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Higher immune-related gene expression in major depression is independent of CRP levels: results from the BIODIP study

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Abstract

Compelling evidence demonstrates that some individuals suffering with major depressive disorder (MDD) exhibit increased levels of inflammation. Most studies focus on inflammation-related proteins, such as serum or plasma C-reactive protein (CRP). However, the immune-related modifications associated with MDD may be not entirely captured by CRP alone. Analysing mRNA gene expression levels, we aimed to identify broader molecular immune-related phenotypes of MDD. We examined 168 individuals from the non-interventional, case-control, BIODIP study, 128 with a diagnosis of MDD and 40 healthy controls. Individuals with MDD were further divided according to serum high-sensitivity (hs)CRP levels (n=59 with CRP <1, n=33 with CRP 1-3, and n=36 with CRP >3 mg/L). We isolated RNA from whole blood and performed gene expression analyses using RT-qPCR. We measured the expression of 16 immune-related candidate genes: A2M, AQP4, CCL2, CXCL12, CRP, FKBP5, IL-1-beta, IL-6, ISG15, MIF, GR, P2RX7, SGK1, STAT1, TNF-alpha, and USP18. Nine of the 16 candidate genes were differentially expressed in MDD cases vs. controls, with no differences between CRP-based groups. Only CRP mRNA was clearly associated with serum CRP. In contrast, plasma (proteins) IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-16, IL-17A, IFN-gamma and TNF-alpha, and neutrophils counts, were all differentially regulated between CRP-based groups (higher in CRP >3 vs. CRP <1 and/or controls), reflecting the gradient of CRP values. Secondary analyses on MDD individuals and controls with CRP values <1 mg/L (usually interpreted as “no inflammation”) confirmed MDD cases still had significantly different mRNA expression of immune-related genes compared with controls. These findings corroborate an immune-related molecular activation in MDD, which appears to be independent of serum CRP levels. Additional biological mechanisms may then be required to translate this mRNA signature into inflammation at a protein and cellular levels. Understanding these mechanisms will help to uncover the true immune abnormalities in depression, opening new paths for diagnosis and treatment.

Introduction

Immunopsychiatric studies provide compelling evidence of immune-related biological changes in individuals with major depressive disorder (MDD) [1, 2]. However, there is still uncertainty around the precise biological or molecular mechanisms underpinning this relationship. Meta-analytic evidence confirms higher levels of inflammatory biomarkers in people with MDD compared with non-depressed controls, especially when assessed using serum/plasma C-reactive protein (CRP) [3]. Moreover, these alterations tend to be more pronounced in people with treatment-resistant depression (TRD) [4, 5]. Recent work from our group in the UK Biobank has also demonstrated that the increased serum CRP is present in depression even after adjusting for potential confounders such as smoking, body mass index (BMI), exposure to childhood trauma, adverse socioeconomic circumstances and ill physical health, and should thus be considered a ‘core’ biological feature of depression [2].

Together, these lines of evidence indicate that inflammation may be on the causal pathways to MDD and a promising target for treatment [6, 7]. However, recent meta-analyses highlighted inconclusive results in the potentially beneficial antidepressant effect of commercially available anti-inflammatory medications in MDD [8–10]. A major cause of uncertainty is that the real proportion of MDD individuals who show immune alterations is yet to be elucidated, and that, most importantly, there are still no clear biomarkers to identify a person with ‘immune-related depression’ that is more likely to respond to anti-inflammatories. Different findings in terms of inflammation prevalence and response to anti-inflammatory interventions may emerge in samples selected based on different biomarkers of inflammation.

As mentioned above, most of the published research in MDD focuses on serum/plasma CRP. CRP is produced in the liver in response to increased levels of inflammatory cytokines, mainly interleukin (IL)-6 [11]. It is a reliable and easy-to-measure acute-phase response protein, widely used as a biomarker of inflammation, reflecting both peripheral and central inflammation [12]. According to the Centers for Disease Control and Prevention and the American Heart Association, values of CRP below 1 mg/L are considered as normal, and therefore identify a low-risk category for cardiovascular diseases, while values above 3 mg/L are suggestive of a high cardiovascular risk [13]. Increased concentrations of CRP have been consistently associated with MDD [2, 14, 15]. In addition, some clinical trials with anti-

inflammatory compounds have demonstrated their effectiveness only in depressed people with increased CRP levels. For example, Raison and colleagues demonstrated a higher response rate in TRD individuals treated with the tumor necrosis factor (TNF)-alpha antagonist, infliximab, compared with placebo, but only in those with high baseline plasma CRP (>5 mg/L) [16]. Similarly, another randomized controlled trial (RCT) from our group using minocycline on subjects with non-responsive MDD and CRP levels >1 mg/L found a significant greater improvement in MDD scores only in participants receiving minocycline with serum CRP levels ≥ 3 mg/L [17]. Consequently, CRP is frequently the only biomarker of inflammation measured in immunopsychiatric studies [18].

However, CRP alone may not be sufficient to identify the immune-related biological and molecular modifications associated with depression. Firstly, several other biomarkers of inflammation have been described as raised in people with depression [18]. For example, individuals with MDD also have higher concentrations of other serum/plasma immune-related proteins, such as C-C motif chemokine ligand 2 (CCL2), IL-6, IL-10, and TNF-alpha [19–21]. Furthermore, protein markers of inflammation – including CRP – provide a picture of the down-stream biological effects of immune activation, and are influenced by many clinical and sociodemographic variables [5, 22]. For example, CRP levels are influenced by age, sex, smoking status, blood pressure, and pharmacological treatments (such as hormone replacement therapy), as well as metabolic variables (such as weight, BMI, and lipid profile) [11]. IL-6 is also relevant to this association between CRP and metabolic variables, being produced in the adipose tissue, and regulating multiple metabolic aspects [23]. Therefore, CRP cannot be simply considered as a biomarker of inflammation, but it may rather represent the expression of other complex and non-specific biological and clinical processes [24]. This raises the question of whether serum/plasma CRP is the best discriminant to identify the immune-related phenotypes of depression. Instead, it is possible that many individuals with an immune-related predisposition to MDD are undiagnosed by using CRP alone, and there may be other markers to capture these modifications.

Of note, the immune-related phenotypes associated with MDD are the result of molecular and transcriptional alterations, as demonstrated in differences in blood mRNA gene expression. Previous research has shown that individuals with MDD have altered patterns of expression of

immune-related genes compared with controls [25–30], but it is still unclear whether this altered immune-related gene expression is fully captured by CRP. For example, in a microarray study performed on 1848 subjects, Jansen and colleagues found 129 genes to be differentially regulated in MDD vs. controls, including genes involved in immune-related pathways, 127 of which were still significant after correction for CRP values [31]. In a previous study from our group on people with MDD (in a sample partially overlapping with that used in the present paper), we demonstrated that whole-blood mRNA gene expression of immune-related genes could distinguish antidepressant pharmacotherapy non-responders and drug-free depressed patients from antidepressant treatment responders and controls [32]. However, these clinically-defined groups only partially mapped onto CRP levels; most strikingly, CRP levels were higher in individuals with TRD (mean of around 5 mg/L) compared with those who were currently depressed but unmedicated (mean of around 3 mg/L), even if most immune-related genes were not different between these two groups. In contrast, CRP levels were similar in drug-free MDD participants and in treatment responders (mean of around 3 and 2 mg/L, respectively), while mRNA levels for immune-related genes were different between these two groups. Another study from the same cohort confirmed that individuals with MDD had significantly increased proinflammatory proteins (CRP and IL-6) and immune cell counts (neutrophils, CD4+ T-cells, and monocytes) compared with controls [33], but these two sets of biomarkers identified different, albeit overlapping, subgroups of depression. Various meta-analyses and our own aforementioned study in the UK Biobank found that only 21-27% of depressed patients have ‘inflamed depression’ according to the CRP >3 mg/L criteria [2, 15], but the evidence reviewed here suggests that the proportion of immune-related MDD cases may be underestimated by analysing CRP only. Indeed, immune-related depression may be a mechanistically heterogeneous condition, rather than a ‘monolithic’ subgroup, with potentially different causal mechanisms and biomarkers [33].

In the aforementioned study by Cattaneo et al. [32] we had shown that mRNA expression of the 13 genes that were differentially regulated between depressed patients and controls was not, or only minimally correlated, with levels of serum CRP. In the present study, we re-analysed the immune-related gene expression from the sample described in [32], but grouped the participants according to clinically-relevant cut-offs of serum CRP (<1, 1-3, and >3 mg/L), and compared CRP-based MDD groups between themselves and vs. healthy controls. All these analyses on CRP-based MDD groups and controls are novel and not published before, and we

have here also included the three genes, AQP4, ISG15 and USP-18, that were not included in the correlational analyses in [32]. Furthermore, we also conducted new analyses, on CRP-based MDD groups and controls, comparing plasma IL-6 and white cell counts, as well as hitherto unpublished plasma cytokines (IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-15, IL-16, IL-17A, interferon (IFN)-gamma, TNF-alpha, and vascular endothelial growth factor (VEGF)-A).

Our aim was to understand whether there was a difference in the immune-related phenotypes of MDD identified by immune gene expression levels vs. those identified by CRP. Specifically, we hypothesised that the immune-related gene expression signature associated with MDD would be (at least partially) independent of CRP levels. To our knowledge, this is the first study to analyse immune-related gene expression in individuals with MDD who have been stratified based on serum CRP levels.

Materials and Methods

Study design and sample characteristics

Data were obtained from the multicentre, non-interventional, case–control, Biomarkers of Depression (BIODEP) study [5]. Participants were recruited and assessed in 5 clinical centres in the UK: Brighton, Cambridge, Glasgow, London (King’s College London), and Oxford. The study was conducted as 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) and conducted according to the Declaration of Helsinki. Further information on the study design, and inclusion and exclusion criteria, are presented in [5, 32] and in the Supplementary Materials and Methods.

Based on serum CRP levels, we identified three sub-groups of MDD cases: 1) with CRP levels below 1 mg/L (CRP <1, n=59), suggestive of no inflammation, 2) with CRP between 1 and 3 mg/L (CRP 1-3, n=33), suggestive of increased inflammation (elevated CRP), and 3) with CRP above 3 mg/L (CRP >3, n=36), suggestive of (at least) low-grade inflammation [15].

Descriptive clinical and sociodemographic characteristics of subgroups are summarised in Table 1.

Biomarkers

Blood was taken from an antecubital vein at a time comprised between 08⁰⁰ and 10⁰⁰ am on the same day of the clinical assessment. Participants were instructed to fast for 8 hours, abstain from strenuous exercise for 72 hours, and lie supine for 30 minutes prior to venous blood sampling.

Whole-blood mRNA

At each recruitment site, whole blood was collected in PaxGene tubes (2.5 mL) and kept at –80°C. RNA isolation and gene expression analyses were performed in a central site (Brescia). Total RNA was isolated using the PAXgene blood miRNA kit according to the manufacturer’s protocol (PreAnalytiX, Hombrechtikon, CHE). Quality and quantity of RNA were assessed through the evaluation of A260/280 and A260/230 ratios by using the Nanodrop

spectrophotometer (NanoDrop Technologies, Delaware, USA) and by Agilent BioAnalyzer (Agilent Technologies). The RNA integrity number (RIN) was above 8 for all the samples, which were stored at -80°C until processing.

