High serum soluble tumor necrosis factor receptor 1 predicts poor treatment response in acute-stage schizophrenia

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Abstract

Inflammation may be involved in the pathophysiology of schizophrenia. However, few cross-sectional or longitudinal studies have examined changes in biomarker expression to evaluate diagnostic and prognostic efficacy in acute-stage schizophrenia. We compared serum inflammatory biomarker concentrations in 87 patients with acute-stage schizophrenia on admission to 105 age-, sex-, and body mass index (BMI)-matched healthy controls. The measured biomarkers were soluble tumor necrosis factor receptor 1 (sTNFR1) and adiponectin, which are associated with inflammatory responses, and pigment epithelium-derived factor (PEDF), which has anti-inflammatory properties. We then investigated biomarker concentrations and associations with clinical factors in 213 patients (including 42 medication-free patients) and 110 unmatched healthy controls to model conditions typical of clinical practice. Clinical symptoms were assessed using the Brief Psychiatric Rating Scale and Global Assessment of Function. In 121 patients, biomarker levels and clinical status were evaluated at both admission and discharge. Serum sTNFR1 was significantly higher in patients with acute-stage schizophrenia compared to matched controls while no significant group differences were observed for the other markers. Serum sTNFR1 was also significantly higher in the 213 patients compared to unmatched controls. The 42 unmedicated patients had significantly lower

PEDF levels compared to controls. Between admission and discharge, sTNFR1 levels decreased significantly; however, biomarker changes did not correlate with clinical symptoms. The discriminant accuracy of sTNFR1 was 93.2% between controls and patients, showing no symptom improvement during care. Inflammation and a low-level anti-inflammatory state may be involved in both schizophrenia pathogenesis and acute-stage onset. High serum sTNFR1 in the acute stage could be a useful prognostic biomarker for treatment response in clinical practice.

Keywords: Schizophrenia; inflammation; biomarker; TNFR1; adiponectin; pigment epithelium-derived factor

Abbreviations: TNF-α, tumor necrosis factor-α; TNFR1, TNF receptor 1; TNFR2, TNF receptor 2; mTNF, membrane-bound TNF-α; sTNF, soluble TNF-α; TACE, TNF-α converting enzyme; sTNFR1, soluble TNFR1; sTNFR2, soluble TNFR2; PEDF, pigment epithelium-derived factor; BMI, body mass index; BPRS, Brief Psychiatric Rating Scale; GAF, Global Assessment of Function; DSM-IV, Diagnostic and Statistical Manual of Mental Disorder, Fourth Edition; FGAs, first-generation antipsychotics; SGAs, second-generation antipsychotics.

1. Introduction

Inflammation may be involved in the pathophysiology of schizophrenia (Najjar et al., 2013). Epidemiologically, maternal infection during pregnancy is associated with increased risk of schizophrenia in offspring (Brown and Derkits, 2010). Anti-inflammatory agents as adjunct treatments with antipsychotics have been shown to improve symptoms and cognitive function in patients with schizophrenia (Akhondzadeh et al., 2007; Nitta et al., 2013; Sommer et al., 2014). Furthermore, several studies have suggested that pro-inflammatory oxidative (Flatow et al., 2013; Marchbanks et al., 2003) and carbonyl stress (Katsuta et al., 2014; Takeda et al., 2015) are involved in the pathophysiology of schizophrenia. Moreover, investigations of inflammatory markers in peripheral blood indicate a chronic inflammatory state in schizophrenia (Dickerson et al., 2015; Joseph et al., 2015; Mondelli et al., 2015).

The tumor necrosis factor-α (TNF-α) receptors TNFR1 and TNFR2 are candidate inflammatory mediators with possible utility as biomarkers for schizophrenia. Serum TNF-α exerts its pro-inflammatory effects by binding to TNFR1 and TNFR2 (Idriss and Naismith, 2000). While TNF-α is usually membrane bound (mTNF), during inflammation it is cleaved into soluble TNF-α (sTNF) by TNF-α converting enzyme (TACE) (Supplemental Figure 1) (Yoshida et al., 2006). TNFRs are subsequently

released into the circulation as soluble TNFRs (sTNFRs) (Levine et al., 2005). Both serum sTNFR1 and sTNFR2 have been detected at high concentrations over prolonged periods in patients with inflammatory diseases (Bastard et al., 2000; Brauner et al., 2000; Kaplanski et al., 2002). Several studies have investigated the associations between neuroinflammation and schizophrenia using sTNFR1 and/or sTNFR2 as biomarkers (Coelho et al., 2008; Hope et al., 2009; Noto et al., 2013; Noto et al., 2015). In particular, sTNFR1 is considered to be a stable and reliable marker of TNF-α activity (Coelho et al., 2008; Hope et al., 2011; Noto et al., 2013) and a marker reflecting both treatment resistance and severe clinical course in outpatients with chronic schizophrenia (Noto et al., 2013; Noto et al., 2015). Adiponectin, the most abundant adipokine, is a key regulator of insulin sensitivity and tissue inflammation (Whitehead et al., 2006; Ziemke and Mantzoros, 2010). It is secreted by adipose tissue and circulated in the blood at high concentration (Whitehead et al., 2006). While only a few studies have examined the inflammatory hypotheses of schizophrenia using peripheral adiponectin levels, these studies have shown significantly higher adiponectin levels in chronic patients than controls (Beumer et al., 2012; Song et al., 2013). It seems reasonable that sTNFR1 and adiponectin, as pro- and anti-inflammatory factors, could also be clinical biomarkers for acute-stage schizophrenia, which has not been previously investigated.

Pigment epithelium-derived factor (PEDF), a secreted glycoprotein, inhibits the AGE–RAGE pathway, which is associated with the activation of pro-inflammatory genes, and is altered in schizophrenia (Takeda et al., 2015). Therefore, PEDF can reduce inflammation by suppressing pro-inflammatory pathways (Famulla et al., 2011; Sheikpranbabu et al., 2010). PEDF has neurotrophic, anti-inflammatory, and anti-oxidative properties (Sanagi et al., 2005; Yabe et al., 2005) and has been shown to protect the central nervous system (Sanagi et al., 2010). PEDF and adiponectin are known to be key adipokines and are related to chronic inflammatory disorders such as diabetes and obesity (Famulla et al., 2011). Therefore, the study of PEDF as a curious anti-inflammatory and anti-oxidative biomarker in acute schizophrenia also seems reasonable.

A recent meta-analysis concluded that interleukin (IL)-1β, IL-6, and transforming growth factor-β (TGF-β) could be considered as state markers and IL-12, interferon-γ (IFN-γ), TNF-α, and soluble IL-2 receptor (sIL-2R) as trait markers for longitudinal observation of acute-stage schizophrenia (Miller et al., 2011). However, it has not yet been established whether changes in sTNFR1 and adiponectin levels reflect the current pathological condition and serve as biological markers for acute-stage schizophrenia in clinical practice.

In the current study, we investigated whether serum levels of sTNFR1, adiponectin, and PEDF can be used as diagnostic/prognostic biomarkers for acute-stage schizophrenia by comparing these patients to healthy matched controls and unmatched controls in a cross-sectional study. Matched controls were used to obviate the influences of age, sex, and body mass index (BMI) as confounders while unmatched controls were used to model the clinical situation. In addition, the associations between inflammatory biomarker changes and clinical symptoms were investigated in a longitudinal study.

