive group 302 compared with controls. For CXCL12, this confirms our hypothesis, based on the RSD

303 animal model ²⁷, that this gene would be reduced in (at least some) patients with 304 depression.

305

The three genes that were not differentially regulated were three of the four interferonresponsive genes, *AQP4*, *ISG15*, and *USP-18*.

308

309 The correlation matrix (Spearman's rho) for 13 differentially expressed genes together 310 with serum CRP and immune cell counts is presented in Figure 1. There were 311 significant, positive correlations between P2RX7, pro-inflammatory cytokines, and 312 FKBP5 mRNAs, and significant negative correlations between all of these genes and 313 *GR* mRNA. Moreover, white cell and neutrophil counts were (not-significantly) positively 314 correlated with FKBP5 (rh0=0.20/0.21) and negatively correlated with GR mRNA (rho=-315 0.21/-0.22). Together, these correlations indicate that, as hypothesised, the 316 inflammasome/inflammatory gene over-expression and resulting immune activation are 317 associated with glucocorticoid resistance and with FKBP5-mediated pro-inflammatory 318 signalling. Interestingly, GR was negatively correlated with FKBP5, but neither was 319 correlated with SGK1, confirming that SGK1 is not a marker of GR resistance. It is also 320 of note that serum CRP (largely produced by the liver) was significantly, positively 321 correlated with CRP mRNA (from the whole blood).

322

323 Binomial logistic models show that a signature comprising P2RX7, IL-1-beta, IL-6,

324 TNF-alpha, CXCL12 and GR, discriminates between TRD and responder patients

325 over and above standard clinical and blood immune assessments

326

Binomial logistics 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**).

332

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.

339

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-Rsquared =0.89.

344

Finally, the third model included the two significant variables from model 1 (Trait Anxiety and neutrophils number) and the 10 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 6 genes remain significant predictors of the allocation of depressed patients to the TRD or responders group even after adjusting for the 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' pseudo-R-squared =0.90 vs. 0.53).

354

A second series of multinomial logistics 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-dree 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.

361

Partial least square discriminant analyses show that P2RX7 best discriminates
 TRD patients vs. all other patients, while GR best discriminates responsive vs. all
 other depressed patients

365

The partial least square discriminant analysis (PLSDA) is presented in **Figure 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,

Iess, 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 principal component analysis (PCA) of the 13 differentially expressed genes presented in Supplementary Material (Figure S1).

378 **DISCUSSION**

379

380 In a study examining whole blood mRNA expression of candidate genes in depressed 381 patients characterised for their depressive symptoms and response to antidepressants, 382 and testing both established and hitherto unmeasured mRNAs, we find evidence of 383 inflammasome activation and glucocorticoid resistance in both drug-free depressed 384 patients and antidepressant-treated TRD patients (less so in antidepressant-treated 385 responsive patients). Moreover, a mRNAs signature of six genes (P2RX7 and CXCL-12, 386 both measured for the first time in psychiatric patients, as well as IL-1-beta, IL-6, TNF-387 alpha and GR) is a significant predictor of allocation of depressed patients to the TRD or 388 responder group in binomial logistics models, even after adjusting for other clinical 389 variables that are different between groups, such as a history of childhood maltreatment 390 and serum CRP.

391

392 Our data confirm our previous findings showing increased whole blood mRNA 393 expression of *IL-6*, *MIF* and *TNF-alpha* in depressed patients vs. controls ¹⁵, with higher 394 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 395 noticeable since the above-mentioned studies are clinical trials with a pre-post 396 assessment ^{15,17}, and thus the biomarkers were measured before starting the 397 398 antidepressants (at a time where patients were all drug-free and their response status 399 was still unknown) and the response was measured prospectively. Admittedly, this was 400 a much better design than the present study, which instead compares patients allocated 401 to different groups based on a combination of current symptomatology and medication 402 use as well historical treatment response. As shown in Table 1, these leads to groups 403 that are different in a number of biological and clinical risk factors. All things considered, 404 it is thus reassuring that we replicate both the increased IL-6, MIF and TNF-alpha in all 405 our depressed groups vs. controls, as well as the increased IL-1-beta, TNF-alpha and 406 *MIF* in TRD vs. responsive.