We had *a-priori* decided that the maximum number of measurable genes, based on mRNA quantity and technical restrictions, was $n=16$. As previously described [32], we measured: alpha-2-macroglobulin (A2M), IL-1-beta, IL-6, macrophage inhibiting factor (MIF), TNF-alpha, CRP, aquaporin 4 (AQP4), C-X-C Motif Chemokine Ligand 12 (CXCL12), CCL2, interferon-stimulated gene 15 (ISG15), P2X purinoceptor 7 (P2RX7), SGK1, signal transducer and activator of transcription 1 (STAT1), ubiquitin specific peptidase 18 (USP18), nuclear receptor subfamily 3 group C member 1 (NR3C1, the glucocorticoid receptor (GR) encoding gene), and FK506 binding protein 51 (FKBP5). This panel was originally selected based on previous research to include both genes which are associated with inflammation and glucocorticoid resistance and others we wanted to explore in clinical research after promising preclinical data. Seven genes had been previously measured in people with depression (FKBP5, IL-1-beta, IL-6, MIF, GR, SGK1, and TNF-alpha) [34–36]. Nine had never been examined in depression before: CRP was selected because of the scarce evidence on whole-blood CRP mRNA expression, and we were interested in understanding its relationship with serum CRP and its reliability as a gene expression marker; A2M is another acute-phase protein which is related to depression through gene environment interactions [37]; AQP4, ISG15, STAT1 and USP-18 are associated with IFN-alpha-induced depression in people with chronic viral hepatitis [38], and this evidence has been confirmed using *in vitro* models of human hippocampal neurogenesis [39]; CCL2 and CXCL12 are chemokines linked to ‘repeated social defeat’ (RSD), an animal model of inflammation and glucocorticoid resistance induced by chronic stress [40]; and P2RX7 mediates stress-induced activation of the inflammasome and is considered a target of new antidepressant strategies using receptor antagonist [41]. Gene expression analyses were performed using a 384-wells reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) system (Biorad). All the samples were assayed in duplicate and randomised in different plates, also adding a calibrator, for a quality control of potential differences in the efficiency of reactions. Commercially-available Taqman primer and probes were used, by using Taqman assays, available at the Thermo Fisher website (<https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr->

[assays/taqman-gene-expression.html](#)). Assays already had been tested for efficiency by Thermo Fisher Scientific (catalogue numbers available on request).

The expression levels of each candidate gene were normalised to the geometric mean of the expression of three reference housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and beta-2-microglobulin), previously selected as the most stable in the whole blood of people with depression [32, 35]. We further confirmed these housekeeping genes were stable across the different study groups in analysis, and were not correlated with serum CRP. Relative target mRNA gene expression was determined using the Pfaffl method (comparing between depression cases and controls) [42]. Gene expression analyses were conducted by researchers who were blind to group allocation.

Serum high-sensitivity (hs)CRP

The CRP levels were assayed using a turbidimetry method on Beckman Coulter AU analysers, with anti-CRP-antibodies coated on latex particles [5]. We included $n=7$ individuals with CRP levels ≥ 10 mg/L; this is in line with recent evidence suggesting the inclusion of this population may help to provide a better estimate of the association between CRP and depression [43].

Other immune markers

Information on plasma cytokines and immune cells subpopulations are presented in the Supplementary Materials and Methods.

Statistical analyses

Statistical analyses were performed using the IBM Statistical Package for Social Sciences, version 27 (SPSS Inc., USA). We tested the Gaussian assumption of the analysed variable by graphical inspection (boxplots and Q-Q plots) and decided the subsequent analyses accordingly. Student's t -test (for Gaussian distributed variables) or Mann–Whitney U test (for non-Gaussian distributed variables) were used to compare continuous features among 2 groups. Analysis of variance (ANOVA, for Gaussian distributed variables), or Generalised Linear Model (GLM) and Kruskal–Wallis H test (for non-Gaussian distributed variables) were used to compare continuous features among more than 2 groups. Fisher's exact test or chi-squared

test were used to compare categorical variables. We applied generalized linear models, with a Tweedie distribution with log link for variables with a zero-inflated distribution, and with a Poisson loglinear distribution for cell counts. Correlations between serum CRP levels and other immunological variables and immune-related candidate genes were assessed using Spearman's correlation coefficient (ρ). Analysis of covariance (ANCOVA) was conducted to control for the effects of covariates (age, sex and BMI). The Bonferroni correction was applied to ANOVAs, ANCOVAs and GLMs, to control for the effect of multiple group comparisons. Gene expression p-values were further adjusted for the 16 genes analysed using a Benjamini-Hochberg False Discovery Rate (FDR) cut-off of .05 (q-value).

Results

Immune-related genes are differentially expressed in MDD cases compared with controls, independently of CRP values

The main clinical features of the CRP-based groups are presented in Table 1. There were no significant differences in clinical measures, except for HAMD-17 scores that were highest in the CRP >3 group (HAMD-17 around 16) but numerically very similar to the mean of the CRP <1 group (HAMD-17 around 15), while the lowest mean was in the CRP 1-3 group (HAMD-17 around 12). There were also no significant differences in the presence of, or the response to, antidepressant treatment (MDD treatment responsive vs. MDD treatment non-responsive vs. MDD drug-free; $p=.210$). As expected, mean serum CRP values were different between CRP >3 vs. others, and between CRP 1-3 vs. others, but not between CRP <1 and controls. Mean BMI was also higher in CRP >3 vs. CRP <1 and controls, and in CRP 1-3 vs. CRP <1 (Table 1).

We performed group analyses of mRNA gene expression between the four subgroups of participants, namely the three groups of MDD cases (CRP <1, 1-3, and >3 mg/L) and the controls. We found a consistent up-regulation of mRNA transcripts from 5 pro-inflammatory genes in all three MDD CRP-based groups compared with controls (A2M, IL-1-beta, IL-6, MIF, and TNF-alpha), with no difference between CRP-based groups. The increases ranged +15-25% vs. controls, across the different transcripts and groups. The GR was also down-regulated in all the three MDD CRP-based groups vs. controls (around -14 to -15%), while the other glucocorticoid-related gene, FKBP5, was up-regulated, again in all CRP-based groups vs. controls (+20-26%).

The mRNA transcripts for two other pro-inflammatory genes (CCL2 and STAT1) were significantly higher in both the CRP <1 and >3 groups vs. controls (ranging +14-20%), but not in the 'intermediate' CRP 1-3 group vs. controls, that is, the findings did not reflect the gradient of CRP levels. The only transcripts that followed serum CRP levels was CRP mRNA, as levels were higher in the CRP 1-3 and >3 vs. controls group (approximately +14 and +25%, respectively), and in CRP >3 vs. CRP <1 (+19% vs. CRP <1). There was some evidence of higher expression of another glucocorticoid-related gene, SGK1, in MDD cases with CRP >3 vs. both controls (+7%) and MDD cases with CRP 1-3 mg/L (+4%), but the Bonferroni's post-

hoc comparisons (following the significant ANOVA) only reached trend significance. No significant differences were found in the expression levels of the remaining candidate genes (AQP4, CXCL12, ISG15, P2RX7, and USP18). ANCOVA analyses to control for the effects of age, sex, and BMI, or for oral contraceptives, did not affect the findings (see Table 2 and Supplementary Results). Additional sensitivity analyses excluding the (n=7) individuals with serum CRP ≥ 10 mg/L did not change our results (see also Supplementary Results). Significance levels were also unaffected by the number of transcripts (n=16) analysed (all $p < .05$ survived the FDR-adjusted q threshold of .05)

We further looked at correlations between mRNA levels and plasma levels of IL-6 and TNF-alpha (the only cytokines for which we had both mRNA and protein measures) and we found a significant, weak correlation between mRNA and plasma levels for IL-6 only ($\rho = 0.16$, $p = .045$). As in the original paper by Cattaneo et al. [32], mRNA expression of the genes was not, or only minimally correlated, with levels of serum CRP, except for CRP mRNA (see Supplementary Results).

Serum CRP levels predict plasma and cellular immune biomarkers of depression

Comparisons between the MDD CRP-based groups and controls for plasma cytokines levels and white cell counts are presented in Table 3. In contrast with mRNAs, most of these variables (IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-16, IL-17-A, IFN-gamma, TNF-alpha, and neutrophils) were differentially regulated between groups, reflecting the gradient of CRP values, that is, with values that were significantly higher for the CRP > 3 group vs. CRP < 1 and/or controls, with differences sometimes reaching +200-300%. Moreover, CRP levels were also correlated with most of these immune biomarkers using Spearman's correlations. Further details are provided in Table 3, the Supplementary Results and Supplementary Table 3.

MDD cases with normal CRP values (<1 mg/L) have significantly different mRNA expression of immune-related genes compared with controls selected for CRP <1 mg/L

Since we found that immune-related gene expression was up-regulated in MDD independently of serum CRP levels, we performed secondary analyses limited to MDD subjects and controls with serum hsCRP values below 1 mg/L (usually interpreted as “no inflammation”). This

comparison included 85 individuals (of the total 168) with CRP <1 mg/L (59 MDD cases vs. 26 controls). There were no significant differences between groups in sociodemographic, clinical and immune characteristics, including BMI and values of plasma cytokines or white cell counts (Table 4).

Interestingly, the mean mRNA expressions differed significantly between groups for 11 out of the 16 candidate genes (Table 5). Consistent with our findings in the whole sample, we found an up-regulation of pro-inflammatory and glucocorticoid-related genes in MDD cases vs. controls (A2M, CCL2, IL-1-beta, IL-6, MIF, FKBP5, SGK1, STAT1 and TNF-alpha), as well as the downregulation of GR, as we described above. In addition, we found a significant down-regulation of CXCL12 in the MDD group. These are the same genes we have shown above to be differentially expressed in all subgroups of MDD cases vs. controls, except for CXCL12 which was not different in the previous comparisons.

Not surprisingly, we found no differences in CRP mRNA between the two groups, and they had comparable serum CRP values (0.5 vs. 0.4 mg/L). No significant differences were found in the expression levels of other genes (AQP4, ISG15, P2RX7, and USP18). Findings remained significant after the inclusion of sex, age, and BMI as covariates, and after the FDR correction.

Discussion

In the present study, we found that the different immune-related mRNA gene expression in MDD cases compared with controls is independent of serum CRP levels and is present even in individuals with CRP <1 mg/L. These findings corroborate the presence of an immune-related molecular signature in many individuals with MDD, and they query the ability of CRP to fully capture the immune-related phenotypes of depression.

As mentioned in the Introduction, most of the published literature uses serum or plasma CRP to identify inflammation in MDD, and some clinical trials with anti-inflammatory medications have recruited participants based on CRP levels. However, this paper finds that the immune-related modifications associated with MDD are wider than those captured by CRP, and that people with depression show mRNA evidence of immune activation even when their CRP

values is <1 mg/L. Specifically, out of the 16 genes analysed, 7 are differentially expressed in all the CRP-based subgroups of individuals with MDD vs. controls, but with no difference between the CRP-based groups, while two other genes are higher in both the low and high CRP groups vs. controls, but not in the intermediate CRP group. Further corroborating our findings, 11 of the 16 genes are differentially regulated even when we compare 'not inflamed' MDD vs. controls, all with CRP <1 mg/L. Results are robust to the effects of age, sex, and BMI as potential confounders, and stringent statistical adjustment for multiple comparisons. In contrast, plasma and cellular immune biomarkers follow a similar pattern of serum CRP.

