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Increased Levels of Plasma Tumor Necrosis Factor-α Mediate Schizophrenia Symptom Dimensions and Neurocognitive Impairments and Are Inversely Associated with Natural IgM and Paraoxonase 1 Activity

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Abstract

Accumulating evidence suggests that TNF-α-mediated immune-neurotoxicity contributes to cognitive impairments and the overall severity of schizophrenia (OSOS). There are no data whether peripheral IL-6 and IL-4 may affect the phenome of schizophrenia above and beyond the effects of TNF-α and whether those cytokines are regulated by lowered natural IgM to malondialdehyde (MDA) and paraoxonase 1 enzyme activity. We assessed the aforementioned biomarkers in schizophrenia patients with (n=40) and without (n=40) deficit schizophrenia and 40 healthy controls. Deficit schizophrenia was best predicted by a combination of increased IL-6 and PON1 status (QQ genotype and lowered CMPAase activity) and lowered IgM to MDA. Partial Least Squares bootstrapping shows that 41.0% of the variance in negative symptoms, psychosis, hostility, excitation, mannerism, psychomotor retardation, and formal thought disorders was explained by increased TNF-α and PON1 status (QQ genotype and lowered CMPAase activity), lowered IL-4 and IgM to MDA as well as male sex and lowered education. We found that 47.9% of the variance in verbal fluency, word list memory, true recall, Mini-Mental State Examination, and executive functions was predicted by increased TNF-α and lowered IL-4, IgM to MDA and education. In addition, both TNF-α and IL-4 levels were significantly associated with lowered IgM to MDA, while TNF-α was correlated with PON1 status. These data provide evidence that the symptomatic (both the deficit subtype and OSOS) and cognitive impairments in schizophrenia are to a large extent mediated by the effects of immune-mediated neurotoxicity as well as lowered regulation by the innate immune system.

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Introduction

The first author of this paper published in 1995 the first comprehensive immune theory of schizophrenia, i.e. the macrophage-T lymphocyte theory, which summarized that repetitive immune hits including maternal infections activate peripheral immune pathways including M1 macrophagic (increased interleukin-6, IL-6) and T helper (Th)-1 (increased IL-2 signaling) cytokines, which play a pivotal role in the pathophysiology of schizophrenia through aberrations in neurodevelopmental trajectories, neuroinflammation, nitro-oxidative damage and activation of indoleamine 2,3-dioxygenase (IDO) with an elevated production of neurotoxic tryptophan catabolites (TRYCATs) [1].

Since 1995, evidence has accumulated showing that the neurotoxic effects of activated neuro-immune and neuro-inflammatory pathways including M1 (IL-1, IL-6, tumor necrosis factor-α or TNF-α), Th-1 (IL-2, interferon-γ or IFN-γ) and Th-2 cytokines/chemokines (e.g. CCL11 or eotaxin) and noxious TRYCATs (e.g. picolinic and xanthurenic acid) may affect neurocognitive functions indicating disorders in specific brain areas, which may also mediate overall severity of schizophrenia (OSOS) as well as severity of symptom domains, including PHEM (psychosis, hostility, excitation, and mannerism) and negative symptoms, psychomotor retardation and formal thought disorders [2-10]. It is noteworthy that the cumulative effects of increased TNF-α signaling [increased levels of TNF-α and its two soluble receptors, namely the soluble TNF receptor 1 (sTNFR1) and sTNFR2], IL-1β signaling (increased IL-1β and sIL-1RA) and chemokine signaling (CCL2 and CCL11) appear to significantly predict OSOS and shape deficit schizophrenia as a qualitatively distinct class relative to healthy controls [11,12].

Moreover, new data show that schizophrenia is not only accompanied by activation of the immune-inflammatory response system (IRS), but also by activation of compensatory immune-

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regulatory mechanisms including an activation of Th-2 (indicated by an increase in peripheral blood IL-4 and IL-5) and T regulatory (Treg, indicated by increased interleukin-10) immune cells as well as increased levels of some acute phase reactants (e.g. haptoglobin) and TRYCATs, which have immunosuppressive effects [3]. The aggregation of all pathways and factors that downregulate the primary IRS was conceptualized as the compensatory immune-regulatory system (CIRS) [3,13,14]. As such, the older macrophage-lymphocyte theory of schizophrenia [1] is superseded by the IRS-CIRS theory [3], which considers that the different schizophrenia phenotypes including first-episode psychosis and acute phase, treatment-resistant, chronic and deficit schizophrenia are characterized by an intercorrelated activation of IRS and CIRS pathways whereby the dampening effects of the latter are insufficient to normalize the IRS resulting in a net peripheral immune-inflammatory response.

Furthermore, deficits in the CIRS also appear to contribute to OSOS as well as to different symptom domains of schizophrenia and the deficit syndrome [15-18]. Firstly, while M1, Th-1 and Th-17 cytokines are significantly increased in first-episode psychosis, the levels of the immune-regulatory soluble cytokine receptors including sTNFR2, sIL-1 receptor antagonist (sIL-1RA) and sIL-2R are not increased indicating that lowered CIRS effects are associated with activated immune pathways [15]. Secondly, deficit schizophrenia is characterized by impairments in innate immune resilience as indicated by lowered natural IgM directed against oxidative specific epitopes (OSEs) and lowered levels of paraoxonase 1 (PON1) which both protect against immune activation, nitro-oxidative stress, and increased bacterial translocation [16, 18]. Natural IgM antibodies directed to malondialdehyde (MDA) are produced by innate-like B1 and marginal zone B cells and are an important component of the innate immune system with housekeeping, anti-inflammatory, anti-oxidative and immune-regulatory functions [19-24]. PON1 is an antioxidant,

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anti-inflammatory and detoxifying enzyme, which is considered to be part of the innate immune system [18,25]. PON1 activity is also lowered in patients with first-episode psychosis and is significantly associated with increased plasma levels of IL-4, IL-6, and IL-10, but not TNF-α [26]. Nevertheless, there are no data to indicate whether TNF-α levels would be elevated in patients with deficit schizophrenia versus those with non-deficit schizophrenia and controls, and whether IL-6 (another M1 macrophagic cytokine) or IL-4 (a Th-2 immune-regulatory cytokine) would shape the phenomenology of schizophrenia above and beyond the effects of TNF-α. In addition, lowered IgM to MDA and PON1 activity could also be associated with increased levels of TNF-α, IL-6, and IL-4.

Hence, the current study aimed to examine whether TNF-α, IL-6 and IL-4 predict deficit schizophrenia, OSOS, and its symptoms domains as well as neurocognitive deficits and whether the levels of these cytokines are inversely associated with IgM to MDA and PON1 activity.

Methods

Participants

This study enrolled 120 participants, namely 40 healthy controls and 80 schizophrenia outpatients. We recruited Thai nationals of both sexes and aged 18-65 years. The healthy volunteers were recruited by word of mouth from the same catchment area of the schizophrenia patients in Bangkok, Thailand. The schizophrenia patients were all admitted as outpatients to the Department of Psychiatry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All patients met diagnostic criteria of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-TR). Moreover, patients were in a stable phase of illness and did not show psychotic flare-ups for one year. Patients were divided into two groups,

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those with and without deficit schizophrenia as defined using the criteria of the Schedule for deficit schizophrenia [27]. We excluded patients and controls with a current or lifetime diagnosis of a) major medical illness including diabetes type 1, psoriasis, inflammatory bowel disease, rheumatoid arthritis, chronic obstructive pulmonary disease; b) neurodegenerative and neuroinflammatory disease including stroke, Parkinson's disease, multiple sclerosis; c) use of therapeutic doses of antioxidants (e.g. 1.2 g NAC/day) or ω3-polyunsaturated fatty acids (e.g. 1.2 g EPA/day) 6 months prior to the study; d) current or lifetime use of immunomodulatory drugs including glucocorticoids. Controls were excluded for any current or lifetime diagnosis of axis I DSM-IV-TR disorders. Patients were excluded for a current or lifetime diagnosis of axis I disorders other than schizophrenia including major depressive episode, generalized anxiety disorder, bipolar disorder, autism spectrum disorders, schizoaffective disorder, and substance use disorders (except tobacco use disorder).