2. Methods

2.1. Participants

We enrolled patients who were diagnosed with schizophrenia and admitted to the Juntendo Koshigaya Hospital (Saitama) or Juntendo Hospital (Tokyo) due to acute exacerbation of symptoms. Clinical interviews conducted by at least three experienced psychiatrists confirmed that all patients met the criteria for schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorder, Fourth Edition (DSM-IV). Both disease relapse and first-episode drug-naïve patients were included. Patients with schizophreniform disorder, schizoaffective disorder, psychosis not otherwise specified, or schizoid personality disorder were excluded. Controls were enrolled from healthy

volunteers with no current or previous psychiatric disorder according to the Structured Clinical Interview for DSM-IV (SCID-IV). All participants met the following criteria: 1) no current infections or allergies, 2) no more than grade 2 obesity (BMI < 35, WHO: Global Database on Body Mass Index, http://apps.who.int/bmi/index.jsp?introPage=intro_3.html), 3) no current use of anti-inflammatory agents, 4) no history of autoimmune disorders, 5) no history of diabetes, lipid disorders, or kidney dysfunction, 6) no previous head trauma with disturbed consciousness, and 7) no history of smoking, alcoholism, or substance use disorder.

Improvement of the patient's condition by appropriate medication is a priority of the Juntendo University Schizophrenia Projects (JUSP) (Ohnuma et al., 2008); therefore, pharmacotherapy was not controlled. This study was approved by the Ethics Committee of the Juntendo University School of Medicine (2015014). All participants provided written informed consent prior to study inclusion.

2.2. Evaluation of clinical symptoms

Clinical data were obtained by interviewing patients and/or families and from hospital records. Clinical symptoms were assessed at admission and discharge using the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962) and the Global

Assessment of Function (GAF), respectively. All administered antipsychotics were converted to the chlorpromazine (CP) equivalent dose (Inada and Inagaki, 2015).

2.3. Measurements of serum inflammatory biomarkers

Blood samples were taken in the morning after fasting to control for the influences of diet and exercise. The studies were conducted in serum rather than plasma because we could only establish reliable immunoassays for these three inflammatory biomarkers in serum. Serum sTNFR1 levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The detection limit was 10 pg/ml. Serum adiponectin levels were measured using a latex particle-enhanced turbidimetric immunoassay (LTIA) (Nishimura and Sawai, 2006). The detection limit was 0.2 μg/ml. Serum PEDF levels were measured using competitive ELISA (Fukami et al., 2010). The detection limit was 0.045 ng/ml. Details of these measurement protocols are provided in Supplemental Methods.

2.4. Statistical analysis

All statistical analyses were conducted using SPSS version 22 (IBM Corp.,

Armonk, NY). Chi-square tests were conducted to assess differences in demographic background between groups. Differences in serum sTNFR1, adiponectin, and PEDF levels between the unpaired groups were examined using the two-tailed Mann–Whitney *U* test for two-group comparisons and the Kruskal–Wallis test for comparisons of three or more groups. To evaluate the diagnostic biomarkers, discriminant analyses were performed with schizophrenia versus control as the dependent variable and the measured biomarkers as independent variables. The differences in the biomarkers in paired samples of each patient between the time of admission and the time of discharge were examined by the Wilcoxon matched-pairs signed-rank test. The correlations between clinical features, such as BMI and daily CP equivalent dose (continuous variables), and the measured serum biomarkers were analyzed using Pearson's correlation test. Correlations between the clinical symptom scale scores for the BPRS and GAF (discrete variables taking integer values) and measured serum biomarkers were analyzed using Spearman's correlation test. Multiple linear regression analyses were performed using the forced entry method to investigate the relationship between the measured biomarker levels in the patients with schizophrenia and several correlative independent variables. The level of statistical significance was set at $P < 0.05$. However, we applied the Bonferroni correction to multiple comparisons among three biomarkers,

resulting in a level of statistical significance for these comparisons of $P < 0.017$ (i.e., 0.05/3).

3. Results

3.1. Inflammatory biomarkers in patients with acute-stage schizophrenia

First, we investigated the differences in biomarker expression between 87 patients with acute-stage schizophrenia and 105 healthy controls matched for the potential confounders of sex, age, and BMI (Table 1). Thus, no significant difference was observed in sex ratio, mean age, or BMI between patients and controls. Serum sTNFR1 levels were significantly higher in patients at admission than in matched controls while no significant group differences were observed in serum adiponectin and PEDF levels (Table 1, Figure 1A-C). To confirm whether these inflammatory biomarkers could be useful in clinical practice, we compared serum marker concentrations between all 213 enrolled patients with acute-stage schizophrenia and 110 healthy unmatched controls aged 16–76 years (Table 2). In this case, mean age and BMI were higher in the patients while no significant difference was observed in sex ratio (Table 2). As in the matched comparison, serum sTNFR1 was significantly higher in patients while no significant difference was observed in adiponectin or PEDF (Table 2).

Thus, elevated sTNFR1 may represent a possible diagnostic biomarker for acute-phase schizophrenia.

3.2. Relationships between inflammatory biomarkers and clinical variables at admission in clinical practice

We then assessed possible correlations between biomarkers and clinical variables. In analyses including all participants, a significant positive correlation was found between serum sTNFR1 and PEDF levels ($r = 0.312$, $P < 0.001$) and a significant negative correlation between adiponectin and PEDF levels ($r = -0.304$, $P < 0.001$). No significant correlation was found between sTNFR1 and adiponectin ($r = -0.003$, $P = 0.954$). In the controls, BMI was significantly correlated with serum concentrations of adiponectin ($r = -0.422$, $P < 0.001$) and PEDF ($r = 0.477$, $P < 0.001$) whereas age was not correlated with any biomarker concentration (all $P > 0.05$). In the patients with acute schizophrenia, however, age was significantly correlated with both sTNFR1 ($r = 0.274$, $P < 0.001$) and adiponectin ($r = 0.243$, $P < 0.001$). In addition, BMI was negatively correlated with adiponectin ($r = -0.419$, $P < 0.001$) and positively correlated with PEDF ($r = 0.306$, $P < 0.001$).

Serum sTNFR1 and PEDF concentrations positively correlated with daily CP

equivalent dose at admission (r = 0.176, P = 0.010 and r = 0.205, P = 0.003, respectively). Serum sTNFR1 and adiponectin levels also positively correlated with the duration of illness (r = 0.267, $P < 0.001$ and r = 0.198, P = 0.004, respectively). The other clinical variables including BPRS and GAF scores were not significantly associated with serum biomarker concentrations. As expected, age and duration of illness showed a strong positive correlation ($r = 0.669$, $P < 0.001$).

We performed multiple linear regression analyses with the three biomarkers as dependent variables and the clinical parameters showing significant correlations as independent variables (age, daily CP equivalent dose, duration of illness, and BMI) in patients. In this analysis, sTNFR1 level (adjusted $R^2 = 0.114$, F = 7.74, $P < 0.001$) was significantly related to age ($\beta = 0.371$, $P < 0.001$) and adiponectin level (adjusted $R^2 = 0.254$, F = 18.5, $P < 0.001$) to both duration of illness and BMI ($\beta = 0.297$, P = 0.001 and $\beta = -0.406$, $P < 0.001$, respectively). Serum PEDF concentration (adjusted $R^2 = 0.078$, F = 5.42, $P < 0.001$) was significantly related to BMI ($\beta = 0.245$, $P < 0.001$).