The panel of immune-related candidate mRNAs in the present study includes proinflammatory genes and genes associated with glucocorticoid resistance. The proinflammatory A2M, IL-1-beta, IL-6, MIF, and TNF-alpha genes are all up-regulated in all MDD subgroups compared with controls. Additionally, in all MDD cases compared with controls we observe a down-regulation of GR and an up-regulation of FKBP5, suggestive of glucocorticoid resistance [44]. These results confirm our previous findings on whole-blood mRNA in MDD subjects compared with controls, in two completely different samples [35, 36] and in another overlapping sample [32], all without stratification for CRP levels. Interestingly, CRP mRNA is the only gene to significantly differ within the CRP-based MDD subgroups, further supporting our results. We additionally found significantly increased CCL2 and STAT1 expression in two MDD subgroups (CRP <1 and >3 mg/L) compared with controls, but *not* in the CRP 1-3 mg/L subgroup, that is, showing no linear relationship with CRP levels. Indeed, mRNA gene expression is not, or only minimally correlated, with serum CRP, except for CRP mRNA. In contrast, classic plasma inflammation-related (protein) cytokines (IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-17A, TNF-alpha) and absolute neutrophils count are different within the CRP-based MDD groups, following the same pattern of CRP, that is, higher in the CRP >3 group (vs. CRP <1 and/or controls).

Taken together, these findings suggest that additional biological processes need to be activated to translate this immune-related mRNA signature (indicating a predisposition to inflammation) into inflammation at a protein and cellular levels. These processes might be related to clinical and sociodemographic factors that regulate serum/plasma CRP, as also discussed in our UK Biobank paper [2], such as age, sex, BMI, smoking, socioeconomic status, childhood stressors

and ill physical health, as well as lifestyle factors such as exercise or diet [2, 45–47]. It is plausible that such clinical and sociodemographic factors might affect translation of the immune signal from mRNA to proteins, rather than regulate gene expression *per se* – as also supported by the low or absent correlations between serum and mRNA levels of the same (IL-6 and TNF-alpha) cytokine. This may occur through regulation of additional biological processes, for example those relevant to vascular alterations (such as atherosclerosis) that are well captured by CRP levels [13]. Indeed, in previous research from our group on individuals with coronary heart disease (CHD), we showed that serum CRP levels are higher in depressed compared with non-depressed individuals [48], and that higher CRP levels in non-depressed CHD patients are associated with the future development of depression [49].

Interestingly, the depression features *per se* do not seem to drive these translational processes: subjects in the MDD CRP >3 group show the highest HAMD-17 scores (mean around 16), but this is numerically very similar to the mean of the CRP <1 mg/L group (around 15), while the lowest mean is in the CRP 1-3 group (around 12); this suggests that higher CRP is not unequivocally associated with more severe or treatment-resistant depression. Indeed, the distribution of non-responders across the three MDD CRP-based groups is not statistically different.

Moreover, it is unclear if the presence of central inflammation could be relevant to these translational processes. Previous studies in depression have found that CRP in serum/plasma is correlated with both CRP in the cerebrospinal fluid (CSF) and CSF pro-inflammatory cytokines [12], and that blood CRP, IL-6 and neutrophils levels are correlated with dysconnectivity of a brain functional network [50]. However, studies using positron emission tomography (PET) of translocator protein (TSPO), a widely used *in vivo* measure of microglial activation, have found no correlation between serum CRP levels and PET binding of TSPO, in both subjects with MDD and controls [51, 52], although TSPO expression may reflect other non-inflammatory conditions and its diagnostic value has been questioned [53].

Of course, our findings should *not* be interpreted as saying that serum CRP is not a clinically-relevant biomarker in depression. We have mentioned above that two studies [16, 17] find an

antidepressant response to infliximab or to minocycline *only* in depressed people with CRP values above 3-5 mg/L. However, it is possible that adding different biomarkers to CRP might identify people who might show an even better response to an anti-inflammatory intervention. For example, we have demonstrated that serum IL-6 is a better predictor of response to minocycline than serum CRP, being able to identify responders in both sexes, while CRP is relevant only in females [54]. Of note, two studies in two different samples of depressed patients [35, 36] find that high mRNA expression of pro-inflammatory genes longitudinally predicts lack of response to antidepressants, irrespectively of their pharmacological classes, while another study finds that high CRP levels only predict lack of response to noradrenergic antidepressants [55]. This again suggests that the subgroup of depressed people identified by high CRP is not completely overlapping with the subgroup identified by high mRNA expression of immune-related genes. Future studies should compare the ability of different biomarkers, including immune-related mRNA expression levels to predict the response to an anti-inflammatory. Moreover, given the complexity of immune processes, we advocate moving away from the concept of a single immunological marker (whether it is CRP or another) able to detect the entire biological modifications involved in the immune-related MDD phenotypes, and instead use a systems immunology approach [56]. Hence, the immune-related phenotypes of MDD should not be considered as singular and homogenous phenotypes (a conceptual flaw that may hinder research in the field) but rather as dynamic processes that encompass a wide range of factors (including transcriptional ones) that we have not fully elucidated yet.

The use of peripheral whole blood for measuring mRNAs of immune-related genes has several advantages, such as the relative ease of drawing and an immediate stabilisation of RNA. Moreover, transcripts measured in peripheral blood have been associated with gene expression in other body compartments, including the brain, further validating its use in psychiatric research [57]. Indeed, peripheral blood may be considered as a ‘sentinel’ tissue, which provides a reliable indication of the overall state of the organism [31]; this is particularly important in conditions such as MDD, where the immune alteration is not clearly located in a precise body compartment. Nevertheless, the biological link between whole-blood mRNAs and proteins levels is not always clear. For example, CRP is primarily produced by the hepatocytes and regulated at a transcriptional level in the liver [58, 59], but CRP mRNA has been detected in other tissues, such as the adipose tissue and in macrophages from atherosclerotic plaques, where it is up-regulated by inflammation [60, 61]. Notably, one study [61] found a correlation

between the mRNA and protein levels in the plaque, but no correlation with levels in the serum, while another study found a correlation between mRNA from tumor tissues (renal cell carcinoma) and plasma CRP levels [62]. Thus, it is possible that CRP mRNA from peripheral tissues can circulate in the whole blood and reach the liver or other cellular compartments where it can be translated.

One limitation of the candidate-mRNA approach is that it is limited to a restricted number of genes, selected based on previous knowledge. Even though the 16 genes explored in this study have been carefully selected based on research from us and other research groups, many others could have been of interest in this context, such as those involved in the TLR4/I κ B/NF- κ B pathway or the NLRP3 inflammasome, which could be relevant to stress- and inflammation-related depression [63]. Moreover, the qPCR technique measures “relative” gene expression and is limited by the use of internal controls (or housekeeping genes). While these are chosen through rigorous experimental validation as transcripts that are stable under the experimental conditions of interest, they might still be affected in other situations; for example, beta-2-microglobulin is altered in neurological and psychiatric conditions, such as multiple sclerosis and schizophrenia [64, 65]. With the use of whole-genome omics techniques, such as mRNA sequencing and spatial transcriptomics, it is possible to identify the expression levels of every single gene in the entire genome, and avoid the limitation of testing candidate genes, as in our study [66], although interestingly a recent paper analysed the transcriptomic profile using RNA sequencing in PBMCs [67], and the authors found no evidence of differential gene expression in MDD cases compared with controls. Of course, our study differs in tissue (whole blood vs. isolated PBMCs in [67]) and in the molecular approach (qPCR vs. sequencing in [67]); however, it is also possible that the rapid stabilisation of the whole-blood mRNA in the present study (compared with the longer interval needed for PBMCs separation) can, at least partially, explain these different findings.

The main limitation of this paper is that we use previously published mRNA data, although all the analyses presented here are new, and have been integrated with unpublished data on plasma cytokines. In addition, the small sample size may limit the generalisation of the present results. Clearly, replication of these findings in an independent and larger sample is paramount. Also, our MDD samples grouped people with mixed treatment state; however, key analyses based

on treatment exposure and response have been published before [32]. Another potential limitation of the present study is its cross-sectional design, as this does not allow us to measure the changes of these immune variables longitudinally and in association with changes in symptoms; it is possible that the overlap between the mRNA and protein signals changes with time, or that one or the other signal is better in predicting future response to antidepressants. In addition, being our sample mainly representative of white ethnic groups, future research should investigate more diverse populations.

In conclusion, the results of the present study confirm immune-related molecular abnormalities in MDD, which are independent of serum CRP levels. These data support the inclusion of different immune markers, in addition to CRP, in future studies, and the comparison between different biomarkers in their ability to identify clinically-relevant characteristics of depressed patients, such as the ability to respond to anti-inflammatory adjuvant treatments. This will ultimately help to identify molecular mechanisms and pathways involved in MDD, which may be targeted in more tailored and personalised strategies.

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Author contribution statement

LS, AC, CF, LT, NM, NC, LP, PJC, JC, NAH, DJ, WCD, VM, ETB and CMP have substantially contributed to the conception or design of the work. All the authors have approved the final version to be published and agreed 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 and resolved. All the authors and the members of the NIMA Consortium (as detailed in the Supplementary Material) have substantially contributed to the acquisition, analysis, or interpretation of data for the work.

Conflict of Interest

The authors declare that they have no known conflict of interest that could have appeared to influence the work reported in this paper. Prof. Pariante have received research funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 853966-2, as part of the EU-PEARL project. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA. Prof. Pariante is also funded by a Senior Investigator award from the National Institute for Health Research (NIHR); the Medical Research Council (grants MR/L014815/1, MR/J002739/1 and MR/N029488/1); the European Commission (EARLYCAUSE grant SC1-BHC-01-2019); the NARSAD; the Psychiatry Research Trust; and the Wellcome Trust (SHAPER, Scaling-up Health-Arts Programme to scale up arts interventions, grant 219425/Z/19/Z). Less than 10% of his support in the last 10 years derives from commercial collaborations, including consultation and speakers fees from Boehringer Ingelheim, Eli Lilly, Compass, Eleusis, GH Research, Lundbeck, and Värde Partners. Dr Mondelli is also funded by MQ: Transforming Mental Health (Grant: MQBF/1 and MQBF/4) and the Medical Research Foundation (Grant: MRF-160-0005-ELP-MONDE).