All participants, as well as the guardians of patients (parents or other close family members) provided written informed consent to take part in the study. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No 298/57), which is in compliance with the International Guideline for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization on Good Clinical Practice (ICH-GCP).

Clinical assessments

Socio-demographic and clinical data were collected from all participants. A semi-structured interview was employed to collect socio-demographic data, onset and duration of

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illness, the family history of schizophrenia, and medical and psychiatric history. The Mini-International Neuropsychiatric Interview (M.I.N.I.) was employed in a validated Thai translation [28] to make the diagnosis of schizophrenia. The SDS was employed to make the diagnosis of primary deficit schizophrenia [27] and patients without deficit syndrome according to SDS criteria were classified as non-deficit schizophrenia. Severity of negative symptoms was measured with a) the total score on the SDS scale; b) the total score on the Scale for the Assessment of Negative Symptoms (SANS) [29]; and c) the total score on the negative subscale of the Positive and Negative Syndrome Scale (PANSS) [30]. Moreover, we employed some items of the Brief Psychiatric Rating Scale [31] and the Hamilton Depression Rating Scale [32] to compute composite scores refecting formal thought disorders (FTD), psychomotor retardation (PMR) and the PHEM symptoms psychosis, hostility, excitation and mannerism [7,8,10, 11,16]. In all controls and patients we also assessed the CERAD-Neuropsychological tests [33] to assess memory impairments and the overall neuropsychological deficit in schizophrenia and CANTAB tests [34] in order to estimate executive functions. We assessed the following CANTAB tests: the one touch stockings of Cambridge probability solved on first choice, which assesses spatial planning; spatial working memory between errors and strategy, which probe self-monitoring ability, maintenance of data in the visuospatial sketchpad, executive working memory ability and task strategy used by the central executive. Executive functions were estimated by extracting the first principal component of these three CANTAB test scores explaining 81.7% of the variance [7,8]. We also assessed the Mini-Mental State Examination (MMSE) which offers a measurement of overall neurocognitive functioning by probing orientation, naming, memory, concentration, and constructional praxis. In the current study we used three CERAD tests, namely a) Verbal Fluency Test (VFT) to probe cognitive flexibility, fluency, language, verbal productivity and thus semantic

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memory; b) Word List Memory (WLM) to assess verbal episodic memory, learning ability and working memory for verbal information; and c) Word List Recall, True Recall (True Recall) in order to assess verbal episodic memory–recall. We employed DSM-IV-TR criteria to make the diagnosis of tobacco use disorder (TUD). Body mass index (BMI) was computed as body weight (kg) / length (m^2).

Assays

Blood was sampled at 8:00 a.m. after an overnight fast for the assay of cytokines, IgM levels directed to MDA and PON1 status. Blood was immediately centrifuged and serum was aliquoted and frozen at −80 °C until thawed for the assays of cytokines and other biomarkers. TNF-α, IL-4 and IL-6 (R&D Systems, Inc, Minneapolis, MN, USA) were measured using the Bio-Plex® 200 System (Bio-Rad Laboratories, Inc.). In brief: 50 µl of serum (1:2 dilution in calibrator diluent) was mixed with 50 µl of microparticle cocktail containing the cytokines (R&D Systems, Inc, Minneapolis, MN, USA) per well of a 96-well plate provided by manufacturer and incubated for 2 hours at room temperature on a shaker at 800 rpm. The mixture was then washed 3 times with wash buffer and 50 µl diluted Biotin Antibody cocktail was added and then incubated for 1 hour. Wells were washed 3 times before another 50 µl of diluted Streptavidin-PE was added and further incubated for 30 minutes. Finally, wells were washed 3 times and 100 µl of wash buffer was added and left at room temperature for 2 minutes before being read with Bio-Plex® 200 System (Bio-Rad Laboratories, Inc.). The intra-assay CV values were <7.0%. The sensitivity of the assays were 0.38 pg/mL for IL-6, 0.62 pg/mL for TNF-α, and 0.07 pg/mL for IL-4. All levels of the three cytokines were greater than the sensitivity levels of the assays and, consequently, no left-censoring of data was employed. IgM concentrations against conjugated MDA were measured using an

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indirect enzyme-linked immunosorbent assay (ELISA) [16]. The methods to link MDA to delipidated bovine serum albumin was explained previously [35,36]. The intra-assay coefficient of this assays was < 6%. The PON1 status was measured as described previously. In brief "a blood sample of 10 mL was withdrawn from all individuals. To stratify individuals in the functional genotypes of the PON1 Q192R polymorphism (QQ, QR, and RR), the substrates used were phenyl acetate (PA, Sigma, USA) under high salt condition and 4-(chloromethyl)phenyl acetate (CMPA, Sigma, USA), which is an alternative to the use of the toxic paraoxon. PON1 activities were determined by the rate of hydrolysis of CMPA (CMPAase, which is influenced by the PON1 Q192R polymorphism) as well as by the rate hydrolysis of phenyl acetate under low salt condition (AREase, which is less influenced by the PON1 Q192R polymorphism). Analysis were conducted in a microplate reader (EnSpire, Perkin Elmer, USA) [18,25,26,37]. We computed two different PON1 status indices namely PON1 index1, which combines the additive model of the Q192R genotypes (with QQ=2, QR=1 and RR=0) with CMPAase activity (whereby an increased index indicates the QQ genotype and accompanying lowered CMPAse activity); and PON1 index2, which combines the enzymatic activities of CMPAase and AREase independently from the genotype [18].

Statistical analysis

We used analysis of variance (ANOVA) to assess differences in scale variables between categories and analysis of contingency tables (χ^2-tests) to assess relationships between categorical variables. Boxplots indicating minimum, maximum, Q1, Q3, and median values, out-values (shown as circles) and far-out or extreme values (shown as stars) were used to display the results of cytokine measurements. Univariate GLM analysis was used to examine the associations

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between biomarkers and diagnosis while controlling for the effects of sex, age, BMI and the drug status. We used protected pair-wise comparisons among treatment means to assess differences in demographic and clinical data and biomarkers among controls and patients with and without deficit schizophrenia. P-corrections for false discovery rate (FDR) were employed to adjust for multiple comparisons [38]. Multiple regression analysis was used to delineate the significant biomarkers (entered as explanatory variables) predicting severity of symptom domains and neurocognitive test scores, while allowing for the intervening effects of age, sex and education. Results of regression analyses were always checked for multicollinearity using the collinearity diagnostics tolerance and VIF. Automatic stepwise binary logistic regression analysis was used to delineate the significant predictors (biomarkers) of deficit schizophrenia (the dependent variable with controls + non-deficit schizophrenia as reference group) while allowing for the effects of age, sex and education. Results of regression analyses were bootstrapped (5000 samples) and we show the bootstrapped results if these would differ from the non-bootstrapped results. All tests were two-tailed and a p-value of 0.05 was used for statistical significance. We used IBM SPSS 25 windows version to analyze the data.