3.3 Differences in inflammatory biomarkers between patients with and without medication and healthy controls

Of the 213 patients with schizophrenia, 42 were medication-free for at least

four weeks prior to presentation, including four first-episode patients who were drug-naïve at admission. We investigated differences in biomarker levels between the 171 patients on medication, the 42 medication-free patients, and the 110 unmatched healthy controls (Table 3). Serum sTNFR1 and PEDF levels differed significantly among the groups and post-hoc analysis revealed significantly lower serum sTNFR1 in the medication-free patients compared to those on medication. The medication-free patients also showed significantly lower serum PEDF than controls. Those on medication had significantly higher sTNFR1 levels than controls, as did the total patient group. Serum adiponectin levels did not differ significantly among groups. Mean age was significantly higher in both medicated and medication-free patients compared to controls whereas BMI was significantly higher only in medicated patients (Table 3).

Compared to patients on medication, medication-free patients had significantly higher age at onset, longer duration of education, longer duration of untreated psychosis (DUP), shorter duration of illness, lower number of admissions, and lower GAF scores (Table 3).

We also investigated associations between biomarkers and the types of antipsychotics prescribed [first-generation antipsychotics, FGAs (15%); second-generation antipsychotics, SGAs (53%); and concomitant use of both types, FGAs +

SGAs (32%)]. Both serum sTNFR1 ($\chi^2 = 16.3$, $P = 0.001$) and PEDF ($\chi^2 = 8.68$, $P = 0.034$) differed significantly among the drug treatment groups and post-hoc analysis revealed that sTNFR1 and PEDF levels were significantly higher in patients receiving FGAs + SGAs than in medication-free patients (sTNFR1: 1218.7 ± 474.1 pg/ml vs. 1006.0 ± 363.7 pg/ml, $\chi^2 = -3.52$, $P < 0.001$; PEDF: 12.8 ± 3.83 μg/ml vs. 10.9 ± 4.47 μg/ml, $\chi^2 = -2.64$, $P = 0.008$). Similarly, sTNFR1 levels were significantly higher in patients receiving FGAs compared to medication-free patients (1178.6 ± 270.5 pg/ml vs. 1006.0 ± 363.7 pg/ml, $\chi^2 = -3.40$, $P = 0.001$). Adiponectin levels did not differ significantly among groups ($\chi^2 = 2.89$, $P = 0.409$). No significant correlations were found between biomarkers and daily CP equivalent dose in any of the drug treatment groups (all $P > 0.017$).

3.4. Changes in inflammatory biomarker levels between admission and discharge

Serum biomarker concentrations and clinical parameters were compared between admission and discharge in 121 patients with schizophrenia (Table 4) while the remaining 92 patients were unexpectedly discharged without examination upon patient or family request due to symptomatic improvement. As expected, GAF scores and positive, negative, and total BPRS scores improved significantly between admission and

discharge. The daily CP equivalent dose also increased significantly. Serum sTNFR1 levels significantly decreased from admission to discharge whereas adiponectin and PEDF levels did not change (Table 4). Although the sTNFR1 level in patients was reduced by discharge, it was still higher than in controls (1035.3 ± 261.7 pg/ml vs. 923.2 ± 158.2 pg/ml, Mann–Whitney U; $\chi^2 = -3.68$, $P < 0.001$).

Changes (Δ) in biomarker levels and symptom scores were calculated as (discharge level – admission level) / (admission level). There were no significant correlations between changes in biomarker concentrations (ΔsTNFR1, Δadiponectin, or ΔPEDF) and test scores (Δpositive, Δnegative, and Δtotal BPRS scores and ΔGAF score) (all $P > 0.017$). Changes in biomarker concentrations were not significantly correlated with changes in CP equivalent dose in the short term (mean: 103.8 days) (all $P > 0.017$). Furthermore, duration of hospitalization did not show any correlations with changes in biomarker concentrations, test scores, or CP dose (all $P > 0.017$).

3.5. Serum sTNFR1 in deterioration cases

Because studies have shown that sTNFR1 levels are higher in treatment-resistant schizophrenia (Noto et al., 2013; Noto et al; 2015), we compared sTNFR1 levels between patients with improved total BPRS score from admission to discharge

and with score deterioration, indicating treatment resistance. Among the 121 cases that were followed from admission to discharge, improvement in total BPRS was observed in 113 cases (93%) and deterioration in 8 cases (7%). Serum sTNFR1 levels at admission were significantly higher in the deterioration cases than in the improved cases (1427.5 ± 489.6 pg/ml vs. 1101.5 ± 420.2 pg/ml; Mann–Whitney U; $\chi^2 = 2.70$, $P = 0.007$) whereas no significant difference was found in any clinical variable (e.g., age, BMI, duration of illness, or daily CP equivalent dose) between improvement and deterioration cases (all $P > 0.05$).

3.6. Discriminant analysis using inflammatory biomarkers

Discriminant analysis was performed to assess whether increased sTNFR1 levels and/or decreased PEDF levels accurately discriminated between the 87 patients with schizophrenia and the 105 matched controls. Serum sTNFR1 and PEDF predicted schizophrenia with 57.5% sensitivity, 66.7% specificity, and 62.4% accuracy in cross-validated grouped cases (eigenvalue: 0.164, canonical correlation: 0.375, Wilks' lambda: 0.859, $P < 0.001$) (Table 5A). Using only sTNFR1, the sensitivity, specificity, and accuracy were 52.9%, 67.6%, and 60.9% of the cross-validated grouped cases (eigenvalue: 0.108, canonical correlation: 0.313, Wilks' lambda: 0.902, $P < 0.001$). A

similar analysis was also performed to assess discrimination accuracy between all 213 enrolled patients and the 110 unmatched controls. Serum sTNFR1 and PEDF discriminated schizophrenia with 56.9% sensitivity, 70.9% specificity, and 61.5% accuracy in the cross-validated grouped cases (eigenvalue: 0.115, canonical correlation: 0.321, Wilks' lambda: 0.897, $P < 0.001$) (Table 5B). Using only sTNFR1, the sensitivity, specificity, and accuracy were 51.9%, 75.5%, and 59.9% of the cross-validated grouped cases (eigenvalue: 0.081, canonical correlation: 0.274, Wilks' lambda: 0.925, $P < 0.001$).

While discriminant analysis revealed relatively modest accuracy for the entire patient and control groups, sTNFR1 demonstrated relatively high efficacy for discriminating the 8 deterioration cases from the 110 controls with sensitivity of 75.0%, specificity of 94.5%, and accuracy of 93.2% of the cross-validated grouped cases (eigenvalue: 0.431, canonical correlation: 0.549, Wilks' lambda: 0.699, $P < 0.001$) (Table 5C). In addition, a discriminant analysis between the 8 deterioration and 113 improvement cases using sTNFR1 also showed high discriminative efficacy with sensitivity, specificity, and accuracy of 82.8%, 62.5%, and 81.0% of the cross-validated grouped cases, respectively (eigenvalue: 0.037, canonical correlation: 0.189, Wilks' lambda: 0.964, $P = 0.038$) (Table 5D).

4. Discussion

In the cross-sectional phase of this study, we investigated differences in serum sTNFR1, adiponectin, and PEDF concentrations between patients with acute-stage schizophrenia and healthy controls with and without adjustment for confounders such as age and BMI. We also evaluated correlations between these markers and various clinical parameters. In addition, the associations between changes in these inflammatory biomarkers and clinical symptoms were investigated in patients followed from admission to discharge. We demonstrated that serum sTNFR1 levels were significantly higher in patients with acute-stage schizophrenia than in healthy controls with or without adjustment for age and BMI. Higher sTNFR1 alone and sTNFR1 plus PEDF had moderate efficacy for discriminating patients from controls (accuracy > 60%) but sTNFR1 demonstrated relative high efficacy for discriminating patients exhibiting deterioration during the course of inpatient care from controls (>90% accuracy) and from improved cases (>80% accuracy). Thus, elevated serum sTNFR1 may represent a useful adjunct biomarker for acute-phase schizophrenia and may be a valuable prognostic marker for treatment response.