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Tables

Table 1: Clinical and sociodemographic characteristics

	MDD serum hsCRP <1 mg/L n= 59	MDD serum hsCRP 1-3 mg/L n= 33	MDD serum hsCRP >3 mg/L n= 36	Controls n= 40	Group tests (Statistics and p values) and post-hoc analyses
Age (years) <i>mean (± SD)</i>	n=59 34.02 (±7.70)	n=33 34.30 (±6.96)	n=36 36.97 (±7.24)	n=40 34.18 (±7.50)	H=4.57, p=.206
Sex <i>n (%)</i>	n=59 Females: 38 (64.4%)	n=33 Females: 24 (72.7%)	n=36 Females: 24 (66.7%)	n=40 Females: 26 (65%)	$\chi^2=0.73^*$, p=.866
Ethnicity <i>n (%)</i>	n=59 White: 49 (83.1%)	n=33 White: 28 (84.8%)	n=36 White: 36 (100%)	n=40 White: 38 (95%)	Fisher's exact test** = 9.69, p=0.015
Height (cm) <i>mean (± SD)</i>	n=58 170.42 (±8.55)	n=33 169.68 (±9.66)	n=34 171.94 (±9.82)	n=39 170.73 (±8.09)	F=0.381, p=.767
BMI (Kg/m²) <i>mean (± SD)</i>	n=58 23.62 (±3.07)	n=33 28.68 (±5.87)	n=34 32.91 (±7.64)	n=39 25.38 (±4.92)	H=45.99, p<.001 MDD CRP>3 vs. controls and vs. MDD CRP<1 MDD CRP 1-3 vs. MDD CRP<1
Contraceptive medications (in females) <i>n (%)</i>	n=38 9 (23.7%)	n=24 5 (20.8%)	n=24 7 (29.2%)	n=26 8 (30.8%)	$\chi^2=0.87^*$, p=.832
Exposure and response to antidepressant treatment <i>n (%)</i>	n=59 MDD treatment responsive: 15 (25.4%) MDD treatment non-	n=33 MDD treatment responsive: 12 (36.4%) MDD treatment non-	n=36 MDD treatment responsive: 8 (22.2%) MDD treatment non-	n=40	$\chi^2=5.86^*$, p=.210 Across the MDD subgroups only (no controls)

	responsive: 24 (40.7%) MDD drug-free: 20 (33.9%)	responsive: 17 (51.5%) MDD drug-free: 4 (12.1%)	responsive: 17 (47.2%) MDD drug-free: 11 (30.6%)		
HAMD-17 <i>mean (± SD)</i>	n=59 14.88 (±7.56)	n=33 12.27 (±7.50)	n=36 16.06 (±7.34)	n=40 0.65 (±1.17)	H=6.58, p=.037 MDD CRP>3 vs. MDD CRP 1-3 Across the MDD subgroups only (no controls)
BDI <i>mean (± SD)</i>	n=59 19.97 (±12.32)	n=33 21.55 (±12.86)	n=36 24.19 (±11.97)	n=40 1.50 (±1.87)	H=2.71, p=.259 Across the MDD subgroups only (no controls)
CTQ <i>mean (± SD)</i>	n=59 49.85 (±13.69)	n=33 52.45 (±15.76)	n=36 53.75 (±17.36)	n=40 40.15 (±11.05)	H=1.04, p=.596 Across the MDD subgroups only (no controls)
State anxiety <i>mean (± SD)</i>	n=59 47.44 (±14.51)	n=33 45.21 (±13.05)	n=36 47.89 (±12.13)	n=40 26.70 (±6.16)	H=0.93, p=.627 Across the MDD subgroups only (no controls)
Trait anxiety <i>mean (± SD)</i>	n=59 56.85 (±13.08)	n=33 53.82 (±14.70)	n=36 57.03 (±11.24)	n=40 27.73 (±5.20)	H=0.86, p=.650 Across the MDD subgroups only (no controls)
Serum hsCRP (mg/L) <i>mean (± SD)</i>	n=59 0.52 (±0.22)	n=33 1.66 (±0.65)	n=34 10.25 (±16.59)	n=40 1.11 (±1.47)	H=114.97, p<.001 MDD CRP>3 vs. others MDD CRP 1-3 vs. controls and vs. MDD CRP<1
BDI= Beck Depression Inventory; BMI=body mass index; CRP=C-reactive protein; CTQ= Childhood Trauma Questionnaire; HAMD-17= Hamilton Rating Scale for Depression (17-item); hs=high-sensitivity; IL=					

interleukin. Contraceptive medications include any hormonal contraceptive treatment (oral, intramuscular, transdermal, and vaginal).

SD=standard deviation; F=ANOVA *F* value; H=Kruskal–Wallis *H* value; post-hoc analyses use Bonferroni correction (specific groups reported have statistically different mean scores (larger or smaller) compared with others); χ^2 =Pearson Chi-Square; *0%; **37.5%, expected count less than 5. Significant tests ($p < 0.05$) are in bold.

Table 2: Immune-related mRNA candidate gene expression

	MDD serum hsCRP <1 mg/l n= 59	MDD serum hsCRP 1-3 mg/L n= 33	MDD serum hsCRP >3 mg/L n= 36	Controls n= 40	Group tests ANOVA (Statistics and p values) and post-hoc analyses	Group tests with covariates ANCOVA (Statistics and p values) and post-hoc analyses
A2M <i>Mean expression levels ±SD (95%CI)</i>	n=59 1.24 ±0.18 (1.20-1.29)	n=33 1.25 ±0.16 (1.19-1.30)	n=36 1.25 ±0.21 (1.18-1.32)	n=40 1.02 ±0.23 (0.95-1.10)	F=13.56, p<.001 Controls vs. others	F=13.40, p<.001 Controls vs. others
AQP4 <i>Mean expression levels ±SD (95%CI)</i>	n=59 1.08 ±0.24 (1.02-1.14)	n=31 0.99 ±0.25 (0.90-1.08)	n=35 1.06 ±0.22 (0.99-1.14)	n=38 1.03 ±0.19 (0.97-1.09)	F=1.16, p=.328	F=0.91, p=.437
CRP <i>Mean expression levels ±SD (95%CI)</i>	n=59 1.08 ±0.22 (1.03-1.14)	n=33 1.17 ±0.15 (1.12-1.22)	n=36 1.29 ±0.22 (1.22-1.37)	n=40 1.03 ±0.21 (0.96-1.09)	F=13.56, p<.001 MDD CRP >3 vs. MDD CRP <1; MDD CRP >3 vs. controls; MDD CRP 1-3 vs. controls <i>Trend: MDD CRP >3 vs. MDD CRP 1-3</i>	F=7.46, p<.001 MDD CRP >3 vs. MDD CRP <1; MDD CRP >3 vs. controls; MDD CRP 1-3 vs. controls
CCL2 <i>Mean expression levels ±SD (95%CI)</i>	n=59 1.14 ±0.16 (1.09-1.18)	n=33 1.09 ±0.19 (1.02-1.15)	n=36 1.15 ±0.17 (1.09-1.20)	n=40 1.03 ±0.10 (0.99-1.06)	F=5.31, p=.002 MDD CRP >3 vs. controls; MDD CRP <1 vs. controls	F=5.13, p=.002 MDD CRP >3 vs. controls; MDD CRP <1 vs. controls
CXCL12 <i>Mean expression levels ±SD (95%CI)</i>	n=59 1.01 ±0.20 (0.95-1.06)	n=33 1.02 ±0.15 (0.97-1.08)	n=36 1.08 ±0.20 (1.01-1.14)	n=40 1.06 ±0.25 (0.90-1.14)	F=1.16, p=.328	F=2.82, p=.041 MDD CRP >3 vs.

						MDD CRP<1
FKBP5	n=59	n=33	n=36	n=40	F=19.37, p<.001	F=16.72, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.23 ±0.12 (1.20-1.26)	1.20 ±0.13 (1.16-1.25)	1.26 ±0.14 (1.21-1.31)	1.04 ±0.19 (0.97-1.10)	Controls vs. others	Controls vs. others
GR	n=59	n=33	n=36	n=40	F=16.22, p<.001	F=18.21, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	0.896 ±0.14 (0.86-0.93)	0.899 ±0.12 (0.86-0.94)	0.901 ±0.13 (0.86-0.94)	1.05 ±0.08 (1.02-1.08)	Controls vs. others	Controls vs. others
IL-1-beta	n=59	n=33	n=36	n=40	F=7.87, p<.001	F=6.37, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.23 ±0.18 (1.19-1.28)	1.26 ±0.26 (1.17-1.35)	1.28 ±0.31 (1.18-1.39)	1.07 ±0.09 (1.04-1.10)	Controls vs. others	Controls vs. others
IL-6	n=58	n=33	n=35	n=38	F=16.44, p<.001	F=15.40, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.28 ±0.20 (1.23-1.33)	1.27 ±0.19 (1.21-1.34)	1.25 ±0.14 (1.20-1.30)	1.06 ±0.07 (1.03-1.08)	Controls vs. others	Controls vs. others
ISG15	n=59	n=33	n=36	n=40	F=0.27, p=.849	F=0.11, p=.956
<i>Mean expression levels ±SD (95%CI)</i>	1.01 ±0.25 (0.95-1.08)	0.99 ±0.31 (0.88-1.10)	1.03 ±0.26 (0.94-1.12)	0.99 ±0.24 (0.91-1.06)		
MIF	n=59	n=33	n=36	n=40	F=19.11, p<.001	F=19.75, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.236 ±0.20 (1.18-1.29)	1.242 ±0.17 (1.18-1.30)	1.243 ±0.15 (1.19-1.29)	1.00 ±0.14 (0.96-1.05)	Controls vs. others	Controls vs. others
P2RX7	n=59	n=33	n=36	n=40	F=1.07, p=.364	F=1.09, p=.357
<i>Mean expression levels ±SD (95%CI)</i>	1.15 ±0.36 (1.05-1.24)	1.10 ±0.29 (1.00-1.20)	1.12 ±0.32 (1.01-1.23)	1.03 ±0.26 (0.95-1.12)		
SGK1	n=59	n=33	n=36	n=40	F=3.22, p=.024	F=4.03, p=.009
<i>Mean expression levels ±SD (95%CI)</i>	1.10 ±0.13 (1.07-1.14)	1.06 ±0.11 (1.02-1.10)	1.13 ±0.14 (1.08-1.18)	1.06 ±0.08 (1.03-1.09)	<i>Trend: MDD CRP>3 vs. MDD CRP 1-3; MDD CRP>3 vs. controls</i>	MDD CRP>3 vs. MDD CRP 1-3; MDD CRP>3 vs. controls

STAT1	n=59	n=31	n=36	n=40	F=4.78, p=.003	F=4.69, p=.004
<i>Mean expression levels ±SD (95%CI)</i>	1.17 ±0.18 (1.12-1.21)	1.14 ±0.18 (1.08-1.21)	1.20 ±0.15 (1.15-1.25)	1.06 ±0.18 (1.00-1.11)	MDD CRP>3 vs. controls; MDD CRP<1 vs. controls	MDD CRP>3 vs. controls; MDD CRP<1 vs. controls
TNF-alpha	n=59	n=33	n=36	n=40	F=32.25, p<.001	F=32.06, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.28 ±0.11 (1.25-1.31)	1.30 ±0.13 (1.25-1.34)	1.31 ±0.11 (1.28-1.35)	1.06 ±0.17 (1.00-1.11)	Controls vs. others	Controls vs. others
USP18	n=59	n=33	n=36	n=40	F=1.01, p=.392	F=0.67, p=.569
<i>Mean expression levels ±SD (95%CI)</i>	1.01 ±0.20 (0.96-1.06)	1.00 ±0.22 (0.92-1.08)	1.07 ±0.24 (0.99-1.15)	0.99 ±0.25 (0.91-1.07)		

A2M= alpha-2-macroglobulin; AQP4= aquaporin 4; CCL2= C-C Motif Chemokine Ligand 2; CXCL12= C-X-C Motif Chemokine Ligand 12; CRP=C-reactive protein; FKBP5= FK506 binding protein 51; GR= glucocorticoid receptor; IL= interleukin; ISG15= interferon-stimulated gene 15; MIF=macrophage inhibiting factor; P2RX7= P2X purinoceptor 7; SGK1= serum/glucocorticoid regulated kinase 1; STAT1= signal transducer and activator of transcription 1; TNF= tumor necrosis factor; USP18= ubiquitin specific peptidase 18.

CI=confidence interval; SD=standard deviation; F=ANOVA and ANCOVA F value, post-hoc analyses use Bonferroni correction (specific groups reported have statistically different mean scores (larger or smaller) compared with others). Covariates are sex, age, and BMI. Significant tests ($p < 0.05$) are in bold; trends ($0.05 < p < 0.09$) in italics.