We employed Partial Least Squares (PLS) path structural equation modeling [39] to assess causal paths from the immune biomarkers (entered as input variables) to the clinical and cognitive parts of the phenome of schizophrenia. The biomarkers were entered as single indicators predicting latent vectors (LV) extracted from all 9 symptomatic (named OSOS) and all 5 neurocognitive (named cognitive LV) scores (see Figure 1 for variables). Age, sex and education were entered as additional input indicators predicting OSOS and cognitive LVs. We performed complete and consistent PLS analysis using 5000 bootstrap samples only when the inner and outer model complied with prespecified quality data, namely a) adequacy of model fit SRMR < 0.080; b) the

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OSOS and cognitive LVs showed good composite reliability > 0.7, Cronbach's alpha > 0.7, rho_A > 0.8 with an AVE (average variance extracted > 0.500); c) all loadings on the LV are > 0.600 (at $p<0.001$); and d) the construct crossvalidated communalities or redundancies are adequate [39]. Consequently, path coefficients and p-values are computed using 5000 bootstraps. Moreover, we conducted Confirmatory Tetrad analysis to evaluate whether the reflective models were not misspecified.

Results

Socio-demographic data

Table 1 shows the demographic data of the patients (divided into those with and without deficit SCZ) and controls. There were no significant differences in age, TUD and education between the three study groups. There were somewhat more males in both schizophrenia groups than in controls. The BMI was lower in deficit schizophrenia than in non-deficit schizophrenia. The unemployment rate increased from controls → non-deficit schizophrenia → deficit schizophrenia. **Electronic Supplementary File (ESF) Figure 1** shows the mean z scores of the symptom domains and neurocognitive functions. The results of the comparisons have been presented somewhere else [10,17,18]. All symptom scores and cognitive tests scores are significantly different between the three study groups with the highest symptoms scores and lowest neurocognitive test scores in deficit schizophrenia (increased loadings on the executive principal component denote lowered functions).

Biomarkers in deficit schizophrenia

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Table 1 shows the measurement of the 6 biomarkers examined in the present study. **Electronic Supplementary File (ESF)** shows the boxplots of the three cytokine levels. The three cytokine levels were analyzed using univariate GLM analysis with age, sex, BMI, use of risperidone (n=32), clozapine (n=10), haloperidol (n=7), perphenazine (n=20), antidepressants (n=25), mood stabilizers (n=11) and hypnotics (n=26) as covariates. **ESF Figure 2** shows the box plots of the raw TNF-α values and Table 1 shows the adjusted values (z scores after Ln transformation) after covarying for the different background variables. We found that TNF-α was significantly higher in deficit schizophrenia than in the two other study groups, and higher in non-deficit schizophrenia than in controls while the effect size (partial eta squared) of diagnosis was 0.229. **ESF Figure 3** shows that 2 patients with deficit schizophrenia and 1 with non-deficit schizophrenia showed extreme IL-6 values although after Ln transformation these cases could no longer be identified as outliers. IL-6 was significantly higher in deficit schizophrenia than in patients with non-deficit schizophrenia and normal controls. **ESF Figure 4** shows the box plot of the raw IL-4 measurements, while Table 1 shows the adjusted values in z scores. IL-4 levels were significantly lower in non-deficit schizophrenia as compared with deficit schizophrenia. The IgM levels directed against MDA were significantly lower in deficit schizophrenia as compared with controls and patients with non-deficit schizophrenia. The PON1 index1 was significantly higher in deficit schizophrenia than in the two other groups (indicating a higher frequency of the QQ genotype and lowered PON1 CMPAase activity).

In this regression analysis, we found a significant and positive association between IL-6 and BMI ($t=+4.07$, $p<0.001$), but not with the other cytokines. Therefore, we have used the residualized IL-6 values in subsequent analyses which have the effects of BMI partialled out. None of the cytokines was associated with age, sex or TUD. There was a significant suppressant effect

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of risperidone on IL-6 (F=5.08, df=1/105, p=0.026; partial eta squared: 0.046) and TNF-α (F=6.06, df=1/110, p=0.015; partial eta squared: 0.053) and of perphenazine on TNF-α (F=7.56, df=1/110, p=0.007; partial eta squared: 0.064). In the total study sample, there were significant correlations between TNF-α and IL-6 (r=0.381, p<0.001) and IL-4 (r=0.307, p<0.001) but not between IL-6 and IL-4.

Table 2 shows the results of binary logistic regression analysis with deficit schizophrenia as dependent variable (and controls + non-deficit schizophrenia as reference group). The first regression shows that TNF-α was significantly associated with deficit schizophrenia ($\chi 2$=18.00, df=1, p<0.001; Nagelkerke=0.196; sensitivity=40.0% and specificity=92.3%). Regression #2 shows that also the residualized IL-6 values (after regression on BMI) were significantly associated with deficit schizophrenia ($\chi 2$=6.62, df=1, p=0.010; Nagelkerke=0.079). In the third regression, we examined whether a combination of different biomarkers could predict deficit schizophrenia and found that the latter was best predicted by IL-6, IgM to MDA and PON1 index1 ($\chi 2$=43.17, df=3, p<0.001; Nagelkerke=0.455, sensitivity=63.9% and specificity=91.8%; AUC ROC=0.856 ±0.041, p<0.001).

Prediction of severity of illness by biomarkers

In order to examine the associations between biomarkers and severity of illness (OSOS) and the different symptoms subdomains we have performed multiple regression analyses with symptoms as dependent variables and all 6 biomarkers as explanatory variables while also allowing for the effects of age, sex and education. **Table 3**, regression #1 shows that 35.9% of the variance in the OSOS index was explained by the regression on TNF-α, PON1 index 1 (both positively), PON1 index 2 and education (both inversely) and male sex. Regression #2 shows that

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34.0% of the variance in the total SANS score was explained by the regression on TNF-α, PON1 index1 (both positively), IgM to MDA and education (both inversely) and male sex.

We found that 29.3% of the variance in the negative subscore of the PANSS may be explained by TNF-α, PON1 index1 (both positively), IL-4 (inversely) and male sex. Hostility and mannerism were to a large extent (29.3% - 21.8%) predicted by TNF-α (positively), IL-4 and education (both inversely) and male sex. Regression #6 shows that 26.1% of the variance in excitation was explained by TNF-α (positively), and IL-4, IgM to MDA and education (all three inversely). The last regression shows that 34.2% of the variance in PMR is explained by TNF-α and PON1 index1 (both positively), and IgM to MDA (inversely).

Prediction of neurocognitive deficits by biomarkers

In order to examine the associations between biomarkers and severity of neurocognitive deficits we performed automatic stepwise multiple regression analyses with cognitive test scores as dependent variables and all 6 biomarkers as explanatory variables while allowing for the effects of background variables. **Table 4**, regression #1 shows that 31.9% of the variance in WLM was explained by education (positively) and TNF-α and male sex (both inversely). VFT was best predicted by education (positively) and the residualized IL-6 values (after regression on BMI) which together explained 19.9% of the variance. True Recall was best predicted by education (positively) and TNF-α and male sex (both inversely), explaining 31.5% of its variance. We found that 41.9% of the variance in MMSE was explained by the regression on education (positively), and residualized IL-6 and male sex (both inversely).