The mean serum sTNFR1 concentration of patients with acute-phase

schizophrenia in the present study (1022.8 ± 199.3 pg/ml with adjustment and 1124.6 ± 398.2 pg/ml without adjustment for confounders) was similar to that of non-schizophrenic patients with systemic inflammation due to obesity (mean: 1098 pg/ml) (Bastard et al., 2000) but markedly lower compared to patients with severe inflammatory diseases such as septic shock (median: 7577 pg/ml) (Brauner et al., 2000), hepatitis C (mean: 2219 pg/ml) (Kaplanski et al., 2002), and IgA nephropathy (median: 1412 pg/ml) (Sonoda et al., 2015). Thus, low-grade inflammation could be involved in the pathophysiology of schizophrenia. Indeed, our results are consistent with several smaller-scale studies showing higher peripheral sTNFR1 levels in patients with schizophrenia compared to controls (Coelho et al., 2008; Hope et al., 2009; Morch et al., 2016; Noto et al., 2013; Noto et al., 2015).

However, the serum sTNFR1 level in acute-stage schizophrenia did not appear to reflect cross-sectional clinical severity as there were no correlations with BPRS and GAF scores; however, sTNFR1 levels were correlated with duration of illness, daily CP equivalent dose, and, in particular, age. This latter finding is consistent with a previous report on the association between inflammatory markers, such as TNF-α, sTNFR1, and sTNFR2, and age in psychiatric patients (Haack et al., 1999). In contrast, in the present study, controls exhibited no such association between age and serum sTNFR1. The first

schizophrenic crisis usually occurs during early adolescence (van Os and Kapur, 2009), so age at admission and duration of illness naturally show a strong positive association, as seen in the present study. However, accurate identification of age at onset in schizophrenia is often difficult, especially in cases of emerging negative symptoms and cognitive dysfunction. In such cases, the age of onset tends to be defined by the appearance of psychotic symptoms (Kahn and Sommer, 2015). Thus, estimated age of onset may be older than the actual age of the disease's biological onset. Therefore, if we investigate these biomarkers in patients at their first clinical onset (emerging symptoms), it is impossible to infer whether inflammatory pathophysiology in patients with schizophrenia at that time is the cause or consequence of schizophrenia. Accordingly, the estimated duration of illness may be shorter than the actual duration. In general, duration of illness appears to be of greater significance for disease severity than simple aging. Starting at disease onset, patients with schizophrenia may have suffered from a variety of psychological stressors in addition to prolonged symptoms such as declining psychosocial situation, conflicts stemming from loss of insight or behavioral control, and side-effects of antipsychotics (Supplemental Figure 2A). Chronic psychological stressors have been shown to evoke a stable inflammatory state in patients with both psychotic and post-traumatic stress disorder (Eraly et al., 2014; Mondelli et al., 2011;

Mondelli et al., 2010). The medication-free patients in the present study had a significantly shorter duration of illness and lower serum sTNFR1 than those on medication. Thus, although sTNFR1 levels may not be elevated at the time of disease onset, they may increase with duration of illness and concomitant accumulation of stressors (Supplemental Figure 2A).

In the longitudinal analysis, sTNFR1 levels decreased significantly with intensive therapy over the relatively short period from admission to discharge (Supplemental Figure 2A). This improvement was associated with an increase in daily CP equivalent dose. Nevertheless, changes in serum sTNFR1 did not correlate with changes in positive, negative, and total BPRS scores, GAF score, or daily CP equivalent dose during hospitalization. These results suggest that sTNFR1 may not be a direct therapeutic marker of improved symptoms. In addition, the type and dose of antipsychotics may not directly influence sTNFR1 because previous studies have also shown inconsistent results regarding the influence of antipsychotics on the inflammatory system (Drzyzga et al., 2006; Haack et al., 1999; Kato et al., 2011). Thus, changes in serum sTNFR1 may be more strongly influenced by the relief of psychological stress than by abatement of symptoms or medication. Patients with schizophrenia suffer from various kinds of social dysfunction such as disorder

development, including relapse (Rajkumar and Thara, 1989), and maladjustment to the community environment (Johnstone et al., 1990). Interpersonal communication of social functioning is also notably impaired in patients with schizophrenia (Yasuyama et al., 2016). Patients who are hospitalized with an acute exacerbation could be relieved of some of the above psychosocial stresses experienced in the social community. Considering that sTNFR1 levels were significantly decreased during hospitalization, hospitalization may not necessarily be a stressor.

A limitation of the present study is that the vast majority (93%) of patients who were followed longitudinally showed improvement with consistent treatment intervention from admission to discharge while only a few (7%) exhibited deterioration. Despite this small number, the sTNFR1 level at admission had 93.2% accuracy for discriminating deterioration cases from controls. Similarly, sTNFR1 level also discriminated between deterioration cases and improvement cases with 81.0% accuracy, although this was no longer significant after Bonferroni correction. Thus, serum sTNFR1 concentration could be a useful prognostic biomarker for intensive therapy response in acute-stage schizophrenia. Indeed, two previous cross-sectional studies have suggested that sTNFR1 could be a diagnostic marker for treatment-resistant schizophrenia (Noto et al., 2013; Noto et al., 2015), although neither study measured

sTNFR1 levels in the acute stage. Taken together, we suggest that high sTNFR1 levels could be a state marker for treatment-resistant schizophrenia and predict poorer response to treatment in acute-stage schizophrenia. To confirm this hypothesis, a greater number of patients showing deterioration in symptoms under treatment must be included in future study.

In contrast to sTNFR1, there were no significant differences in serum adiponectin and PEDF levels between the total number of patients with acute-stage schizophrenia and healthy controls, whether matched or unmatched for confounders. Alternatively, previous studies reported that adiponectin levels were significantly higher in patients with schizophrenia, including both drug-naïve and chronic stable patients (Beumer et al., 2012; Song et al., 2013). This inconsistency may result from differences in enrollment of patients or because these previous studies did not control for age and BMI. A recent meta-analysis concluded that there were significant differences in adiponectin levels among patients taking different SGAs; however, again, this study did not consider BMI, the factor with the greatest influence on adiponectin (Bartoli et al., 2015). In particular, SGAs, such as olanzapine and clozapine, are associated with severe metabolic dysfunction that could result in low adiponectin levels.