Table 3: Comparison of plasma protein levels across subgroups

	MDD serum hsCRP <1 mg/L n= 59	MDD serum hsCRP 1-3 mg/L n= 33	MDD serum hsCRP >3 mg/L n= 36	Controls n= 40	Group tests (Statistics and p values) and post-hoc analyses
Plasma IL-6 (pg/mL) <i>mean ± SD (IQR)</i>	n=59 0.48 ±0.29 (0.41)	n=32 0.94 ±0.76 (0.69)	n=36 1.72 ±1.63 (1.27)	n=40 0.56 ±0.40 (0.31)	Wald Chi- Square=78.86, p<.001 MDD CRP>3 vs. others MDD CRP 1-3 vs. MDD CRP<1
Plasma IL-7 (pg/mL) <i>mean ± SD (IQR)</i>	n=59 2.60 ±1.48 (1.55)	n=33 3.96 ±3.78 (3.58)	n=36 4.44 ±3.28 (3.35)	n=39 3.66 ±2.96 (1.53)	Wald Chi- Square=17.71, p<.001 MDD CRP>3 vs. MDD CRP<1 and MDD CRP 1-3 vs. MDD CRP<1
Plasma IL-8 (pg/mL) <i>mean ± SD (IQR)</i>	n=59 2.92 ±1.38 (1.75)	n=33 3.14 ±2.10 (1.41)	n=36 4.01 ±1.95 (2.77)	n=40 4.01 ±3.02 (2.33)	Wald Chi- Square=13.83, p=.003 MDD CRP>3 vs. MDD CRP<1 and controls vs. MDD CRP<1
Plasma IL-10 (pg/mL) <i>mean ± SD (IQR)</i>	n=59 0.24 ±0.12 (0.13)	n=30 0.28 ±0.21 (0.27)	n=36 0.39 ±0.50 (0.25)	n=39 0.27 ±0.14 (0.18)	Wald Chi- Square=17.02, p<.001 MDD CRP>3 vs. controls and vs. MDD CRP<1
Plasma IL- 12/IL-23p40 (pg/mL) <i>mean ± SD (IQR)</i>	n=59 152.0 ±109.9 (88.04)	n=33 143.8 ±66.48 (82.26)	n=36 175.6 ±91.62 (119.7)	n=40 128.4 ±58.46 (88.85)	Wald Chi- Square=8.00, p=.046 MDD CRP>3 vs. controls
Plasma IL-15 (pg/mL) <i>mean ± SD</i>	n=59 2.86 ±0.63	n=33 2.83 ±0.59	n=36 3.0 ±0.79	n=40 2.88 ±0.63	F=0.46, p=.711

Plasma IL-16 (pg/mL) <i>mean ± SD (IQR)</i>	n=59 154.6 ±47.19 (60.72)	n=33 153.0 ±54.38 (79.71)	n=36 195.1 ±85.28 (77.86)	n=39 153.5 ±55.28 (78.35)	Wald Chi-Square=14.76, p=.002 MDD CRP>3 vs. others
Plasma IL-17A (pg/mL) <i>mean ± SD (IQR)</i>	n=56 3.26 ±3.68 (1.85)	n=32 2.18 ±1.53 (1.33)	n=35 3.77 ±4.25 (2.81)	n=39 2.36 ±1.36 (1.34)	Wald Chi-Square=14.69, p=.002 MDD CRP>3 vs. controls and vs. MDD CRP 1-3 <i>Trend: MDD CRP 1-3 vs. MDD CRP<1</i>
Plasma IFN gamma (pg/mL) <i>mean ± SD (IQR)</i>	n=59 4.70 ±5.41 (2.76)	n=32 4.83 ±6.28 (2.83)	n=34 9.64 ±16.82 (3.29)	n=40 3.43 ±2.24 (1.71)	Wald Chi-Square=33.60, p<.001 MDD CRP>3 vs. others
Plasma TNF alpha (pg/mL) <i>mean ± SD (IQR)</i>	n=59 2.14 ±0.66 (0.68)	n=33 2.30 ±0.80 (0.92)	n=36 2.66 ±0.62 (0.89)	n=40 2.27 ±0.63 (2.39)	Wald Chi-Square=14.44, p=.002 MDD CRP>3 vs. MDD CRP<1 <i>Trend: MDD CRP>3 vs. controls</i>
Plasma VEGF-A (pg/mL) <i>mean ± SD (IQR)</i>	n=59 69.42 ±58.41 (36.73)	n=33 77.23 ±56.59 (37.33)	n=36 80.28 ±38.82 (67.22)	n=40 86.74 ±79.11 (60.78)	Wald Chi-Square=3.50, p=.321
White cell count (x10⁹/L) <i>mean ± SD (IQR)</i>	n=59 6.24 ±1.53 (2.0)	n=33 6.62 ±1.50 (2.5)	n=34 7.79 ±2.49 (3.0)	n=40 5.94 ±1.38 (2.2)	Wald Chi-Square=0.44, p=.803
Lymphocytes absolute (x10³/μL) <i>mean ± SD (IQR)</i>	n=59 1.93 ±0.53 (0.81)	n=33 1.99 ±0.51 (1.57)	n=34 2.22 ±0.73 (0.89)	n=40 1.85 ±0.42 (0.57)	<i>H=6.74, p=.081</i> <i>Trend: MDD CRP>3 vs. controls</i>

Neutrophils absolute (x10³/μL) <i>mean ± SD (IQR)</i>	n=59 3.67 ±1.21 (1.73)	n=33 4.03 ±1.34 (2.14)	n=34 4.87 ±1.88 (1.75)	n=40 3.51 ±1.17 (1.36)	H=16.54, p<.001 MDD CRP>3 vs. controls and vs. MDD CRP<1
Basophils absolute (x10³/μL) <i>mean ± SD (IQR)</i>	n=59 0.03 ±0.02 (0.01)	n=33 0.03 ±0.02 (0.02)	n=34 0.03 ±0.02 (0.03)	n=40 0.02 ±0.01 (0.02)	Wald Chi-Square=3.74, p=.291
Eosinophils absolute (x10³/μL) <i>mean ± SD (IQR)</i>	n=59 0.20 ±0.26 (0.16)	n=33 0.17 ±0.11 (0.20)	n=34 0.23 ±0.21 (0.19)	n=40 0.15 ±0.09 (0.12)	Wald Chi-Square=5.92, p=.115
Monocytes absolute (x10³/μL) <i>mean (± SD)</i>	n=59 0.42 (±0.15)	n=33 0.40 (±0.14)	n=34 0.45 (±0.13)	n=40 0.40 (±0.14)	F=0.81, p=.438
<p>IFN=interferon; IL= interleukin; TNF= tumor necrosis factor; VEGF= vascular endothelial growth factor.</p> <p>IQR=interquartile range; SD=standard deviation; F=ANOVA F value; H=Kruskal–Wallis <i>H</i> value; post-hoc analyses use Bonferroni correction (specific groups reported have statistically different mean scores (larger or smaller) compared with others). Significant tests (p < 0.05) are in bold; trends (0.05<p<0.09) in italics.</p>					

Table 4: Clinical and sociodemographic characteristics of MDD cases and controls with serum hsCRP levels <1 mg/L

	MDD cases n=59	Controls n=26	Group tests
Age, years <i>Mean (±SD)</i>	n=59 34.02 (±7.70)	n=26 33.31 (±7.13)	U=779.5, p=.905
Sex <i>n (%)</i>	n=59 Females: 38 (64.4%)	n=26 Females: 17 (64.4%)	$\chi^2=0.008^*$, p=.931
Ethnicity <i>n (%)</i>	n=59 White: 49 (83.1%)	n=26 White: 24 (92.3%)	$\chi^2=1.275^{**}$, p=.259
Height (cm) <i>mean (±SD)</i>	n=58 170.42 (±8.55)	n=26 171.19 (±8.84)	t=0.380, p=.705
Weight (Kg) <i>mean (±SD)</i>	n=58 69.07 (±13.58)	n=26 69.72 (±15.52)	U=788.5, p=.738
BMI (Kg/m²) <i>mean (±SD)</i>	n=58 23.62 (±3.07)	n=26 23.65 (±4.15)	t=0.039, p=.969
Serum hsCRP (mg/L) <i>mean (±SD)</i>	n=59 0.52 (±0.22)	n=26 0.43 (±0.20)	U=949.5, p=.078
Plasma IL-6 (pg/mL) <i>mean (±SD)</i>	n=59 0.48 (±0.29)	n=26 0.51 (±0.43)	U=705.0, p=.554
Plasma IL-7 (pg/mL) <i>mean (±SD)</i>	n=59 2.60 (±1.48)	n=26 3.63 (±2.99)	U=645.0, p=.245
Plasma IL-8 (pg/mL) <i>mean (±SD)</i>	n=59 2.92 (±1.38)	n=26 3.67 (±2.41)	U=656.0, p=.290
Plasma IL-10 (pg/mL) <i>mean (±SD)</i>	n=59 0.24 (±0.12)	n=25 0.29 (±0.15)	U=581.0, p=.126
Plasma IL-12/IL-23p40 (pg/mL) <i>mean (±SD)</i>	n=59 152.0 (±109.9)	n=26 127.5 (±60.61)	U=844.0, p=.463
Plasma IL-15 (pg/mL)	n=59 2.86 (±0.63)	n=26 2.80 (±0.69)	U=834.0, p=.523

<i>mean (± SD)</i>			
Plasma IL-16 (pg/mL) <i>mean (± SD)</i>	n=59 154.6 (±47.19)	n=26 155.39 (±54.74)	U=807.0, p=.703
Plasma IL-17 (pg/mL) <i>mean (± SD)</i>	n=56 3.26 (±3.68)	n=26 2.55 (±1.35)	U=651.0, p=.443
Plasma IFN gamma (pg/mL) <i>mean (± SD)</i>	n=59 4.70 (±5.41)	n=26 3.51 (±2.29)	U=726.0, p=.696
Plasma TNF alpha (pg/mL) <i>mean (±SD)</i>	n=59 2.14 (±0.66)	n=26 2.18 (±0.56)	t=0.304, p=.762
Plasma VEGF (pg/mL) <i>mean (± SD)</i>	n=59 69.42 (±58.41)	n=26 93.40 (±91.61)	U=880.0, p=.281
White cell count (x10⁹/L) <i>mean (±SD)</i>	n=59 6.24 (±1.53)	n=26 5.84 (±1.45)	U=880.0, p=.281
Lymphocytes absolute (x10³/μL) <i>mean (± SD)</i>	n=59 1.93 (±0.53)	n=26 1.90 (±0.46)	U=790.5, p=.823
Neutrophils absolute (x10³/μL) <i>mean (± SD)</i>	n=59 3.67 (±1.21)	n=26 3.35 (±1.27)	U=918.0, p=.150
Basophils absolute (x10³/μL) <i>mean (± SD)</i>	n=59 0.03 (±0.02)	n=26 0.02 (±0.01)	U=846.0, p=.781
Eosinophils absolute (x10³/μL) <i>mean (± SD)</i>	n=59 0.20 (±0.26)	n=26 0.14 (±0.07)	U=808.5, p=.554
Monocytes absolute (x10³/μL) <i>mean (± SD)</i>	n=59 0.42 (±0.15)	n=26 0.41 (±0.15)	U=747.0, p=.849
BMI=body mass index; CRP=C-reactive protein; Hs=high-sensitivity; IL= interleukin; TNF= tumor necrosis factor; VEGF= vascular endothelial growth factor.			