Prediction of cytokines by markers of the innate immune system

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In order to examine whether the concentrations of the cytokines are associated with the innate immune biomarkers we conducted multiple regression analysis with the TNF-α, IL-6 or IL-4 as dependent variables and PON1 index1 and index2 and IgM to MDA as explanatory variables while allowing for the effects of age, sex and BMI. **Table 5** shows that 17.6% of the variance in TNF-α concentrations were explained by IgM to MDA and PON1 index2 (both inversely). We found that 20.9% of the variance in IL-4 was explained by IgM to MDA and age (both inversely). There were no associations between IL-6 and any of the innate biomarkers.

Results of PLS analysis

Figure 1 shows the results of PLS analysis with the OSOS and cognitive LVs as output variables and the significant biomarkers as well as sex and education as input variables. In addition, IgM to MDA predicted IL-4 and TNF-α values. The model fit was adequate with SRMR=0.043. The construct reliabilities of the OSOS and cognitive LVs were very good with Cronbach's alpha > 0.869, composite reliability > 0.905, rho_A > 0.877 and AVE > 0.656. All loadings on the OSOS LV were > 0.723 and the cognitive LV > 0.734. The cognitive and OSOS LVs showed adequate discriminatory validity (heterotrait-monotrait ratio <0.771). Blindfolding showed adequate construct crossvalidated redundancies for the OSOS LV (0.281) and cognitive LV (0.279), while confirmatory tetrad analysis showed that both LVs were not misspecified as reflective models. Consistent PLS analysis with 5000 bootstraps showed that 41.0% of the variance in the OSOS LV was explained by the regression on TNF-α, PON1 index1 and male sex (all positively associated) and IL-4, IgM MDA and education (all three inversely associated); 47.9% of the variance in the cognitive LV was explained by the regression on TNF-α (positively associated) and IL-4, IgM MDA and education (inversely associated). There were significant direct effects of IgM MDA on

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both TNF-α and IL-4 (inversely associated). There were significant indirect effects of IgM MDA on the OSOS LV mediated by TNF-α (t=-2.52, p=0.012) and IL-4 (t=+2.02, p=0.044). There were significant direct effects of IgM MDA on OSOS LV (t=02.81, p=0.005) and cognitive LV (t=+2.53, p=0.011).

Discussion

The first major finding of this study is that the pro-inflammatory cytokines IL-6 and TNF-α are significantly higher in patients with deficit schizophrenia than in those without deficit and normal controls, while patients without deficit show increased TNF-α values relative to controls. These data extent previous papers which showed that the deficit syndrome is accompanied by increased levels of TNF-α as compared to healthy controls and that levels of IL-1β and CCL2 are increased in deficit schizophrenia [11,12]. As such, (stable phase) deficit schizophrenia is characterized by increased activity of the M1 macrophage phenotype when compared with controls (i.e. increased TNF-α, IL-6, IL-1β, MCP-1) and patients with non-deficit schizophrenia (i.e. increased TNF-α and IL-6). These findings provide further evidence that deficit schizophrenia is accompanied by signs of activated immune-inflammatory pathways including increased levels of IgA directed against neurotoxic tryptophan catabolites, and increased IgA/IgM directed to the LPS of Gram-negative bacteria when compared to healthy controls and patients with non-deficit schizophrenia [9,17]. It is interesting to note that the differences in TNF-α were more pronounced than those in IL-6 with a distance of 1.841 and 1.127 SDs between patients and controls in TNF-α and IL-6, respectively.

All in all, those findings indicate that deficit schizophrenia has a neuro-immune pathophysiology whereby different mediators produced by the immune system may exert

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neurotoxic activities and hence neuroprogression [3,7,8]. For example, TNF-α may induce neurotoxic effects via glutamate release by indirect effects on AMPA receptors or upregulating glutaminase thereby causing excitotoxicity [40,41]. These neurotoxic effects of TNF-α may result in an increased synaptic excitatory versus inhibitory ratio thereby underpinning the link between neuroinflammation and excitotoxic processes [42]. Furthermore, IL-6 and, especially increased IL-6 trans-signaling, may also contribute to neuroprogression [43]. Thus, IL-6 trans-signaling mediates neuronal degeneration, whilst classical IL-6 signaling may have neuroprotective or regenerative effects [43,44]. As such, TNF-α and IL-6-driven neuroprogressive responses may play a role in the pathophysiology of the deficit syndrome especially in a milieu where neurotoxic pathways are concomitantly activated including the TRYCAT pathway and increased bacterial translocation [9,17].

The current study also reports that IL-4 is higher in deficit schizophrenia as compared with non-deficit schizophrenia, whereas no differences relative to controls could be detected. Previously, increased IL-4 levels were detected in first-episode psychosis and in acute schizophrenic episodes [3,15,45]. Our findings may indicate that deficit schizophrenia is accompanied by the simultaneous upregulation of M1 and Th-2 cytokines which is in accordance with the IRS-CIRS theory of schizophrenia [3]. This theory considers that increases in IL-4 levels are a compensatory response, which may downregulate the primary IRS response including M1 and Th-1-like responses in spite of the fact that IL-4 may also show some neurotoxic effects. For example, IL-4 and IL-13 may have neurotoxic effects by increasing oxidative stress [46] while IL-4 may increase the production of IFN-γ thereby activating M1 macrophages [47].

The second major finding of this study is that increased levels of TNF-α significantly predicted negative symptoms, PHEM symptoms, and psychomotor retardation above and beyond

the effects of the innate immune system (IgM to MDA and PON1 status). These findings are in agreement with Al-Hakeim et al. [11,12] who found that, in Iraqi patients, TNF-α is associated with the same symptom domains. These findings extend reports that other neuro-immune markers are associated with those symptoms including IgA responses to TRYCATs, IgA responses to Gram-negative bacteria and signs of breakdown of the gut paracellular pathways including the tight junctions [9,17,48]. As such, the increased neurotoxic effects of TNF-α and IL-6 and perhaps IL-4 coupled with the above-mentioned pathways may have detrimental effects on brain circuits subserving different symptom domains of schizophrenia [8,9,17,48]. Another novel finding is that increased levels of IL-4 have suppressant effects on the 4 PHEM symptoms, whilst IL-4 was not associated with negative symptoms. These findings are in agreement with the well-known immune-regulatory role of Th-2 cells which, at least in part, could be mediated by the production of IL-4 thereby down-regulating the detrimental effects of activated Th-1 and M1 pathways [3,14]. Therefore, our results suggest that the adverse effects of TNF-α on PHEM symptoms are in part down-regulated by increased IL-4 levels, and hence that IL-4 may have a more protective (i.e. compensatory or regulatory) role in deficit schizophrenia.

The third major finding of the current study is that increased levels of TNF-α significantly predicted lowered scores on episodic memory probes while increased levels of IL-6 were (moderately) associated with verbal fluency test scores and a more general neuropsychological deficit as measured with the MMSE. Those findings extend those of Al-Hakeim et al. [11,12] who found that, in Iraqi patients with deficit schizophrenia, TNF-α was significantly and inversely associated with different test scores of the Brief Assessment of Cognition in Schizophrenia, including list learning (verbal episodic memory), category instances (semantic fluency), and controlled word association (letter fluency). In the present study TNF-α was associated with

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episodic memory deficits, while in the study of Al-Hakeim et al. [11] increased TNF-α was associated with deficits in episodic and semantic memory. Both studies did not find any significant associations between executive functions and increased TNF-α values and, therefore, it appears that increased TNF-α may specifically target memory. Moreover, the current study suggests that TNF-α and IL-6 may have different effects on episodic versus semantic memory circuits. In any case, the results of the present study indicate that the effects of both cytokines coupled with noxious TRYCATs and increased LPS load could underpin the impairments in neuronal circuits, which determine the cognitive deficits including in the prefronto-striato-thalamic, prefronto-parietal, prefronto-temporal, and dorsolateral prefrontal cortex, hippocampus and amygdala [49,50]. There is also evidence that the combined effects of these biological pathways as well as the deficits in memory and executive functions may explain the onset of the negative and PHEM symptom domains of schizophrenia [7,8,11,12,17].