To the best of our knowledge, this is the first study to investigate PEDF in the

pathophysiology of schizophrenia. PEDF exhibits various neuroprotective, anti-inflammatory, and anti-oxidative effects (Yamagishi et al., 2010). Previous studies found increased PEDF expression in the cerebral fluid of patients with Alzheimer's disease (Yamagishi et al., 2004), frontotemporal dementia (Davidsson et al., 2002), and amyotrophic lateral sclerosis (Kuncl et al., 2002), which may be the result of compensatory up-regulation against the inflammatory mechanisms of these neurodegenerative diseases. However, another study found that PEDF induced pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, through activation of nuclear factor-kappa B (NF-κB) in microglia (Yabe et al., 2005), indicating that PEDF has anti- and pro-inflammatory properties. Interestingly, in the present study, PEDF levels were significantly lower in the 42 medication-free patients compared to controls without high sTNFR1 levels. BMI has been reported to influence PEDF levels (Yamagishi et al., 2010), which is consistent with the positive association for the entire patient cohort on multiple linear regression analysis. However, no significant difference was observed in BMI between the medication-free patients and controls. Thus, lower PEDF levels in unmedicated patients with markedly shorter disease course (Table 2) may reflect a preparatory state in the pathophysiology of schizophrenia. Accordingly, long periods of severe stress during the disease process could induce a chronic inflammatory state as

reflected by increased serum sTNFR1 in medicated patients. Therefore, it is not unexpected that treatment-resistant schizophrenics suffering from chronic severe symptoms would exhibit higher sTNFR1 levels (Noto et al., 2013; Noto et al., 2015). Our discriminant analysis extends these results by demonstrating the possible prognostic significance of sTNFR1 elevation. In contrast, PEDF did not change during longitudinal observation or correlate with clinical course (Supplemental Figure 2B).

There are several limitations to the present study. The interval between biomarker measurements in the longitudinal analysis was not controlled and patients exhibited substantial variation in duration of hospital stay. In addition, we focused on daily CP equivalent dose because we could not control for specific types of antipsychotics used in clinical practice. In observational study for acute-stage schizophrenia, it was impossible to control the types and doses of antipsychotics because of the need to change pharmacotherapy according to changing severe symptoms, sometimes at the expense of emergent severe side effects. However, the role of inflammation in schizophrenia will remain unexplained if research is not conducted in clinical practice in acute-stage schizophrenia. Future observational study should be conducted for patients with chronic schizophrenia (or relatively mild patients) in which pharmacotherapy can be fixed. In addition, identifying possible associations between

specific antipsychotic types and inflammatory biomarkers requires larger retrospective or animal model studies. We consider that investigation into the expression and/or activity of some parameters such as peripheral blood mononuclear cells (PBMCs) and NF-κB is worthwhile. Unfortunately, in the present study, we did not collect these molecules in parallel from the subjects. In future study, we aim to investigate further canonical pro-inflammatory nuclear factors in patients with schizophrenia.

5. Conclusion

Acute-stage schizophrenia may be associated with low-level chronic inflammation. Low serum PEDF levels, indicative of diminished anti-inflammatory potential, could be involved in the onset or early progression of the disease. Subsequently, increasing psychological stress with illness progression could induce a chronic inflammatory state. Higher serum sTNFR1 during the acute stage of schizophrenia may be a valuable prognostic biomarker for treatment response in clinical practice.

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Figure Legends

Fig. 1. Serum levels of inflammatory markers in patients with schizophrenia at admission (SZ) and healthy controls (C) matched for age, sex ratio, and BMI

A. sTNFR1, B. Adiponectin, C. PEDF.

Values were compared using the two-tailed Mann–Whitney *U* test. Error bars denote standard deviation.

BMI, body mass index; sTNFR1, soluble tumor necrosis factor receptor 1; PEDF, pigment epithelium-derived factor.

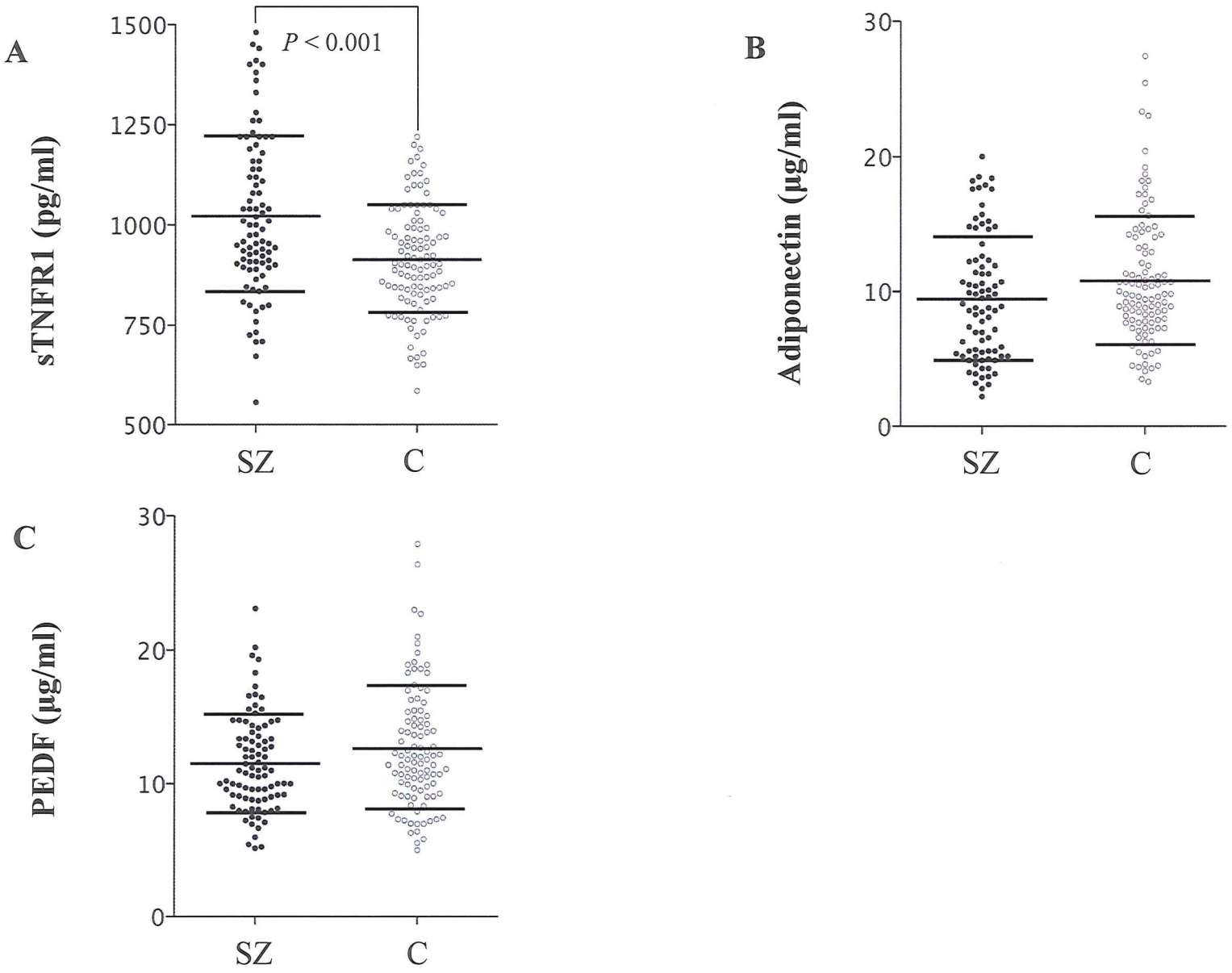

A
sTNFR1 (pg/ml)
1500
1250
1000
750
500
P < 0.001
SZ
C
B
Adiponectin (µg/ml)
30
20
10
0
SZ
C
C
PEDF (µg/ml)
30
20
10
0
SZ
C

Table 1. Comparison of demographic variables and serum inflammatory biomarker levels between patients with schizophrenia and matched healthy controls at admission

	Patients with schizophrenia	Healthy controls	Statistical test and *P* value	
			Mann–Whitney *U*	
	(n = 87)	(n = 105)	χ^2	*P*
Variables				
Sex, M/F	48/39	53/52	0.42	0.517
Age, mean (y)	27.7 ± 5.86 (16–36)	28.1 ± 4.14 (22–39)	−0.25	0.801
BMI	22.5 ± 4.59 (10.4–33.6)	21.3 ± 2.63 (17.0–31.9)	−1.54	0.124
Inflammatory biomarkers				
sTNFR1 (pg/ml)	1022.8 ± 199.3 (556–1480)	912.8 ± 135.1 (585–1220)		
	989 (894, 1160)	901 (826.5, 1002)	−3.88	**<0.001**
adiponectin (μg/ml)	9.47 ± 4.57 (2.20–20.0)	10.7 ± 4.72 (3.30–27.4)		
	8.90 (5.40, 12.3)	9.70 (7.70, 13.2)	1.70	0.089
PEDF (μg/ml)	11.5 ± 3.60 (5.14–23.1)	12.7 ± 4.52 (5.00–27.9)		
	11.0 (9.01, 13.9)	11.8 (9.47, 15.1)	1.55	0.120

Continuous demographic variables are presented as mean ± standard deviation and (range). Inflammatory biomarkers are presented as both mean ± standard deviation (range) in upper lines and as median (quartiles) in lower lines.