SD= standard deviation; t=Student's t value. U=Mann-Whitney *U* value. *0%; **25%, expected count less than 5. Significant tests ($p < 0.05$) are in bold.

Table 5: Immune-related mRNA candidate gene expression in MDD cases and controls with serum hsCRP levels <1 mg/L

	MDD cases n=59	Controls n=26	Group tests (Statistics and p values)	Group tests with covariates ANCOVA (Statistics and p values)
A2M <i>Mean expression levels (± SD)</i>	n=59 1.24 (±0.18)	n=26 1.03 (±0.25)	t=4.60, p<.001	F=21.75, p<.001
AQP4 <i>Mean expression levels (± SD)</i>	n=59 1.08 (±0.24)	n=25 1.06 (±0.20)	t=0.47, p=.642	F=1.88, p=.666
CRP <i>Mean expression levels (± SD)</i>	n=59 1.08 (±0.22)	n=26 1.02 (±0.22)	t=1.30, p=.198	F=1.68, p=.199
CCL2 <i>Mean expression levels (± SD)</i>	n=59 1.14 (±0.16)	n=26 1.04 (±0.11)	t=-2.88, p=.005	F=7.96, p=.006
CXCL12 <i>Mean expression levels (± SD)</i>	n=59 1.14 (±0.16)	n=26 1.21 (±0.25)	t=-2.27, p=.026	F=5.46, p=.022
FKBP5 <i>Mean expression levels (± SD)</i>	n=59 1.23 (±0.12)	n=26 1.01 (±0.21)	t=6.21, p<.001	F=36.63, p<.001
GR <i>Mean expression levels (± SD)</i>	n=59 0.90 (±0.14)	n=26 1.06 (±0.09)	t=-5.51, p<.001	F=30.11, p<.001
IL-1-beta <i>Mean expression levels (± SD)</i>	n=59 1.23 (±0.18)	n=26 1.07 (±0.09)	t=4.32, p<.001	F=18.92, p<.001
IL-6 <i>Mean expression levels (± SD)</i>	n=58 1.28 (±0.20)	n=26 1.05 (±0.07)	t=5.64, p<.001	F=31.29, p<.001
ISG15 <i>Mean expression levels (± SD)</i>	n=59 1.01 (±0.25)	n=26 1.06 (±0.11)	t=-0.64, p=.523	F=0.57, p=.452
MIF <i>Mean expression levels (± SD)</i>	n=59 1.24 (±0.20)	n=26 1.04 (±0.14)	t=4.40, p<.001	F=18.81, p<.001
P2RX7 <i>Mean expression levels (± SD)</i>	n=59 1.15 (±0.36)	n=26 1.11(±0.27)	t=0.45, p=.654	F=0.18, p=.673
SGK1 <i>Mean expression levels (± SD)</i>	n=59 1.10 (±0.13)	n=26 1.04 (±0.08)	t=2.21, p=.030	F=5.02, p=.028

STAT1 <i>Mean expression levels</i> (± <i>SD</i>)	n=59 1.17 (±0.18)	n=26 1.05 (±0.19)	t=2.65, p=.010	F=6.92, p=.010
TNF-alpha <i>Mean expression levels</i> (± <i>SD</i>)	n=59 1.28 (±0.11)	n=26 1.03 (±0.12)	t=9.10, p<.001	F=81.15, p<.001
USP18 <i>Mean expression levels</i> (± <i>SD</i>)	n=59 1.01 (±0.20)	n=26 1.02 (±0.27)	t=-0.25, p=.802	F=0.11, p=.738

A2M= alpha-2-macroglobulin; AQP4= aquaporin 4; CCL2= C-C Motif Chemokine Ligand 2; CXCL12= C-X-C Motif Chemokine Ligand 12; CRP=C-reactive protein; FKBP5= FK506 binding protein 51; GR= glucocorticoid receptor; IL= interleukin; ISG15= interferon-stimulated gene 15; MIF=macrophage inhibiting factor; P2RX7= P2X purinoceptor 7; SGK1= serum/glucocorticoid regulated kinase 1; STAT1= signal transducer and activator of transcription 1; TNF= tumor necrosis factor; USP18= ubiquitin specific peptidase 18.

SD=standard deviation; t=Student's t value. U= Mann-Whitney U value. F=ANCOVA F value. Covariates are sex, age, and BMI. Significant tests (p < 0.05) are in bold.

Higher immune-related gene expression in major depression is independent of CRP levels: results from the BIODP study

Supplementary Material

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Supplementary Materials and Methods

Study design and sample characteristics

Data were obtained from the multicentre, non-interventional, case-control, Biomarkers of Depression (BIODEP) study [1]. Participants were recruited and assessed in 5 clinical centres in the UK: Brighton, Cambridge, Glasgow, London (King's College London), and Oxford. The study was conducted as 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) and conducted according to the Declaration of Helsinki. All participants were aged 25-50 years and provided written informed consent (<https://www.neuroimmunology.org.uk/biodep/>). Individuals with a lifetime history of bipolar disorder or non-affective psychosis were excluded. The healthy controls had no current or past diagnosis of any major psychiatric disorder as defined by DSM-5, and no history of antidepressant drug treatment for any indication. Other exclusion criteria applied to both the healthy control and the MDD participant samples were any lifetime medical disorder or current use of medications likely to compromise interpretation of CRP, alcohol or substance use disorder in the preceding 12 months, and current pregnancy or breast feeding.

We included all participants for whom serum CRP and gene expression data were available, in total 168 individuals. We divided our sample in four sub-groups, based on depression status and CRP levels. We identified 128 MDD cases and 40 healthy controls. The diagnosis of MDD was assessed through the Structured Clinical Interview for DSM-5 (SCID-5) [2]. The severity of the current depressive episode was evaluated with the clinician-administered 17-item Hamilton Rating Scale for Depression (HAM-D) [3]. Additional psychopathological scales were the self-reported Beck Depression Inventory (BDI) [4], the Childhood Trauma Questionnaire (CTQ) [5], and the Spielberger State-Trait Anxiety Rating scale [6]. MDD cases included antidepressant-medicated treatment responders, treatment non-responders and currently depressed but unmedicated individuals. Responders had a HAMD-17 score <7 (*not currently depressed*), while on antidepressant medication(s) at standard dosage for at least 6 weeks, non-responders had a HAMD-17 score >13 (*currently depressed*) while on antidepressant medication(s) at standard therapeutic dose for at least 6 weeks, and drug-free had a HAMD-17 score >17 (*currently depressed*), had not been treated with any antidepressant

medication for at least 6 weeks, and had at least one historical failure to a different antidepressant.

Biomarkers

Other immune biomarkers

Plasma immune-related proteins were measured in peripheral blood collected into plasma preparation tubes (BD Cat #362799). Tubes were centrifuged at 1600g for 15 minutes at room temperature and plasma supernatant was frozen at -80°C. Samples were thawed, and markers assayed in duplicate using the Pro-Inflammatory Panel 1 (K15049D) and Cytokine Panel 1 (K150150D) V-PLEX 10-spot immunoassay kits from Meso Scale Discovery, as per the manufacturer's instructions (MSD; Rockville, MD, USA). Analysed biomarkers, with their lower limits of detectability (LLODs, in pg/mL) were: interleukin (IL)-1-alpha (0.09), IL-1-beta (0.05), IL-2 (0.09), IL-4 (0.02), IL-5 (0.14), IL-6 (0.06), IL-7 (0.12), IL-8 (0.07), IL-10 (0.04), IL-12p70 (0.11), IL-12/IL-23p40 (0.33), IL-13 (0.24), IL-15 (0.15), IL-16 (2.83), IL-17A (0.31), interferon (IFN)-gamma (0.37), tumor necrosis factor (TNF)-alpha (0.04), TNF-beta (0.08), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.16), and vascular endothelial growth factor (VEGF)-A (1.12). We included only samples with immune plasma concentrations equal or above the LLOD. We excluded biomarkers with more than 20% of samples below the LLOD (IL-1-beta, IL-2, IL-4, IL-12p70, IL-13, GM-CSF, IL-15), and further excluded those with assay coefficients of variability (CVs) equal or above 30% (IL-1-alpha and TNF-beta). We therefore presented data on IL-6, IL-7, IL-8, IL-10, IL12/IL-23p40, IL-15, IL-16, IL-17A, IFN-gamma, TNF-alpha, VEGF-A. CVs were below 15% for all cytokines, except for IL-10 (19.4%) and IL-17A (24.8%). Four participants had a very high plasma level (equivalent to more than ten standard deviations from the mean) of IL-6, IL-7, IL-10, and IL-16, respectively; these were therefore considered as "extreme" outliers and removed from the analyses.

Absolute counts of total white blood cells, lymphocytes, neutrophils, monocytes, eosinophils, and basophils were measured using Laser Particle Counting on a Coulter Hematology (LH750/DxH800).

Supplementary Results

Immune-related genes are differentially expressed in MDD cases compared with controls, independently of CRP values

We performed ANCOVAs to control for the effects of age, sex, and BMI on the differences in mRNAs expression. Only the analyses of two mRNAs were affected, with non-significant results becoming statistically significant: the difference in CXCL12, which was numerically higher in the MDD CRP >3 group vs. MDD CRP <1 (from $p=.328$ to $p=.041$, Table 2); and the post-hoc trend difference in SGK1 expression between the CRP >3 group vs. both CRP 1-3 and controls (from $p=.078$ and $p=.069$ to $p=.028$ and $p=.034$, respectively). Significance levels were also unaffected by the number of transcripts ($n=16$) analysed (all $p<.05$ survived the FDR-adjusted q threshold of .05).

We further analysed the female participants and controlled for the effect of contraceptive medications. Twenty-nine out of 112 females were taking any hormonal contraceptive medication (Table 1). After the inclusion of this covariate in the ANCOVAs, between group comparisons were only minimally affected, with no effects on the statistical significance of the findings (Supplementary Table 1).

Lastly, we looked at correlations between serum CRP levels and mRNA expression of immune-related candidate genes. In our entire sample, serum CRP levels were positively correlated with CRP mRNA ($\rho=0.40$, $p<.001$), and, with lower correlation coefficients, with TNF-alpha ($\rho=0.27$, $p<.001$), FKBP5 ($\rho=0.19$, $p=.014$) and IL-6 ($\rho=0.16$, $p=.048$) mRNAs. Notably, of these four correlations, only the correlations between serum CRP and CRP and TNF-alpha mRNAs remained significant after the FDR correction ($q<.05$). The transcripts of the other 12 immune-related genes were not significantly correlated with serum CRP levels (Supplementary Table 2).

We also looked at correlations between mRNA and protein levels for CRP, IL-6 and TNF-alpha in the subsample of participants (depressed patients and controls) with CRP <1 mg/L, and

found only one significant (and weak) correlation, between serum CRP and IL-6 mRNA ($\rho=0.22$, $p=.043$).

Serum CRP levels predict plasma and cellular immune biomarkers of depression

Comparisons between the MDD CRP-based groups and controls for plasma cytokines levels and white cell counts are presented in Table 3. In contrast with mRNAs, most of these variables (IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-16, IL-17-A, IFN-gamma, TNF-alpha, and neutrophils) were differentially regulated between groups, reflecting the gradient of CRP values, that is, with values that were significantly higher for the CRP >3 group vs. CRP <1 and/or controls, with differences sometimes reaching +200-300%.