407

408 Meta-analyses of longitudinal studies have shown that antidepressant treatment (on 409 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 410 411 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 412 much more limited; for example, we published ¹⁵ that 8-weeks of antidepressants 413 414 (escitalopram or nortriptyline) decrease IL-6 mRNA, but this is driven by responders 415 only, while TNF-alpha mRNA levels do not change. In the present study we find that 416 levels of IL-6 and TNF-alpha mRNAs are higher in responders than controls, although 417 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.

423 P2RX7 is a purinergic receptor that activates the NLR family pyrin domain containing 3 424 (NLRP3), a pattern-recognition receptor that precipitates the pro-inflammatory cascade ^{26,43}. P2RX7 is ubiguitously expressed in cells of the immune system ⁴⁴, but recent 425 426 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 427 428 associated with other markers of inflammation and with GR expression, as 429 hypothesised, but it is also the strongest predictors of TRD in the PLSDA, and one of 430 the predictive genes in the signature originated by the binomial and multinomial models. 431 While one previous study found increased levels of NLRP3 in the monocytes of depressed patients ⁴⁶, the only evidence so far of a direct involvement of P2RX7 in 432 433 depression comes from genetic studies associating a polymorphism in the gene with severity of depressive symptoms ^{45,47}. 434

435

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

441 correlated with FKBP5 expression confirm these functional links. Indeed, the ultimate 442 role of the reduced *GR* mRNA in our findings is difficult to define, as most clearly 443 exemplified by the fact that responsive patients have GR levels indistinguishable from 444 controls yet have increased IL-6, MIF, TNF-alpha and A2M levels. Moreover, recent 445 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 446 447 mRNA. While the concept of reduced GR function and expression leading to 'glucocorticoid resistance' in depression has been extensively discussed before ^{51–55}, 448 including for TRD patients 56-58, the present study shows that reduced GR mRNA 449 450 expression alone cannot fully explain the increased inflammation. Indeed the aforementioned study by Mellon et al.²¹ found upregulation of immune pathways in 451 452 mononuclear cells from depressed patients in the absence of changes in GR function, 453 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 454 455 here the additional confounding effects of antidepressant treatment. Previous studies 456 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 457 458 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 459 mRNA levels are 'normal' in responsive patients but lower in TRD, even if both groups 460 have similar profiles of antidepressant treatment. In contrast, we find increased levels of 461 462 the GR-target gene, SGK1, in drug-free depressed patients but not in antidepressant-463 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 ⁶². 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.

468

469 CCL2 and CXCL12 are chemokines involved in the RSD model of depression, characterised by increased inflammation and glucocorticoid resistance ²⁷. These mice 470 471 show increased CCL2 in circulation and increased levels of the receptor for CCL2, C-C 472 chemokine receptor type 2 (CCR2), in the brain, leading to monocyte recruitment to the 473 brain and increased microglia activation. Consistently, we find increased CCL2 mRNA 474 expression in TRD and drug-free patients, and other studies found elevated serum 475 CCL2 (also known as Monocyte chemoattractant protein 1, MCP-1) in depressed patients ⁶³. Interestingly, in the present study we find *lower* levels of CCL2 in TRD 476 477 patients than in drug-free patients (even if both are higher than in controls), and we 478 have previously found, in a different sample, lower levels of serum CCL2 (MCP-1) in TRD vs. responsive patients ⁶⁴. Thus, it is possible that *lower* CCL2 in depression 479 480 identifies a more severe, TRD group. Differently from CCL2, CXCL12 inhibits the 481 trafficking of monocytes to the circulation, and in fact CXCL12 levels are reduced in the RSD model ⁵⁵. A recent meta-analysis did not find any studies measuring CXCL12 in 482 depression ⁶³, but it is interesting that we find *reduced CXCL12* in responsive depressed 483 484 patients in our study (and normal levels in the other depressed groups), showing some 485 consistency with the RSD model.