Sex ratio compared by χ^2 test, all other variables by Mann−Whitney U test

sTNFR1, soluble tumor necrosis factor receptor 1; PEDF, pigment epithelium-derived factor; BMI, body mass index

P values with statistical significance are in **bold** font.

Table 2. Comparison of demographic variables, clinical variables, and serum inflammatory biomarkers between patients with schizophrenia and healthy controls at admission in clinical practice

	Patients with schizophrenia	Healthy controls	Statistical test and *P* value	
			Mann–Whitney *U*	
	(n = 213)	(n = 110)	χ^2	*P*
Variables				
Sex, M/F	89/124	55/55	1.98	0.159
Age, mean (y)	40.9 ± 14.2 (16–76)	28.8 ± 5.62 (22–57)	−8.20	**<0.001**
BMI	22.7 ± 4.34 (10.4–34.7)	21.3 ± 2.67 (17.0–31.9)	−2.61	**0.009**
Onset (y)	25.5 ± 10.1 (11–66)	NA		
Duration of education (y)	13.2 ± 2.47 (9–21)	NA		
Family history (Y/N)[a]	64/147	NA		
Duration of illness (y)	15.2 ± 11.7 (0–56)	NA		
DUP (mo)	25.0 ± 51.7 (0–480)	NA		
Number of admissions	2.18 ± 1.91 (1–14)	NA		
CP dose (mg/day)	597.8 ± 616.9 (0–3600)	NA		
BPRS (Total)	57.4 ± 15.0 (23–110)	NA		
(Positive)	18.7 ± 5.32 (7–33)	NA		
(Negative)	13.4 ± 4.53 (4–28)	NA		
GAF	29.4 ± 13.5 (5–85)	NA		
Inflammatory biomarkers				
sTNFR1 (pg/ml)	1124.6 ± 398.2 (556–3560)	923.2 ± 158.2 (585–1640)		
	1040 (900.3, 1227.5)	901.5 (823.5, 1015)	−5.54	**<0.001**
adiponectin (μg/ml)	11.3 ± 6.02 (2.20–36.8)	10.8 ± 4.73 (3.30–27.4)		
	10.4 (6.45, 14.7)	9.80 (7.70, 14.0)	−0.28	0.780
PEDF (μg/ml)	11.9 ± 4.06 (3.10–27.4)	12.6 ± 4.29 (5.00–27.9)		
	11.2 (9.02, 14.4)	11.8 (9.27, 15.1)	1.26	0.208

Continuous data are presented as the mean ± standard deviation and (range). In addition, inflammatory biomarkers are presented as the median (quartiles) at the low part.

BMI, body mass index; NA, not applicable; DUP, duration of untreated psychosis; CP dose, chlorpromazine equivalent dose; BPRS, Brief Psychiatric Rating Scale; GAF, Global Assessment of Functioning; sTNFR1, soluble tumor necrosis factor receptor 1; PEDF, pigment epithelium-derived factor.

P values with statistical significant are in **bold** font.

[a] Family history: Unclear for two patients.

Table 3. Comparison of clinical variables between the patients with and without medication and the healthy controls

	On Medication (M)	Medication-Free (MF)	Healthy Controls (HC)	M vs MF		M vs HC		MF vs HC		Kruskal–Wallis test	
	(n = 171)	(n = 42)	(n = 110)	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Variables											
Sex, M/F	71/100	18/24	55/55							2.01	0.367
Age, mean (y)	40.6 ± 14.9 (16–76)	42.0 ± 11.1 (23–63)	28.8 ± 5.62 (22–57)	1.23	0.220	7.54	**<0.001**	6.24	**<0.001**	68.7	**<0.001**
BMI	22.7 ± 4.32 (10.4–34.7)	22.6 ± 4.48 (15.4–34.1)	21.3 ± 2.67 (17.0–31.9)	−0.40	0.690	2.62	**0.009**	1.39	0.166	6.96	**0.031**
Onset (y)	24.2 ± 9.46 (11–66)	30.7 ± 10.8 (15–53)	NA	−3.56	**<0.001**						
Duration of education (y)	13.0 ± 2.51 (9–21)	13.8 ± 2.20 (9–18)	NA	−2.24	**0.025**						
Family history (Y/N) [a]	51/118	13/29	NA	0.01	0.922						
Duration of illness (y)	16.2 ± 12.1 (0–56)	11.3 ± 9.10 (0–35)	NA	2.29	**0.022**						
DUP (mo)	18.9 ± 33.1 (0–240)	49.6 ± 92.0 (1–480)	NA	−2.05	**0.040**						
Number of admissions	2.36 ± 2.05 (1–14)	1.45 ± 0.86 (1–5)	NA	3.22	**0.001**						
BPRS (Total)	57.2 ± 15.2 (24–110)	58.4 ± 14.5 (23–96)	NA	−0.92	0.357						
(Positive)	18.4 ± 5.38 (7–33)	19.8 ± 4.99 (8–28)	NA	−1.78	0.075						
(Negative)	13.4 ± 4.55 (4–28)	13.5 ± 4.51 (4–25)	NA	−0.32	0.749						
GAF	30.3 ± 13.4 (5–85)	26.0 ± 13.1 (5–70)	NA	2.07	**0.038**						
Inflammatory Biomarkers											
sTNFR1 (pg/ml)	1153.0 ± 401.8 (556–3560)	1006.0 ± 363.7 (709–2560)	923.2 ± 158.2 (585–1640)								
	1070 (924, 1270)	901 (807, 1095)	901.5 (823.5, 1015)	−3.71	**<0.001**	6.35	**<0.001**	0.71	0.476	44.5	**<0.001**
adiponectin (μg/ml)	11.5 ± 6.28 (2.20–36.8)	10.4 ± 4.81 (3.20–20.6)	10.8 ± 4.73 (3.30–27.4)								
	10.5 (6.60, 14.8)	9.60 (6.15, 14.4)	9.80 (7.70, 14.0)							0.53	0.766
PEDF (μg/ml)	12.1 ± 3.93 (4.31–26.4)	10.9 ±4.47 (3.10–27.4)	12.6 ± 4.29 (5.00–27.9)								
	11.5 (9.14, 14.4)	9.76 (7.99, 13.8)	11.8 (9.27, 15.1)	−2.11	0.035	−0.64	0.525	−2.42	**0.016**	6.04	**0.049**

Continuous data are presented as the mean ± standard deviation and (range). In addition, inflammatory biomarkers are presented as the median (quartiles) at the low part. M, patients on medication; MF, medication-free patients; HC, healthy controls; BMI, body mass index; NA, not applicable; DUP, duration of untreated psychosis; CP dose, chlorpromazine equivalent dose; BPRS, Brief Psychiatric Rating Scale; GAF, Global Assessment of Functioning; sTNFR1, soluble tumor necrosis factor receptor 1; PEDF, pigment epithelium-derived factor. *P* values with statistical significant are in **bold** font. Two-group comparisons were examined using the Mann–Whitney *U* test. Three-group comparisons were examined using the Kruskal–Wallis test. No significant difference was found in PEDF levels between M and MF (adjusted $P = 0.105$). [a] Family history: Unclear for two patients on medication.