This effect was particularly evident for plasma IL-6 levels (higher in the CRP >3 group vs. all the others, and in the CRP 1-3 vs. the <1 group), IFN-gamma (higher in the CRP >3 group vs. all the others), IL-7 (higher in the CRP >3 and 1-3 groups vs. the CRP <1) and IL-17A (higher in the CRP >3 group vs. the 1-3 group and controls). The CRP >3 group also had the highest levels of IL-10 and absolute neutrophils (vs. both CRP <1 and controls), TNF-alpha (vs. CRP <1), IL-8 (curiously with equal values to those measured in controls, both significant vs. CRP <1), and IL-12/IL-23p40 (vs. controls only). We found no differences between groups in the levels of IL-15, IFN-gamma, VEGF-A, white cell count, and absolute basophils, eosinophils, and monocytes values.

Again, differently from mRNAs, CRP levels were also correlated with most of these immune biomarkers using Spearman's correlations, including IL-6 ($\rho=0.64$, $p<.001$), IL-7 ($\rho=0.19$, $p=.012$), IL-8 ($\rho=0.18$, $p=.017$), IL-16 ($\rho=0.24$, $p=.002$), IFN-gamma ($\rho=0.17$, $p=.031$), TNF-alpha ($\rho=0.32$, $p<.001$), white cells ($\rho=0.31$, $p<.001$), lymphocytes ($\rho=0.16$, $p=.041$), and neutrophils counts ($\rho=0.33$, $p<.001$) (Supplementary Table 3).

Sensitivity analyses excluding individuals with serum CRP levels ≥ 10 mg/L demonstrate that findings are not driven by these cases alone

We have performed additional sensitivity analyses on our findings excluding the (n=7) subjects with CRP ≥ 10 mg/L. There is no effect on the significance of mRNA expression results, further corroborating our main findings (Supplementary Table 4). Group analyses confirmed a statistically significant difference in the expression of 7 immune-related genes in MDD cases vs. controls, with no differences between the CRP-based MDD sub-groups (<1, 1-3, >3 mg/L). Results are analogous to those obtained in the whole sample. In details, A2M, FKBP5, IL-1-beta, IL-6, MIF, and TNF-alpha were up-regulated, while GR was down-regulated, in all the three subgroups of CRP-based MDD cases compared with controls. CCL2 and STAT1 were significantly up-regulated in the MDD CRP <1 and >3 groups compared with controls, but not in the CRP 1-3 group. No differences in gene expression were found within the MDD groups except for CRP gene (higher in CRP >3 vs. CRP <1, in CRP 1-3 vs. CRP <1, and in CRP >3 vs. controls).

Supplementary Tables

Supplementary Table 1: Immune-related mRNA candidate gene expression in female participants, ANOVA and ANCOVA with the use of contraceptive medications as an additional covariate to age and BMI

	MDD serum hsCRP <1 mg/l n= 38	MDD serum hsCRP 1-3 mg/L n= 24	MDD serum hsCRP >3 mg/L n= 24	Controls n= 26	Group tests ANOVA (Statistics and p values) and post-hoc analyses	Group tests with covariates ANCOVA (Statistics and p values) and post-hoc analyses
A2M <i>Mean expression levels ±SD (95%CI)</i>	n=38 1.24 ±0.20 (1.18-1.31)	n=24 1.27 ±0.16 (1.21-1.34)	n=24 1.22 ±0.24 (1.12-1.32)	n=26 1.07 ±0.22 (0.98-1.15)	F=5.44, p=.002 Controls vs. others	F=4.77, p=.004 MDD CRP 1-3 vs. controls; MDD CRP<1 vs. controls <i>Trend: MDD CRP>3 vs. controls</i>
AQP4 <i>Mean expression levels ±SD (95%CI)</i>	n=38 1.11 ±0.24 (1.03-1.19)	n=22 0.96 ±0.28 (0.84-1.09)	n=24 1.10 ±0.23 (1.00-1.20)	n=25 1.00 ±0.20 (0.92-1.08)	<i>F=2.53, p=.061</i>	F=2.17, p=.097
CRP <i>Mean expression levels ±SD (95%CI)</i>	n=38 1.11 ±0.24 (1.03-1.18)	n=24 1.16 ±0.15 (1.10-1.22)	n=24 1.31 ±0.25 (1.20-1.42)	n=26 1.02 ±0.22 (0.93-1.11)	F=7.45, p<.001 MDD CRP >3 vs. MDD CRP <1; MDD CRP>3 vs. controls	F=4.15, p=.008 MDD CRP>3 vs. controls <i>Trend: MDD CRP>3 vs. MDD CRP<1</i>
CCL2 <i>Mean expression levels ±SD (95%CI)</i>	n=38 1.12 ±0.16 (1.07-1.18)	n=24 1.07 (0.98-1.15)	n=24 1.20 ±0.13 (1.14-1.25)	n=26 1.03 ±0.11 (0.99-1.07)	F=5.40, p=.002 MDD CRP>3 vs. controls; MDD CRP>3 vs. MDD CRP 1-3	F=6.29, p<.001 MDD CRP>3 vs. controls; MDD CRP>3 vs. MDD CRP 1-3

CXCL12 Mean expression levels \pm SD (95%CI)	n=38 1.02 \pm 0.19 (0.95-1.08)	n=24 1.01 \pm 0.16 (0.95-1.08)	n=24 1.11 \pm 0.21 (1.02-1.20)	n=26 1.08 \pm 0.27 (0.97-1.19)	F=1.41, p=.245	F=2.54, p=.061 Trend: MDD CRP>3 vs. MDD CRP<1
FKBP5 Mean expression levels \pm SD (95%CI)	n=38 1.21 \pm 0.11 (1.18-1.25)	n=24 1.20 \pm 0.13 (1.15-1.26)	n=24 1.29 \pm 0.14 (1.23-1.35)	n=26 1.03 \pm 0.24 (0.93-1.12)	F=12.25, p<.001 Controls vs. others	F=10.09, p<.001 Controls vs. others
GR Mean expression levels \pm SD (95%CI)	n=38 0.90 \pm 0.14 (0.85-0.94)	n=24 0.91 \pm 0.12 (0.86-0.96)	n=24 0.90 \pm 0.11 (0.85-0.95)	n=26 1.03 \pm 0.08 (1.00-1.07)	F=8.50, p<.001 Controls vs. others	F=10.82, p<.001 Controls vs. others
IL-1-beta Mean expression levels \pm SD (95%CI)	n=38 1.27 \pm 0.17 (1.22-1.33)	n=24 1.26 \pm 0.28 (1.14-1.38)	n=24 1.29 \pm 0.22 (1.19-1.38)	n=26 1.06 \pm 0.09 (1.02-1.10)	F=7.96, p<.001 Controls vs. others	F=6.56, p<.001 MDD CRP 1-3 vs. controls; MDD CRP<1 vs. controls
IL-6 Mean expression levels \pm SD (95%CI)	n=37 1.26 \pm 0.21 (1.19-1.33)	n=24 1.24 \pm 0.18 (1.17-1.32)	n=23 1.28 \pm 0.11 (1.23-1.32)	n=24 1.07 \pm 0.07 (1.04-1.10)	F=8.62, p<.001 Controls vs. others	F=7.10, p<.001 Controls vs. others
ISG15 Mean expression levels \pm SD (95%CI)	n=38 1.02 \pm 0.28 (0.93-1.12)	n=24 0.98 \pm 0.31 (0.85-1.10)	n=24 1.08 (0.97- 1.18)	n=26 0.96 \pm 0.21 (0.87-1.04)	F=1.06, p=.369	F=0.58, p=.632
MIF Mean expression levels \pm SD (95%CI)	n=38 1.25 \pm 0.23 (1.18-1.33)	n=24 1.23 \pm 0.17 (1.16-1.30)	n=24 1.23 \pm 0.16 (1.17-1.30)	n=26 1.00 \pm 0.16 (0.94-1.06)	F=11.18, p<.001 Controls vs. others	F=11.71, p<.001 Controls vs. others
P2RX7 Mean expression levels \pm SD (95%CI)	n=38 1.19 \pm 0.40 (1.06-1.33)	n=24 1.08 \pm 0.29 (0.96-1.20)	n=24 1.15 \pm 0.36 (1.00-1.30)	n=26 1.00 \pm 0.24 (0.91-1.10)	F=1.82, p=.147	F=1.91, p=.133
SGK1 Mean expression levels \pm SD (95%CI)	n=38 1.11 \pm 0.12 (1.08-1.15)	n=24 1.07 \pm 0.11 (1.02-1.11)	n=24 1.15 \pm 0.14 (1.09-1.21)	n=26 1.08 \pm 0.09 (1.05-1.11)	F=2.51, p=.062	F=2.97, p=.036 Trend: MDD CRP>3 vs.

						<i>MDD CRP 1-3</i>
STAT1 <i>Mean expression levels ±SD (95%CI)</i>	n=38 1.17 ±0.21 (1.10-1.24)	n=22 1.15 ±0.19 (1.07-1.24)	n=24 1.20 ±0.16 (1.14-1.27)	n=26 1.03 ±0.20 (0.95-1.11)	F=4.00, p=.010 MDD CRP>3 vs. controls; MDD CRP<1 vs. controls	F=3.95, p=.010 MDD CRP>3 vs. controls <i>Trend: MDD CRP<1 vs. controls</i>
TNF-alpha <i>Mean expression levels ±SD (95%CI)</i>	n=38 1.27 ±0.11 (1.24-1.31)	n=24 1.28 ±0.12 (1.23-1.33)	n=24 1.33 ±0.11 (1.28-1.37)	n=26 1.09 ±0.19 (1.01-1.17)	F=15.31, p<.001 Controls vs. others	F=13.58, p<.001 Controls vs. others
USP18 <i>Mean expression levels ±SD (95%CI)</i>	n=38 0.97 ±0.20 (0.90-1.03)	n=24 1.00 ±0.24 (0.90-1.10)	n=24 1.09 ±0.25 (0.99-1.20)	n=26 1.03 ±0.26 (0.93-1.14)	F=1.56, p=.204	F=0.41, p=.749
<p>A2M= alpha-2-macroglobulin; AQP4= aquaporin 4; CCL2= C-C Motif Chemokine Ligand 2; CXCL12= C-X-C Motif Chemokine Ligand 12; CRP=C-reactive protein; FKBP5= FK506 binding protein 51; GR= glucocorticoid receptor; IL= interleukin; ISG15= interferon-stimulated gene 15; MIF=macrophage inhibiting factor; P2RX7= P2X purinoceptor 7; SGK1= serum/glucocorticoid regulated kinase 1; STAT1= signal transducer and activator of transcription 1; TNF= tumor necrosis factor; USP18= ubiquitin specific peptidase 18.</p> <p>SD=standard deviation (95% CI=confidence interval); F=ANOVA and ANCOVA F value, post-hoc analyses use Bonferroni correction (specific groups reported have statistically different mean scores (larger or smaller) compared with others). Covariates are age, BMI, and the use of any hormonal contraceptive medication. Significant tests (p < 0.05) are in bold; trends (0.05<p<0.09) in italics.</p>						

Supplementary Table 2: Correlations between serum hsCRP and immune-related candidate gene mRNA expression levels