486

487 Both CRP and A2M mRNAs are elevated in TRD and drug-free depressed patients in 488 our study. There is an extensive literature showing elevated levels of serum (protein) CRP in depression, with more than 13 thousand patients included in recent meta-489 analyses ^{2,4} and evidence of increased CRP also in the cerebrospinal fluid ⁶⁵. 490 491 Interestingly, while the liver is considered the most important source of CRP, CRP mRNA has been detected in macrophages from the lung ⁶⁶ and from atherosclerotic 492 plaques ⁶⁷. Our study not only finds that *CRP* mRNA is expressed in circulating blood 493 494 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 495 496 there are only three studies looking at A2M serum levels in depression, with conflicting findings ^{68–70}. We have recently described higher A2M mRNA in both whole blood 497 498 mRNA of adult humans exposed to early life trauma and the hippocampus of adult rats 499 exposed to prenatal stress, and identified 7 polymorphisms in the A2M gene that show 500 significant gene x environment interactions with childhood stress in predicting depressive symptoms in adulthood ²⁸. Together, this evidence supports a role of A2M in 501 502 depression, but further studies are needed.

503

Finally, we measure here the four interferon-responsive genes, acquaporin-4, ISG15, STAT1 and USP-18, which are elevated in the whole blood ²⁹ and in human neurones following IFN-alpha ³². 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 cohort ²⁰ and in non-responders to citalopram
 ²⁵ found an upregulation of, respectively, STAT3 and JAK2 mRNAs, and another study
 found STAT3 cell signalling alterations in depression ⁷¹.

513

514 The study has two main limitations that must be discussed. Firstly, as mentioned above, 515 this is not a clinical trial with pre-post measures of gene expression or longitudinal 516 ascertainment of antidepressant resistance, and thus cross-sectional comparisons 517 between groups are likely to be influenced by other clinical and sociodemographic 518 variables that differ between groups. Of course, our analyses attempt to adjust for such 519 group differences in the binomial/multinomial logistic regression models. Moreover, we 520 had already measured the mRNA levels of seven of the 16 genes (IL-1-beta, IL-6, TNFalpha, MIF, GR, FKBP5 and SGK1) in drug-free depressed patients ¹⁵ and in 521 'prospectively-defined' TRD patients ^{15,17,18}, and in the present paper we replicate all of 522 these findings. Nevertheless, the cross-sectional design of the present study implies 523 524 that, especially for the genes never measured before, the findings need to be replicated. 525 The second important limitation is that the measurement of mRNA gene expression is in 526 the whole blood rather than sorted immune cells. Of course, the 'whole-blood' approach 527 has the advantages of speed and simplicity of blood collection and handling 'at the 528 bedside', which is essential for the development of clinically useful biomarkers. 529 However, we do not know which cells predominantly contributes to the mRNA findings, 530 and furthermore we lack functional cellular data, for example, to measure 531 inflammasome activation or glucocorticoid resistance. Thus, future studies should

include an in-depth characterisation of immune cells-specific mRNA profiles as well asfunctional methodologies.

534

535 Notwithstanding these limitations, we believe that our paper is relevant to novel 536 approaches for personalised psychiatry and novel targets for immune-related antidepressants therapies. We find that a combination of six genes (P2RX7, IL-1-beta, 537 538 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 539 540 antidepressants. If replicated in larger, longitudinal samples, this signature might be helpful in identifying patients that should be fast-tracked into augmentation regimes -541 542 potentially a step toward overcoming the classic 'trial and error' approach in treating depression. In terms of novel targets, antagonists of P2RX7⁷², JAK⁷³, CCR2⁷⁴ and 543 FKBP5¹⁶ are all novel antidepressant tools supported by our findings. Future studies 544 545 will need to examine if these new treatments work, and whether responses to such new 546 treatments can be improved by selecting patients with abnormal levels of relevant 547 mRNAs.