Table 4. Changes in characteristics in the 121 patients with schizophrenia that were followed up

Variables	Paired-sample patients with schizophrenia (n = 121)			
Sex, M/F	51/70			
Age, mean (y)	41.2 ± 14.2 (17–76)			
Onset (y)	25.9 ± 10.5 (11–53)			
Duration of illness (y)	15.2 ± 11.7 (0–56)			
DUP (mo)	23.0 ± 36.2 (0–264)			
Duration of hospitalization (range), days	103.8 ± 89.7 (5–438)			
			Wilcoxon test	
	At admission	At discharge	*Z*	*P*
Variables				
CP dose (mg/day)	665.9 ± 644.9 (0–3000)	873.5 ± 541.9 (20–2223)	4.15	**<0.001**
BPRS scores (Total)	56.3 ± 15.0 (24–96)	39.7 ± 9.98 (18–64)	−8.90	**<0.001**
(Positive)	18.2 ± 5.33 (7–30)	12.4 ± 3.55 (5–21)	−8.59	**<0.001**
(Negative)	13.1 ± 4.33 (4–28)	10.5 ± 3.34 (4–20)	−6.45	**<0.001**
GAF	30.5 ± 14.0 (5–85)	53.1 ± 15.8 (20–100)	8.94	**<0.001**
BMI	22.7 ± 4.15 (14.7–33.6)	22.5 ± 3.97 (8.89–33.4)	−1.05	0.296
Inflammatory biomarkers				
sTNFR1 (pg/ml)	1123.1 ± 430.6 (655–3560)	1035.3 ± 261.7 (614-2460)		
	1040 (880.5, 1215)	998 (885.5, 1170)	−2.51	**0.012**
adiponectin (μg/ml)	11.4 ± 6.66 (2.20–36.8)	11.2 ± 6.05 (2.60–31.4)		
	10.5 (6.00, 14.7)	10.5 (6.20, 13.9)	−1.06	0.287
PEDF (μg/ml)	12.0 ± 3.71 (5.43–26.4)	12.0 ± 3.94 (3.39–30.2)		
	11.2 (9.14, 14.4)	11.3 (9.17, 13.8)	−1.03	0.303

Continuous data are presented as the mean ± standard deviation and (range). In addition, inflammatory biomarkers are presented as the median (quartiles) at the low part.

DUP, duration of untreated psychosis; CP dose, chlorpromazine equivalent dose; BPRS, Brief Psychiatric Rating Scale; GAF, Global Assessment of Functioning; BMI, body mass index; sTNFR1, soluble tumor necrosis factor receptor 1; PEDF, pigment epithelium-derived factor.

P values with statistical significant are in **bold** font ($P < 0.017$).

Table 5. Discrimination of patients with acute-stage schizophrenia from healthy controls using inflammatory biomarkers

A	87 patients with acute-stage schizophrenia versus 105 healthy matched controls after adjustment for confounders			
(Independent variables)	Sensitivity	Specificity	Accuracy	*P*
sTNFR1 and PEDF	57.5%	66.7%	62.4%	**<0.001**
sTNFR1	52.9%	67.6%	60.9%	**<0.001**
B	All 213 enrolled patients with acute-stage schizophrenia versus 110 healthy unmatched controls in clinical practice			
(Independent variables)	Sensitivity	Specificity	Accuracy	*P*
sTNFR1 and PEDF	56.9%	70.9%	61.5%	**<0.001**
sTNFR1	51.9%	75.5%	59.9%	**<0.001**
C	8 deterioration cases versus 110 healthy controls			
(Independent variable)	Sensitivity	Specificity	Accuracy	*P*
sTNFR1	75.0%	**94.5%**	**93.2%**	**<0.001**
D	8 deterioration cases versus 113 improvement cases			
(Independent variable)	Sensitivity	Specificity	Accuracy	*P*
aTNFR1	82.8%	62.5%	81.0%	0.038

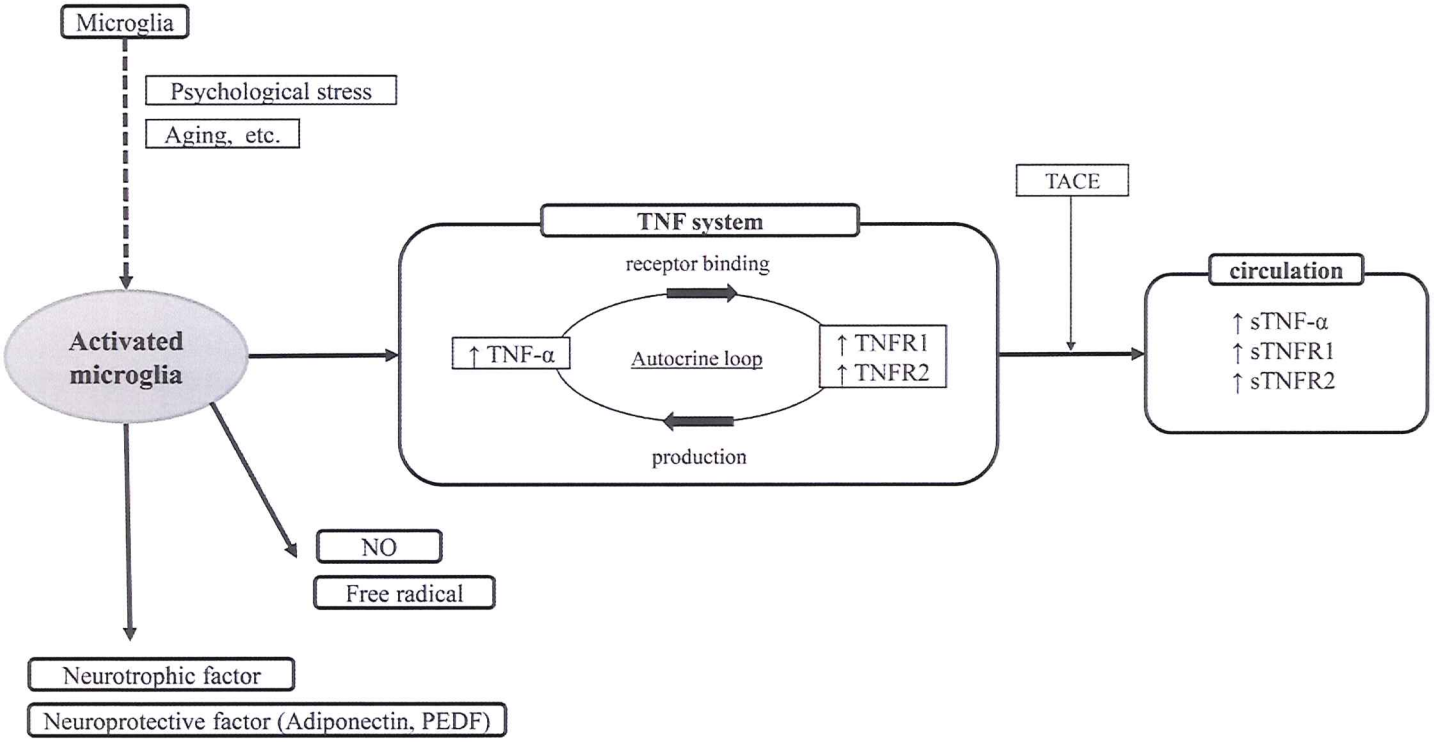
Sensitivity, specificity, and accuracy using sTNFR1 and PEDF together and sTNFR1 alone as the independent variable are shown in A and B.

sTNFR1, soluble tumor necrosis factor receptor 1; PEDF, pigment epithelium-derived factor

P values with statistical significant are in **bold** font ($P < 0.017$).