A2M	$\rho=0.08$, $p=.287$
AQP4	$\rho=-0.05$, $p=.531$
CRP	$\rho=0.40$, $p<.001$
CCL2	$\rho=0.04$, $p=.573$
CXCL12	$\rho=-0.04$, $p=.626$
FKBP5	$\rho=0.19$, $p=.014$
IL-1-beta	$\rho=0.11$, $p=.140$
IL-6	$\rho=0.16$, $p=.048$
ISG15	$\rho=-0.07$, $p=.361$
MIF	$\rho=0.14$, $p=.068$
GR	$\rho=-0.11$, $p=.150$
P2RX7	$\rho=-0.06$, $p=.461$
SGK1	$\rho=0.02$, $p=.759$
STAT1	$\rho=0.11$, $p=.176$
TNF-alpha	$\rho=0.27$, $p<.001$
USP18	$\rho=0.13$, $p=.097$
<p>A2M= alpha-2-macroglobulin; AQP4= aquaporin 4; CCL2= C-C Motif Chemokine Ligand 2; CXCL12= C-X-C Motif Chemokine Ligand 12; CRP=C-reactive protein; FKBP5= FK506 binding protein 51; GR= glucocorticoid receptor; IL= interleukin; ISG15= interferon-stimulated gene 15; MIF=macrophage inhibiting factor; P2RX7= P2X purinoceptor 7; SGK1= serum/glucocorticoid regulated kinase 1; STAT1= signal transducer and activator of transcription 1; TNF= tumor necrosis factor; USP18= ubiquitin specific peptidase 18.</p> <p>ρ = Spearman's correlation coefficient. Significant tests ($p < 0.05$) are in bold.</p>	

Supplementary Table 3: Correlations between serum hsCRP and other plasma and cellular immune biomarkers

Plasma IL-6 (pg/mL)	$\rho=0.64$, $p<.001$
Plasma IL-7 (pg/mL)	$\rho=0.19$, $p=.012$
Plasma IL-8 (pg/mL)	$\rho=0.18$, $p=.017$
Plasma IL-10 (pg/mL)	$\rho=0.12$, $p=.127$
Plasma IL-12/IL-23p40 (pg/mL)	$\rho=0.14$, $p=.062$
Plasma IL-15 (pg/mL)	$\rho=0.01$, $p=.881$
Plasma IL-16 (pg/mL)	$\rho=0.24$, $p=.002$
Plasma IL-17A (pg/mL)	$\rho=0.08$, $p=.331$
Plasma IFN gamma (pg/mL)	$\rho=0.17$, $p=.031$
Plasma TNF alpha (pg/mL)	$\rho=0.32$, $p<.001$
Plasma VEGF-A (pg/mL)	$\rho=0.14$, $p=.067$
White cell count ($\times 10^9/L$)	$\rho=0.31$, $p<.001$
Lymphocytes absolute ($\times 10^3/\mu L$)	$\rho=0.16$, $p=.041$
Neutrophils absolute ($\times 10^3/\mu L$)	$\rho=0.33$, $p<.001$
Basophils absolute ($\times 10^3/\mu L$)	$\rho=0.10$, $p=.201$
Eosinophils absolute ($\times 10^3/\mu L$)	$\rho=0.12$, $p=.131$
Monocytes absolute ($\times 10^3/\mu L$)	$\rho=0.11$, $p=.150$
IFN=interferon; IL= interleukin; TNF= tumor necrosis factor; VEGF= vascular endothelial growth factor.	
ρ = Spearman's correlation coefficient. Significant tests ($p < 0.05$) are in bold.	

Supplementary Table 4: Immune-related mRNA candidate gene expression excluding (n=7) individuals with serum CRP levels ≥ 10 mg/L

	MDD serum hsCRP <1 mg/l n= 59	MDD serum hsCRP 1-3 mg/L n= 33	MDD serum hsCRP >3 (and <10) mg/L n= 29	Controls n= 40	Group tests ANOVA (Statistics and p values) and post-hoc analyses	Group tests with covariates ANCOVA (Statistics and p values) and post-hoc analyses
A2M <i>Mean expression levels \pmSD (95%CI)</i>	n=59 1.24 \pm 0.18 (1.20-1.29)	n=33 1.25 \pm 0.16 (1.19-1.30)	n=29 1.25 \pm 0.21 (1.17-1.33)	n=40 1.02 \pm 0.23 (0.95-1.10)	F=13.64, p<.001 Controls vs. others	F=13.19, p<.001 Controls vs. others
AQP4 <i>Mean expression levels \pmSD (95%CI)</i>	n=59 1.08 \pm 0.24 (1.02-1.14)	n=31 0.99 \pm 0.25 (0.90-1.08)	n=28 1.04 \pm 0.21 (0.96-1.13)	n=38 1.03 \pm 0.19 (0.97-1.09)	F=1.12, p=.344	F=0.66, p=.579
CRP <i>Mean expression levels \pmSD (95%CI)</i>	n=59 1.08 \pm 0.22 (1.03-1.14)	n=33 1.17 \pm 0.15 (1.12-1.22)	n=29 1.24 \pm 0.18 (1.17-1.31)	n=40 1.03 \pm 0.21 (0.96-1.09)	F=7.73, p<.001 MDD CRP >3 vs. MDD CRP <1; MDD CRP >3 vs. controls; MDD CRP 1-3 vs. controls	F=4.85, p=.003 MDD CRP >3 vs. controls; MDD CRP 1-3 vs. controls <i>Trend: MDD CRP >3 vs. MDD CRP <1</i>
CCL2 <i>Mean expression levels \pmSD (95%CI)</i>	n=59 1.14 \pm 0.16 (1.09-1.18)	n=33 1.09 \pm 0.19 (1.02-1.15)	n=29 1.14 \pm 0.16 (1.07-1.20)	n=40 1.03 \pm 0.10 (0.99-1.06)	F=4.77, p=.003 MDD CRP >3 vs. controls; MDD CRP <1 vs. controls	F=4.71, p=.004 MDD CRP >3 vs. controls; MDD CRP <1 vs. controls
CXCL12 <i>Mean expression levels \pmSD (95%CI)</i>	n=59 1.01 \pm 0.20 (0.95-1.06)	n=33 1.02 \pm 0.15 (0.97-1.08)	n=29 1.08 \pm 0.21 (1.00-1.15)	n=40 1.06 \pm 0.25 (0.90-1.14)	F=1.08, p=.361	F=2.83, p=.040 <i>Trend: MDD CRP >3 vs. MDD CRP <1</i>

FKBP5	n=59	n=33	n=29	n=40	F=17.75, p<.001	F=15.86, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.23 ±0.12 (1.20-1.26)	1.20 ±0.13 (1.16-1.25)	1.25 ±0.14 (1.20-1.30)	1.04 ±0.19 (0.97-1.10)	Controls vs. others	Controls vs. others
GR	n=59	n=33	n=29	n=40	F=15.18, p<.001	F=17.02, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	0.896 ±0.14 (0.86-0.93)	0.899 ±0.12 (0.86-0.94)	0.91 ±0.14 (0.86-0.96)	1.05 ±0.08 (1.02-1.08)	Controls vs. others	Controls vs. others
IL-1-beta	n=59	n=33	n=29	n=40	F=7.24, p<.001	F=6.34, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.23 ±0.18 (1.19-1.28)	1.26 ±0.26 (1.17-1.35)	1.27 ±0.34 (1.14-1.40)	1.07 ±0.09 (1.04-1.10)	Controls vs. others	Controls vs. others
IL-6	n=58	n=33	n=28	n=38	F=16.35, p<.001	F=15.37, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.28 ±0.20 (1.23-1.33)	1.27 ±0.19 (1.21-1.34)	1.25 ±0.14 (1.20-1.31)	1.06 ±0.07 (1.03-1.08)	Controls vs. others	Controls vs. others
ISG15	n=59	n=33	n=29	n=40	F=0.15, p=.928	F=0.08, p=.971
<i>Mean expression levels ±SD (95%CI)</i>	1.01 ±0.25 (0.95-1.08)	0.99 ±0.31 (0.88-1.10)	1.02 ±0.27 (0.91-1.12)	0.99 ±0.24 (0.91-1.06)		
MIF	n=59	n=33	n=29	n=40	F=18.33, p<.001	F=18.73, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.236 ±0.20 (1.18-1.29)	1.242 ±0.17 (1.18-1.30)	1.25 ±0.17 (1.18-1.31)	1.00 ±0.14 (0.96-1.05)	Controls vs. others	Controls vs. others
P2RX7	n=59	n=33	n=29	n=40	F=1.00, p=.393	F=0.89, p=.446
<i>Mean expression levels ±SD (95%CI)</i>	1.15 ±0.36 (1.05-1.24)	1.10 ±0.29 (1.00-1.20)	1.10 ±0.36 (0.97-1.24)	1.03 ±0.26 (0.95-1.12)		
SGK1	n=59	n=33	n=29	n=40	F=2.59, p=.055	F=3.26, p=.023
<i>Mean expression levels ±SD (95%CI)</i>	1.10 ±0.13 (1.07-1.14)	1.06 ±0.11 (1.02-1.10)	1.12 ±0.15 (1.07-1.18)	1.06 ±0.08 (1.03-1.09)		<i>Trend: MDD CRP>3 vs. MDD CRP 1-3; MDD CRP>3 vs. controls</i>

STAT1	n=59	n=31	n=29	n=40	F=4.95, p=.003	F=4.91, p=.003
<i>Mean expression levels ±SD (95%CI)</i>	1.17 ±0.18 (1.12-1.21)	1.14 ±0.18 (1.08-1.21)	1.21 ±0.13 (1.16-1.26)	1.06 ±0.18 (1.00-1.11)	MDD CRP>3 vs. controls; MDD CRP<1 vs. controls	MDD CRP>3 vs. controls; MDD CRP<1 vs. controls
TNF-alpha	n=59	n=33	n=29	n=40	F=31.40, p<.001	F=30.95, p<.001
<i>Mean expression levels ±SD (95%CI)</i>	1.28 ±0.11 (1.25-1.31)	1.30 ±0.13 (1.25-1.34)	1.32 ±0.12 (1.28-1.37)	1.06 ±0.17 (1.00-1.11)	Controls vs. others	Controls vs. others
USP18	n=59	n=33	n=29	n=40	F=0.84, p=.473	F=0.47, p=.701
<i>Mean expression levels ±SD (95%CI)</i>	1.01 ±0.20 (0.96-1.06)	1.00 ±0.22 (0.92-1.08)	1.07 ±0.24 (0.98-1.17)	0.99 ±0.25 (0.91-1.07)		

A2M= alpha-2-macroglobulin; AQP4= aquaporin 4; CCL2= C-C Motif Chemokine Ligand 2; CXCL12= C-X-C Motif Chemokine Ligand 12; CRP=C-reactive protein; FKBP5= FK506 binding protein 51; GR= glucocorticoid receptor; IL= interleukin; ISG15= interferon-stimulated gene 15; MIF=macrophage inhibiting factor; P2RX7= P2X purinoceptor 7; SGK1= serum/glucocorticoid regulated kinase 1; STAT1= signal transducer and activator of transcription 1; TNF= tumor necrosis factor; USP18= ubiquitin specific peptidase 18.

CI=confidence interval; SD=standard deviation; F=ANOVA and ANCOVA F value, post-hoc analyses use Bonferroni correction (specific groups reported have statistically different mean scores (larger or smaller) compared with others). Covariates are sex, age, and BMI. Significant tests ($p < 0.05$) are in bold; trends ($0.05 < p < 0.09$) in italics.

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