548

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551

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571

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583	
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585	AUTHOR CONTRIBUTIONS
586	
587	o Substantial contributions to the conception or design of the work: Annamaria
588	Cattaneo, Clarissa Ferrari, Lorinda Turner, Nicole Mariani, Nadia Cattane, Linda
589	Pointon, Philip J. Cowen, Jonathan Cavanagh, Neil A. Harrison, Peter de Boer, Declan
590	Jones ¹ , Wayne C. Drevets, Valeria Mondelli, Edward T. Bullmore, Carmine M. Pariante.
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594	

o Final approval of the version to be published: Annamaria Cattaneo, Clarissa Ferrari,
Lorinda Turner, Nicole Mariani, Daniela Enache, Caitlin Hastings, Melisa Kose, Giulia
Lombardo, Anna P. McLaughlin, Maria A. Nettis, Naghmeh Nikkheslat, Luca Sforzini,
Courtney Worrell, Zuzanna Zajkowska, Nadia Cattane, Nicola Lopizzo, Monica Mazzelli,
Linda Pointon, Philip J. Cowen, Jonathan Cavanagh, Neil A. Harrison, Peter de Boer,
Declan Jones, Wayne C. Drevets, Valeria Mondelli, Edward T. Bullmore, Carmine M.
Pariante.

602

603 o Agreement to be accountable for all aspects of the work in ensuring that questions

related to the accuracy or integrity of any part of the work are appropriately investigated

605 and resolved: Annamaria Cattaneo, Clarissa Ferrari, Lorinda Turner, Nicole Mariani,

Daniela Enache, Caitlin Hastings, Melisa Kose, Giulia Lombardo, Anna P.

607 McLaughlin, Maria A. Nettis, Naghmeh Nikkheslat, Luca Sforzini, Courtney Worrell,

⁶⁰⁸ Zuzanna Zajkowska, Nadia Cattane, Nicola Lopizzo, Monica Mazzelli, Linda Pointon,

609 Philip J. Cowen, Jonathan Cavanagh, Neil A. Harrison, Peter de Boer, Declan Jones,

610 Wayne C. Drevets, Valeria Mondelli, Edward T. Bullmore, Carmine M. Pariante.

611

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615

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841 **FIGURE LEGENDS**

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Figure 1: Correlations (Spearman's rho) between significantly-different genes and
 immune measures

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Coloured coefficients are statistically different from zero at level p<0.05; red = negative
correlations, blue=positive correlations.

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850 Figure 2: Partial Least Squares Discriminant analysis outputs: loading plots

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852 The partial least square discriminant analysis (PLSDA) was conducted to define which 853 genes contribute to discriminate between each of the four groups. The plots depict the 854 loadings of each gene: the larger the loading, the better the gene discriminates the 855 study group from the others. Loadings summarize how the genes are related to each 856 other as well as discriminate between the groups: all genes with positive loadings are 857 positive correlated with each other and negatively correlated with genes with negative 858 loadings; colours indicate the group for which the genes have a maximal median value. 859 Panel A (on the three depressed groups only) shows that: P2RX7, and, less, CXCL12 860 and IL-1-Beta (all in red), best discriminate TRD vs the other depressed groups; CCL2, 861 and, less, FKBP5 and MIF (all in green), best discriminate drug-free vs the other 862 depressed groups; and GR, and, less, IL-6 and A2M (all in blue), best discriminate