The fourth major finding of our study is that the plasma concentrations of TNF-α, but not IL-6, are significantly and inversely associated with lowered levels of natural IgM directed to MDA and PON1 enzyme activities and that IL-4 is inversely associated with the IgM responses to MDA. As explained previously, IgM directed to MDA is a key part of the innate immune system and a first line of defense against inflammatory, oxidant and bacterial stressors [16]. Consequently, a deficit in these natural IgM levels as observed in deficit schizophrenia is probably accompanied by a decreased protection and thus increased vulnerability to immune, oxidative and bacterial loads [16,17]. Lowered PON1 enzymatic activity is another hallmark of deficit schizophrenia that is accompanied by attenuated resilience to immune, oxidative and bacterial responses [18], which may explain the inverse association between TNF-α and PON1 enzymatic activity. Moreover, our

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results extend previous data that IgM responses directed to TRYCATs may regulate TRYCAT pathway activation as assessed by IgA responses to TRYCATs [17].

The fifth major finding of this study is that a latent vector extracted from all symptom domains, which reflects overall severity of schizophrenia (OSOS), was to a large extent predicted by the combined effects of TNF-α (positively), IL-4 and IgM to MDA (both inversely), and PON1 index1 (indicating the QQ genotype and lowered PON1 CMPAase activity associated with this genotype). These findings show that increased neurotoxic effects of TNF-α coupled with lowered immune-regulatory Th-2 functions and deficits in the innate immune system, namely lowered natural IgM and PON1 status, determine to a large extent OSOS. Importantly, PLS analysis showed that the effects of natural IgM antibodies on OSOS symptoms are partly mediated by increased TNF-α levels indicating that lowered protection through deficits in natural IgM increases TNF-associated immune responses in schizophrenia.

The results of the current study should be discussed with respect to its limitations. Firstly, this cross-sectional study followed a case-control approach which precludes the establishment of firm causal inferences. Secondly, there were effects of the “drug state” on TNF-α and IL-6 values with risperidone treatment suppressing IL-6 and TNF-α levels, whilst perphenazine suppressed only TNF-α levels. It is well established that some antipsychotics including risperidone may reduce cytokine levels [15]. Nevertheless, results of inter-group comparisons were corrected for the drug state while the effect sizes of risperidone (0.046-0.053) and perphenazine (0.064) were very low. Thirdly, the assay of a broader panel of cytokines predominantly produced by Th-1 and Th-17 cells could have provided more mechanistic insights.

In conclusion, deficit schizophrenia is characterized by increased TNF-α and IL-6 levels, whilst IL-4 is lower in nondeficit than in deficit schizophrenia. We found that a large part of the

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variance in OSOS was explained by increased TNF-α and PON1 status, lowered IL-4 and IgM to MDA while 47.9% of the variance in neurocognitive functions was predicted by increased TNF-α and lowered IL-4 and IgM to MDA as well as education. Lowered IgM to MDA was inversely associated with TNF-α and IL-4. Those findings provide novel insights on how immune mechanisms may shape schizophrenia phenomenology and more particularly underpin deficit schizophrenia as an unique, biologically valid, phenotype.

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Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

Author's contributions

All the contributing authors have participated in the manuscript. All authors contributed to interpretation of the data and writing of the manuscript. All authors approved the final version of the manuscript.

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Figure 1. Results of Partial least Squares (PLS) path analysis.

This PLS path analysis considered two output variables: a) OSOS: overall severity of schizophrenia entered as a latent vector (LV) extracted from different symptom domains; and b) a cognitive LV extracted from 5 neurocognitive tests scores. Biomarkers, sex, and education were the input variables. Shown are path coefficients with exact p-values (inner model) and loadings with p-values (outer model).

Sex: men=1, women=0; FTD: formal thought disorders; PMR: psychomotor retardation. Negative symptoms were assessed using the total scores on the negative subscale of the Positive and Negative Syndrome Scale (PANSSneg), the Schedule of the Deficit Syndrome (SDS) and the Assessment of Negative Symptoms (SANS).

Executive: executive functions; MMSE: Mini Mental State Examination; VFT: Verbal Fluency test; WLM: Word List Memory.

TNF: tumor necrosis factor; IL: interleukin; IgM MDA: IgM values directed against malondialdehyde; PON1 index1: this index combines the additive model of the Q192R genotype with CMPAase activity.

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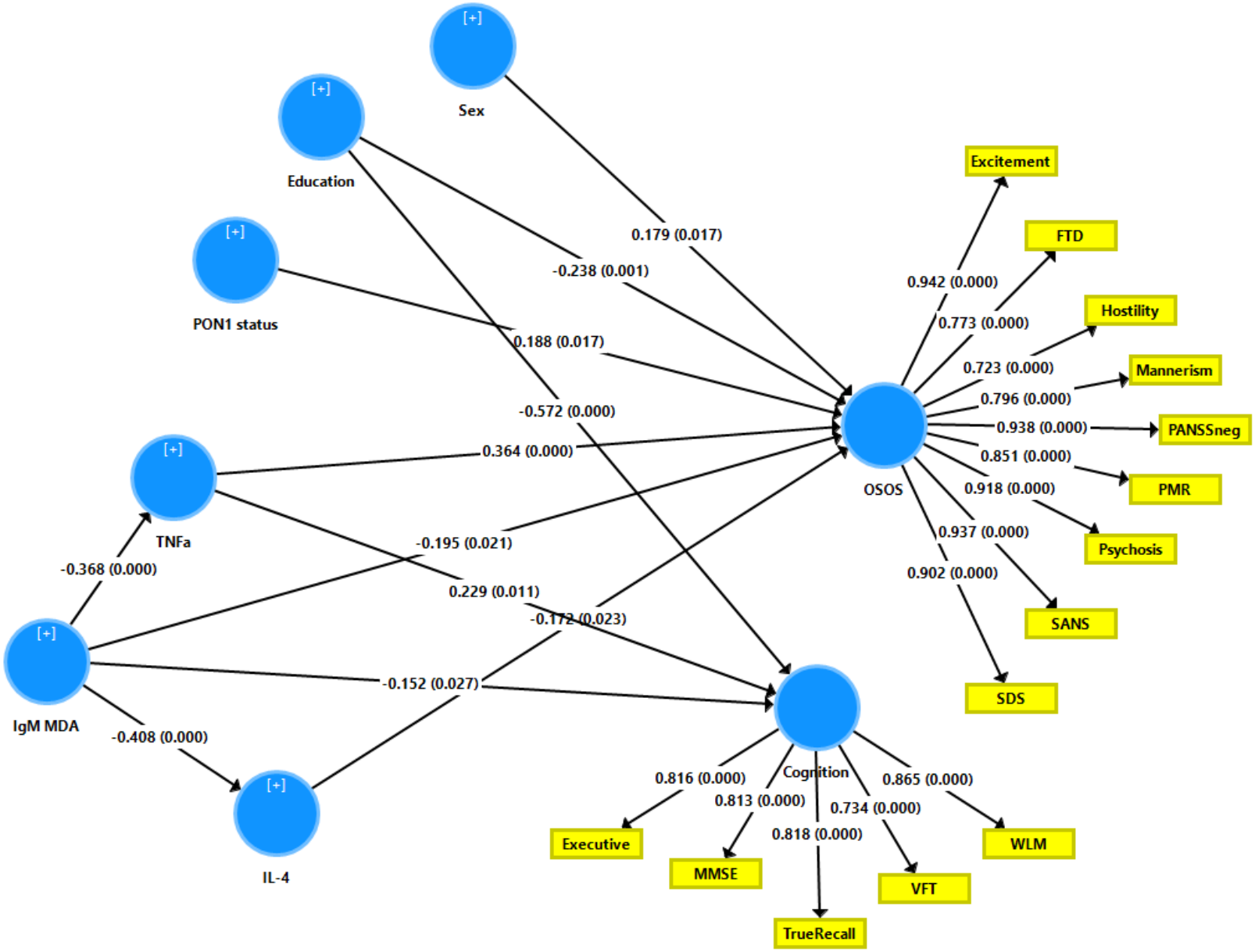