863	responsive vs the other depressed groups. Panel B (on the four groups) shows GR (in
864	black) is the gene that best discriminates controls from all the other depressed groups.
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	Mean [95% o	confidence inte	rval] / N (%)	in category	Group test						
	Healthy controls (Con) N=40	Treatment- responders (Resp) N=36	Drug-free (Free) N=36	Treatment- resistant (TRD) N=58	Statistic	P-value	Post Hoc #				
Age, years [95%CI]	35.1 [32.7-37.5]	36.0 [33.2-38.7]	34.3 [31.8- 36.9]	35.9 [34.0-37.8]	F=0.43	0.73					
Gender, female, N [%]	26 [65.0%]	24 (66.7%)	23 (63.9%)	41 (70.7%)	Chi ² =0.59	0.90					
Education level [below university yes/no %]	9/31 [22.5%/77. 5%]	9/27 (25.0%/75.0 %)	15/21 (41.7%/58 .3%)	22/36 (37.9%/62. 1%)	Chi ² =14.6	0.26					
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 %)	30/28 (51.7/48.3 %)	Chi ² =21.6	0.01					
HAM-D total score [95%CI]	0.7 [0.3-1.0]	3.1 [2.5-3.8]	19.9 [19.0- 20.9]	18.1 [17.3-18.9]	F=683.6	<0.001	Each vs others				
State Anxiety [95%CI]	26.7 [24.7-28.7]	36.8 [33.2-40.4]	52.8 [49.0- 56.6]	49.5 [46.1-52.8]	F=51.19	<0.001	Con <others Resp <i>vs</i> others</others 				
Trait Anxiety [95%CI]	27.8 [26.2-29.5]	44.1 [40.4-47.8]	60.2 [56.8- 63.9]	61.0 [58.2-63.9]	F=114.5	<0.001	Con <others Resp <i>vs</i> others</others 				
Number of failed antidepressants (lifetime) [95%CI]	0.0	0.83 [0.47-1.20]	0.89 [0.45- 1.33)	1.74 [1.30-2.18]	F=15.7	<0.001	Con <others TRD>others</others 				
Duration of exposure to antidepressants (lifetime) [95%CI]	0.0	20.7 [15.8-25.6]	18.9 [12.2- 25.6]	24.6 [20.5-28.8]	F(2,101)=1. 31 (three groups)	0.27					
Total Score CTQ	40.1 [38.2-42.1]	47.6 [45.4-49.9]	54.1 [51.7- 56.6]	53.4 [51.6-55.3]	Wald Chi ² =106.6	<0.001	Con <others Resp <i>vs</i> other</others 				
Smoking % current/past/never	12.8/25.6 /61.6	14.7/17.6 /67.7	11.4/20.0 /68.6	21.1/21.1 /57.8	Chi ² =2.8	0.83					
Alcohol use % current/past/never	59.0/0.0 /41.0	54.3/14.3 /31.4	55.5/13.9 /30.6	63.8/3.4 32.8	Chi ² =9.9	0.13					
BMI, kg/m ²	25.4 [23.8-27.0]	27.6 [25.6-29.7]	26.0 [24.6- 27.3]	28.5 [26.3-30.7]	F=2.35	0.073					
CRP, mg/L	1.1 [0.8-1.6]	2.2 [1.5-3.2]	2.9 [2.0-4.2]	5.0 [3.7-6.7]	Wald Chi ² =40.49	<0.001	TRD>Con TRD>Resp Free>Cont				
Total White Cells	5.9 [5.5- 6.4]	6.2 [5.5-6.9]	6.6 [6.1- 7.2]	7.2 [6.6- 7.7]	F=4.09	0.008	TRD>Con				

870 Table 1. Demographic, clinical and immune data

Lymphocytes absolute	1.9 [1.7- 2 0]	1.9 [1.7-2.1]	1.9 [1.8- 2 1]	2.1 [2.0- 2.3]	F=2.65	0.051	
Monocytes absolute	0.4 [0.35-0.44]	0.43 [0.37-0.49]	0.42 [0.38- 0.47]	0.40 [0.37-0.44]	F=0.46	0.710	
Neutrophils absolute	3.51 [3.14-3.89]	3.64 [3.15-4.41]	4.09 [3.60- 4.57]	4.36 [3.92-4.80]	F=3.30	0.022	TRD>Con
Basophils absolute	0.02 [0.02-0.03]	0.03 [0.02-0.03]	0.03 [0.02- 0.03]	0.03 [0.02-0.03]	Wald Chi ² =9.82	0.611	
Eosinophils absolute	0.15 [0.12-0.19]	0.19 [0.14-0.24]	0.18 [0.14- 0.24]	0.23 [0.18-0.28]	Wald Chi ² =6.22	0.101	

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.

877 Table 2. Candidate gene expression data

	Mean	Expression Levels		Group te	st		
Genes	Healthy controls (Con) N=40	Treatment- responders (Resp) N=36	Drug-free (Free) N=36	Treatment- resistant (TRD) N=58	Statistic	P- value	Post Hoc
A2M	1.02 [0.95-1.09]	1.28 [1.22-1.34]	1.24 [1.17-1.31]	1.23 [1.19-1.27]	F=14.11	<0.001	TRD>Con Free>Con Resp>Con
CRP	1.03 [0.96-1.09]	1.13 [1.07-1.18]	1.18 [1.08-1.29]	1.18 [1.13-1.22]	F=4.54	0.004	TRD>Con Free>Con
IL-1beta	1.07 [1.04-1.10]	1.16 [1.03-1.28]	1.22 [1.18-1.26]	1.32 [1.27-1.37]	F=12.24	<0.001	TRD>Con TRD>Resp Free>Con
IL-6	1.06 [1.03-1.08]	1.32 [1.26-1.38]	1.28 [1.24-1.32]	1.23 [1.17-1.28]	F=19.675	<0.001	TRD>Con Free>Con Resp>TRD Resp>Con
MIF	1.00 [0.96-1.05]	1.13 [1.07-1.20]	1.30 [1.24-1.37]	1.27 [1.23-1.30]	F=29.62	<0.001	TRD>Con TRD>Resp Free>Con Free>Resp Resp>Con
TNF- alpha	1.06 [1.00-1.11]	1.24 [1.21-1.27]	1.30 [1.27-1.33]	1.32 [1.28-1.35]	F=35.09	<0.001	TRD>Con TRD>Resp Free>Con Resp>Con
P2RX7	1.03 [0.95-1.12]	0.79 [0.74-0.84]	1.27 [1.13-1.40]	1.25 [1.20-1.30]	F=29.69	<0.001	TRD>Con TRD>Resp Free>Con Free>Resp Con>Resp
CCL2	1.03 [0.99-1.06]	0.99 [0.94-1.05]	1.25 [1.20-1.29]	1.14 [1.11-1.17]	F=27.485	<0.001	TRD>Con TRD>Resp Free>Con Free>Resp Free>TRD
CXCL12	1.06 [0.98-1.14]	0.93 [0.86-1.00]	1.03 [0.96-1.10]	1.08 [1.04-1.12]	F=4.49	0.005	TRD>Resp Con>Resp
AQP4	1.03 [0.97-1.09]	1.03 [0.96-1.11]	1.03 0.97-1.09]	1.08 [1.01-1.16]	F=0.62	0.605	
ISG15	0.99 [0.91-1.06]	1.03 [0.95-1.12]	0.96 [0.88-1.04]	1.03 [0.95-1.10]	F=0.64	0.59	
STAT1	1.06 [1.00-1.11]	1.08 [1.03-1.14]	1.23 [1.16-1.30]	1.19 [1.15-1.23]	F=9.67	<0.001	TRD>Con TRD>Resp Free>Con Free>Resp
USP18	0.99 [0.91-1.07]	1.02 [0.93-1.10]	1.01 [0.95-1.08]	1.03 [0.98-1.09]	F=0.245	0.865	
FKBP5	1.04 [0.97-1.10]	1.13 [1.08-1.18]	1.27 [1.23-1.30]	1.27 [1.25-1.29]	F=30.31	<0.001	TRD>Con TRD>Resp Free>Con Free>Resp Resp>Con

GR	1.05 [1.02-1.08]	1.01 [0.97-1.05]	0.83 [0.80-0.87]	0.87 [0.84-0.90]	F=40.28	<0.001	TRD <con TRD<resp Free<con Free<resp< td=""></resp<></con </resp </con
SGK1	1.06 [1.03-1.09]	1.05 [1.02-1.08]	1.23 [1.20-1.26]	1.05 [1.02-1.08]	F=32.34	<0.001	Free>Con Free>Resp Free>TRD

879 # Post-Hoc: "one group >/< one group" means that the first category group has score statistically larger/smaller than the second group.