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Table 1: Demographic, clinical and biomarker data of healthy controls (HC) and schizophrenia patients with (DEF) and without (NONDEF) the deficit syndrome.

Variables	HC [A] (n=40)	NONDEF [B] (n=40)	DEF [C] (n=40)	F/ψ/X^2	df	p
Age (years)	37.4 (12.8)	41.3 (10.8)	40.9 (11.4)	1.37	2/117	0.259
Sex (Female/Male)	30/10 [B,C]	18/22 [A]	19/21 [A]	8.99	2	0.011
BMI (kg/m^2)	24.0 (4.3)	26.0 (5.2) [C]	22.9 (4.6) [B]	4.40	2/112	0.015
TUD (No/Yes)	38/2	36/4	39/1	0.13	-	0.346
Employment (No/Yes)	4/36 [B,C]	16/24 [A,C]	30/10 [A,B]	34.83	2	<0.001
Education (years)	14.3 (4.9)	12.8 (4.2)	11.9 (4.1)	3.03	2/115	0.052
TNF-α (z scores) *	-1.611 (0.464) [B,C]	-0.907 (0.307) [A,C]	0.249 (0.248) [A,B]	16.77	2/97	<0.001
IL-6 (z scores) *	-0.884 (0.479) [C]	-0.361 (0.330) [C]	0.217 (0.260) [A,B]	4.99	2/97	0.009
IL-4 (z scores) *	-0.405 (0.496)	-0.907 (0.328) [C]	0.143 (0.265) [B]	8.00	2/97	<0.001
IgM MDA (z score)	-0.27 (0.82) [C]	0.41 (1.00) [C]	-0.67 (0.81) [A,B]	17.87	2/115	<0.001
PON1 status index 1 (z scores)	-0.30 (0.82) [C]	-0.131 (0.93) [C]	0.43 (1.10) [A,B]	6.46	2/117	0.002
PON1 status index 2 (z scores)	0.14 (1.06)	0.01 (0.94)	-0.15 (1.00)	0.89	2/117	0.413

Results are shown as mean (SD), except the cytokine levels, which are shown as mean values (SE)
* Cytokine levels are processed as z scores of Ln transformation and after covarying for body mass index, age, sex, and the drug status of the patients.
BMI: Body Mass Index, TUD: Tobacco Use Disorder
TNF: tumor necrosis factor; IL: interleukin; IgM MDA: IgM values directed against malondialdehyde; PON1 index1: this index combines the additive model of the Q192R genotype (with QQ=2, QR=1 and RR=0) with CMPAase activity; PON1 index2: this index combines the enzymatic activities of CMPAase and AREase.

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Table 2: Results of three different binary logistic regression analyses with the diagnosis of deficit schizophrenia as dependent variable

Varriables	B	SE	Wald	df	p	OR	95% CI
TNF-α	0.928	0.250	13.77	1	<0.001	2.53	1.55-4.13
IL-6	0.577	0.235	6.00	1	0.014	1.78	1.12-2.84
IL-6	0.633	0.279	5.15	1	0.023	1.88	1.09-3.25
IgM MDA	-1.394	0.340	16.79	1	<0.001	0.25	0.13-0.48
PON1 index1	0.916	0.281	10.66	1	0.001	2.50	1.44-4.33

TNF: tumor necrosis factor; IL: interleukin; IgM MDA: IgM values directed against malondialdehyde; PON1 index1: this index combines the additive model of the Q192R genotype (with QQ=2, QR=1 and RR=0) with CMPAase activity.

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Table 3: Results of multiple regression analysis with schizophrenia symptom domains as dependent variables.

Dependent variables	Explanatory variables	β	t	p	F_{model}	df	p	R^2
#1. OSOS	**Model**				11.42	5/102	<0.001	0.359
	TNF-α	0.291	3.55	0.001				
	Sex	0.265	3.32	0.001				
	Education	-0.238	-2.86	0.005				
	PON1 index 1	0.201	2.47	0.015				
	PON2 index 2	-0.168	-2.05	0.043				
#2. SANS	**Model**				10.53	5/102	<0.001	0.340
	TNF-α	0.250	2.88	0.005				
	PON1 index1	0.213	2.57	0.012				
	Sex	0.185	2.24	0.027				
	IgM MDA	-0.204	-2.31	0.023				
	Education	-0.192	-2.30	0.024				
#3. PANSSnegative	**Model**				9.61	3/104	<0.001	0.293
	TNF-α	0.364	4.39	<0.001				
	PON1 index1	0.302	3.65	<0.001				
	Sex	0.199	2.41	0.018				
#4. Psychosis	**Model**				10.58	4/103	<0.001	0.291
	TNF-α	0.381	4.33	<0.001				
	Sex	0.251	3.00	<0.001				
	Education	-0.214	-2.55	0.012				
	IL-4	-0.222	-2.54	0.012				
#5. Hostility	**Model**				10.65	4/103	<0.001	0.293
	Sex	0.361	4.33	<0.001				
	Education	-0.238	-2.83	0.006				
	TNF-α	0.239	2.72	0.008				

	IL-4	-0.192	-2.21	0.030				
#6. Excitation	**Model**				9.18	4/104	<0.001	0.261
	TNF-α	0.272	2.93	0.004				
	Education	-0.236	-2.77	0.007				
	IgM MDA	-0.302	-3.16	0.002				
	IL-4	-0.222	-2.37	0.020				
#7. Mannerism	**Model**				8.46	4/103	<0.001	0.218
	Sex	0.326	3.79	<0.001				
	TNF-α	0.271	2.99	0.003				
	IL-4	-0.189	-2.11	0.038				
	Education	-0.178	-2.05	0.043				
#8. PMR	**Model**				18.06	3/104	<0.001	0.342
	TNF-α	0.317	3.71	<0.001				
	PON1 index1	0.340	4.25	<0.001				
	IgM MDA	-0.211	-2.46	0.015				

OSOS: overall severity of schizophrenia; SANS: Scale for the Assessments of Negative Symptoms, PANSS: Positive and Negative Syndrome Scale; PMR: psychomotor retardation,
TNF: tumor necrosis factor; IL: interleukin; IgM MDA: IgM values directed against malondialdehyde; PON1 index1: this index combines the additive model of the Q192R genotype (with QQ=2, QR=1 and RR=0) with CMPAase activity; PON1 index2: this index combines the enzymatic activities of CMPAase and AREase.