Table 3: Binomial regression models output for detecting the best predictors of

the binomial (two categories: Resp vs TRD) study group variable
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Logistic Models	Explanatory variables	Likelihood r	atio test	Negelkerke's Pseudo-R ²
		Chi ² (degree of	P-value	
		freedom)		
	Trait-Anxiety	23.9 (1)	<0.001	
	State-Anxiety	0.4(1)	0.533	
	CRP	0.2 (1)	0.961	
	Neutrophils absolute	5.9 (1)	0.015	0.53
Mod. i)	Total White Cells	0.3 (1)	0.601	
	Total Score CTQ	0.2 (1)	0.727	
	CXCL12	4.0 (1)	0.038	
	CCL2	4.9 (1)	0.023	
	IL-1beta	3.8 (1)	0.048	
	IL-6	3.6 (1)	0.037	
Mod. ii)	GR	18.4 (1)	<0.001	
	P2RX7	11.5 (1)	0.003	0.89
	SGK1	2.2 (1)	0.125	
	TNF-alpha	3.7 (1)	0.042	
	FKBP5	4.5 (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	
	GR	5.7 (1)	0.017	
	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	
Mod. iii) #	CCL2	3.8 (1)	0.053	0.90
	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	

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888 [#] Explanatory variables of the model iii) were standardized in order to take into account the different 889 variable ranges.

890 Mod.i) considering only (significantly different between group) clinical and blood immune variables; mod 891 ii) considering only (significantly different between group) genes variables; mod iii) considering both

genes and clinical-blood immune variables resulted remained significant in Mod. i) and ii).

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Cas Story	12 FK8P5	1beta	o h	ક્ર જ	- 21	f18-sc	x si	AN	F alph	8 . N	UR NI	(14)	<u>,</u> ℃
A2M 0.11 -0.09 0.13	0.17 0.21	0.19	0.18	-0.09	-0.06	0	0.1	0.17	0.08	0.11	0.11		
CRP 0.08 0.07	0.27 0.24	0.12	0.22	-0.12	0.11	0.09	0.19	0.24	0.39	0.21	0.16		- 0.8
CXCL12 0.08	0.11 0.1	-0.16	0.14	-0.1	0.31	80.0	0.12	0.09	-0.05	0	0.04		- 06
CCL2	0.36 0.27	0.17	0.27	-0.32	0.39	0.28	0.22	0.27	0.05	-0.04	-0.02		0.0
FKE	BP5 0.37	0.33	0.47	-0.44	0.43	0.26	0.3	0.4	0.19	0.21	0.2		- 0.4
	IL-1beta	0.1	0.34	-0.29	0.39	-0.03	0.14	0.31	0.1	0.17	0.19		- 02
		IL-6	0.27	-0.2	0.06	0.09	0.12	0.36	0.15	0.17	0.16		0.2
			MIF	-0.34	0.48	0.19	0.27	0.43	0.16	0.03	0.05		- 0
				GR	-0.32	-0.16	-0.27	-0.33	-0.12	-0.22	-0.21		0.2
				P2)	K7R	0.15	0.25	0.21	-0.05	0	0.06		
					S	GK1	0.25	0.13	0.03	-0.01	-0.04		0.4
						ST	AT1	0.22	0.11	-0.02	-0.03		0.6
						T	VF-al	pha	0.26	0.23	0.25		
							CR	P-se	rum	0.3	0.27		<mark>0.8</mark>
							NE	UTR	OPH	ILS	0.92		

PLS-DA for the 3 patient groups

cxcL12

PLS-DA for the 4 groups