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Table 4: Results of multiple regression analysis with neurocognitive test scores as dependent variables.

Dependent variable	Explanatory variables	β	t	p	F_{model}	df	p	R^2
WLM	**Model**				16.42	3/102	<0.001	0.319
	Education	0.402	4.93	<0.001				
	TNF-α	-0.264	-3.26	0.002				
	Sex	-0.199	-2.45	0.016				
VFT	**Model**				13.21	2/106	<0.001	0.199
	Education	0.403	4.63	<0.001				
	IL-6	-0.221	-2.54	0.012				
True Recall	**Model**				16.06	3/105	<0.001	0.315
	TNF-α	-0.371	-4.56	<0.001				
	Sex	-0.265	-3.25	0.002				
	Education	0.244	2.99	0.004				
MMSE	**Model**				25.21	3/105	<0.001	0.419
	Education	0.602	8.02	<0.001				
	IL-6	-0.153	-2.05	0.043				
	Male sex	-0.152	-2.02	0.046				

WLM: Word List memory; VFT: verbal fluency test; MMSE: Mini Mental State Examination
TNF: tumor necrosis factor; IL: interleukin.

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Table 5: Results of multiple regression analysis with cytokines as dependent variables.

Dependent variable	Explanatory variables	β	t	p	F_{model}	df	p	R^2
TNF-α	**Model**				12.05	2/113	<0.001	0.176
	IgM MDA	-0.378	-4.43	<0.001				
	PON1 index2	-0.195	-2.29	0.024				
IL-4	**Model**				14.30	2/108	<0.001	0.209
	IgM MDA	-0.424	-4.96	<0.001				
	Age	-0.174	-2.04	0.044				

TNF: tumor necrosis factor; IL: interleukin; IgM MDA: IgM values directed against malondialdehyde; PON1 index2: this index combines the enzymatic activities of CMPAase and AREase

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Electronic Supplementary File (ESF)

Increased levels of plasma tumor necrosis factor-α mediate schizophrenia symptoms and neurocognitive impairments and are inversely associated with natural IgM and paraoxonase 1 activity.

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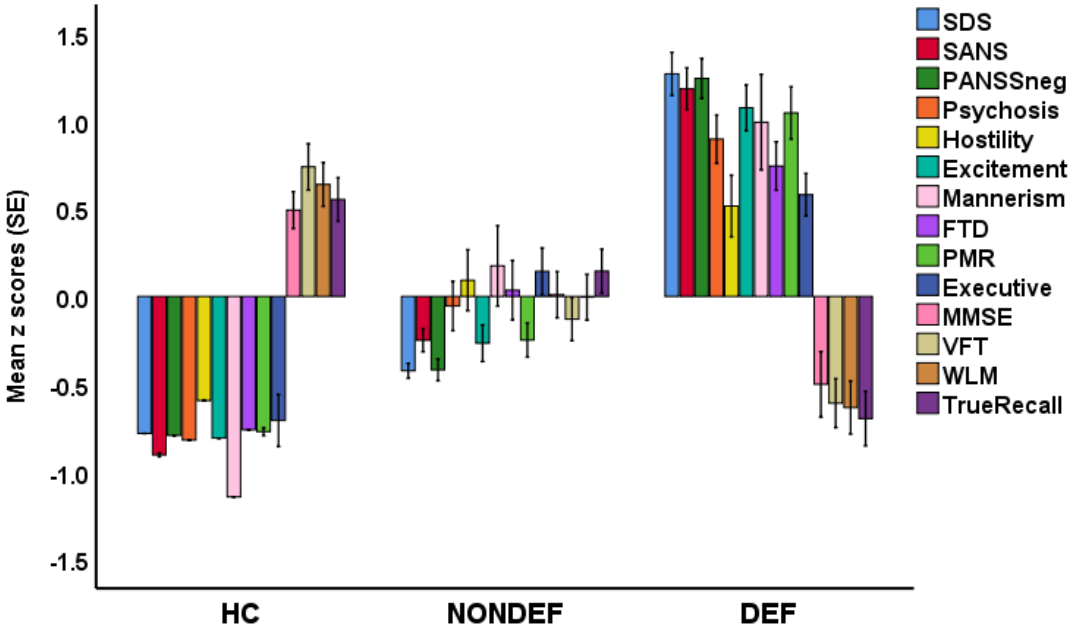

ESF Figure 1. Mean z scores of the symptom domains and neurocognitive functions. All symptom and cognitive tests scores are significantly different between healthy controls (HC) and patients with (DEF) and without (NONDEF) deficit schizophrenia with the highest symptoms scores and lowest neurocognitive scores in deficit schizophrenia.

SDS: Schedule of the Deficit Syndrome; SANS: total score on the Scale for the Assessment of Negative Symptoms; PANSSneg: the total score on the negative subscale of the Positive and Negative Syndrome Scale; FTD: formal thought disoders; PMR: psychomotor retardation; executive: executive functions; MMSE: Mini Mental State Examination; VFT: Verbal Fluency test; WLM: Word List Memory.

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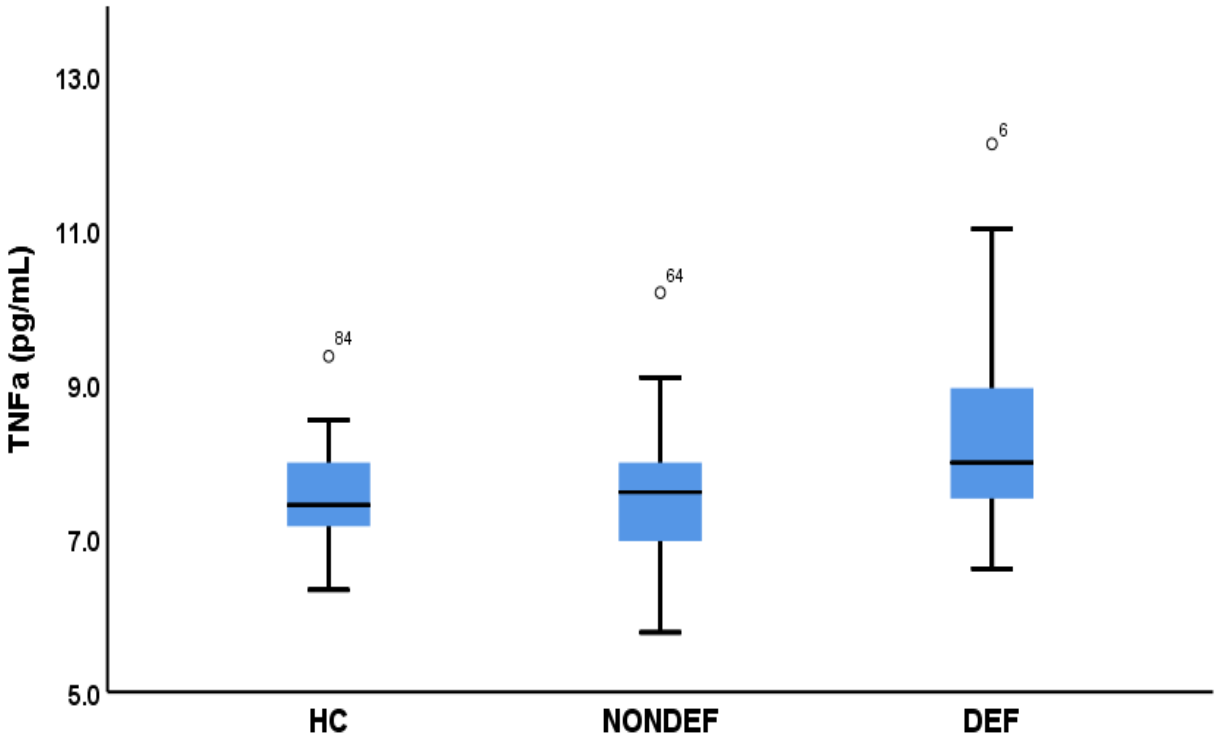

ESF Figure 2. Box plot showing the measurements of tumor necrosis factor (TNF)-α in healthy controls (HC), and patients with (DEF) and without (NONDEF) deficit schizophrenia

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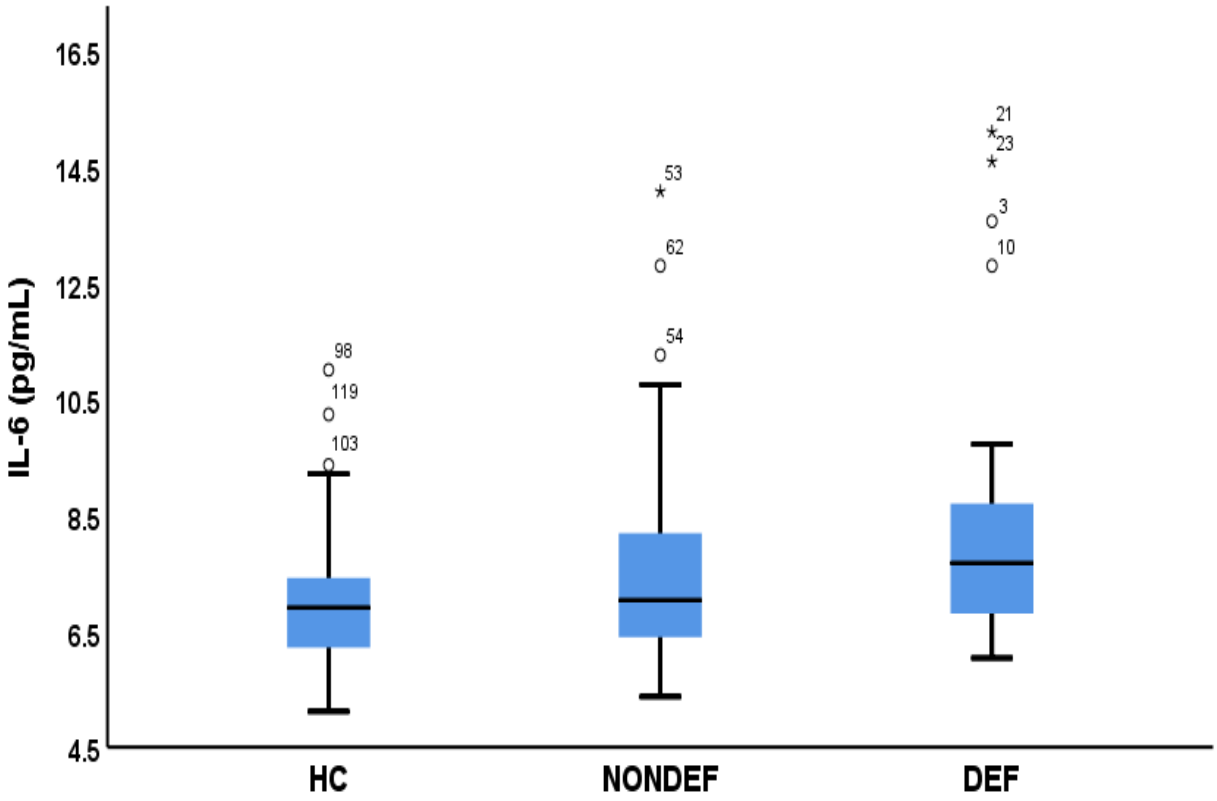
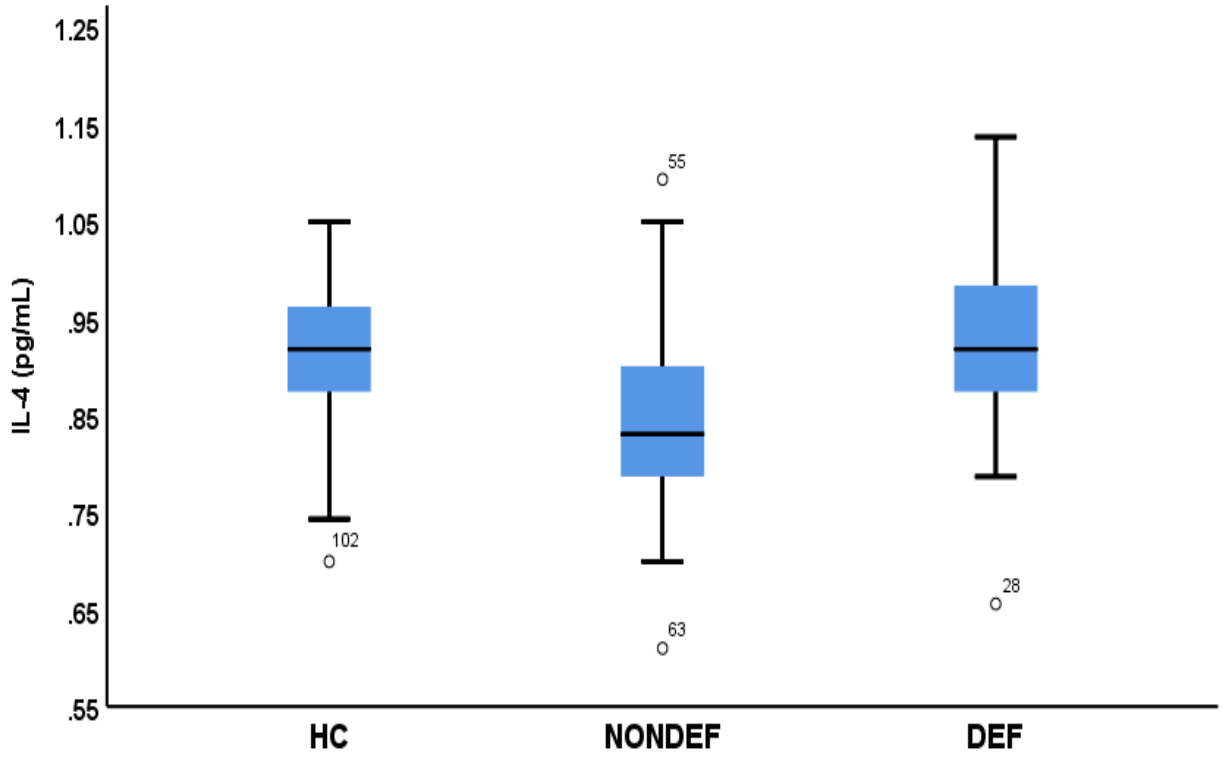


ESF Figure 3. Box plot showing the measurements of interleukin-6 (IL-6) in healthy controls (HC), and patients with (DEF) and without (NONDEF) deficit schizophrenia



ESF Figure 4. Box plot showing the measurements of interleukin-4 (IL-4) in healthy controls (HC), and patients with (DEF) and without (NONDEF) deficit schizophrenia