Highlights

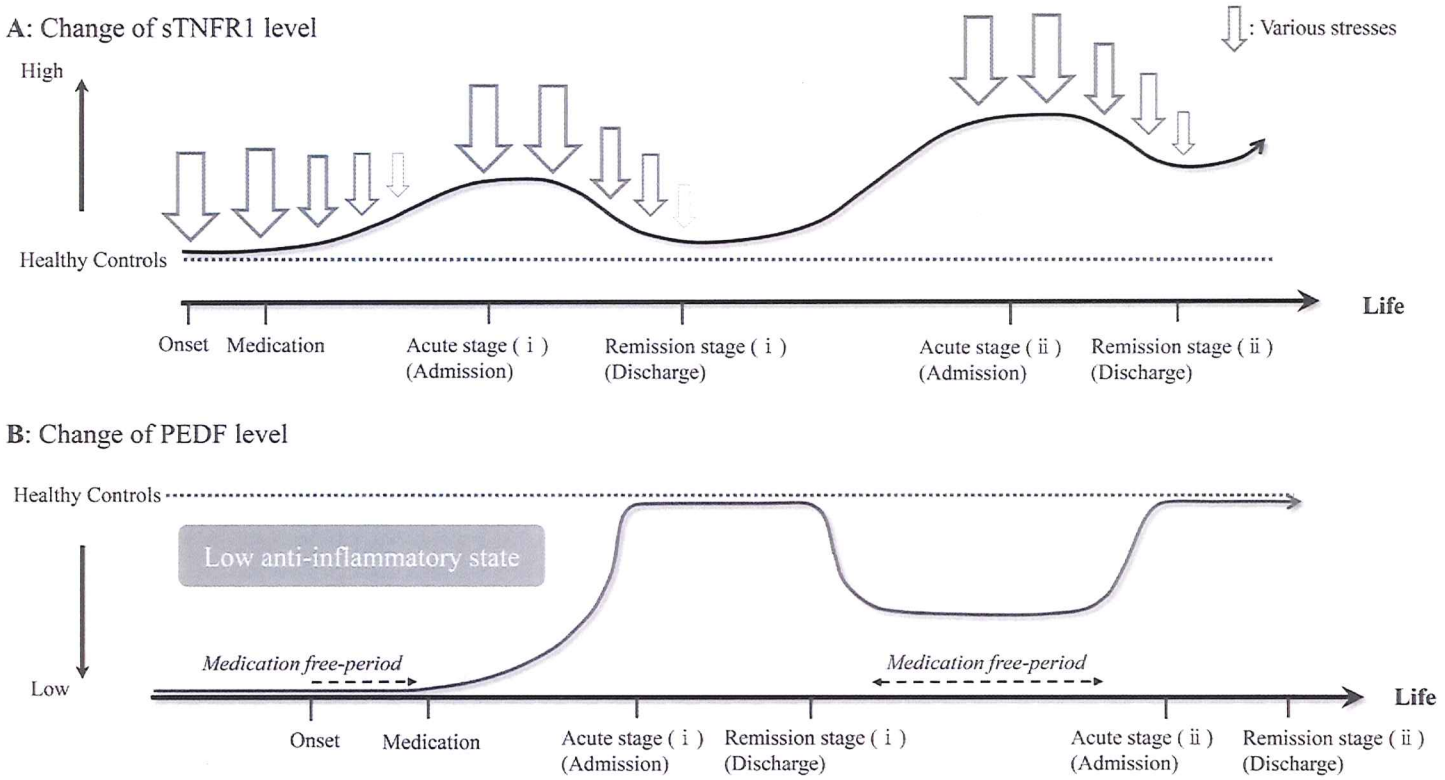
- We compared inflammatory biomarkers between acute-stage schizophrenics and controls
- Serum sTNFR1 was significantly higher in patients with schizophrenia than in controls
- Medication-free patients presented with significantly lower PEDF than controls
- Serum sTNFR1 accurately discriminated treatment-refractory patients from controls
- High sTNFR1 could be a prognostic biomarker for acute-stage schizophrenia



Supplemental Figure 1. Inflammatory mechanism focused on the TNF system

When the brain is exposed to psychological stress or aging, the microglia develop to an activated state. The activated microglia release various inflammatory cytokines, NO, free radicals, neurotrophic factor, and neuroprotective factor. TNF-α binds to TNFR1 and TNFR2, and the TNFRs induce TNF-α production. These "autocrine loops" chronically continue to act on the TNF system. TNF-α, TNFR1, and TNFR2 are cleaved by TACE, and subsequently exist as sTNF-α, sTNFR1, and sTNFR2 in circulation. These systems ultimately induce neuro-inflammation.

TNF, tumor necrosis factor; NO, nitric oxide; TNF-α, tumor necrosis factor-α; TNFR1, tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2; PEDF, pigment epithelium-derived factor; TACE, tumor necrosis factor-α converting enzyme; sTNF-α, soluble tumor necrosis factor-α; sTNFR1, soluble tumor necrosis factor receptor 1; sTNFR2, soluble tumor necrosis factor receptor 2



Supplemental Figure 2. The change in serum sTNFR1 and PEDF levels during the clinical course of patients with schizophrenia

The size of the arrows indicates the levels of various stresses, including psychological stress. All patients with schizophrenia were initially treated with medication at the acute stage (admission). The medication-free patients, including drug-naïve patients and those with relapse at the acute stage, exhibited low PEDF levels (indicating a low anti-inflammatory state) compared to healthy controls, but no difference in sTNFR1 levels. During acute exacerbation, greater stresses (arrows) were associated with higher sTNFR1 levels than in the healthy controls. Subsequently, sTNFR1 levels and psychological stresses decreased in the patients from acute stage to remission with medication treatment. PEDF levels did not change during intensive therapy with antipsychotic medication.
PEDF, pigment epithelium-derived factor; sTNFR1, soluble tumor necrosis factor receptors 1

Supplemental methods

Measurement of serum inflammatory biomarkers

Blood samples were taken in the morning after fasting to control for the influences of diet and exercise. The required sample volume was a total of 5 ml. The samples were immediately centrifuged, and the separated supernatants were stored at −80°C until the analysis.

The studies were conducted in serum rather than plasma because we could only establish reliable immunoassays for these three inflammatory biomarkers in serum. Serum soluble tumor necrosis factor receptor 1 (sTNFR1) levels were measured using a sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The sample was collected using a serum separator tube and clotted at room temperature for 30 min before centrifugation for 15 min. After removing the serum, the sample was assayed immediately or aliquoted and stored at less than −20°C. Excess microplate strips were removed from the plate frame and returned to the foil pouch containing the desiccant and then resealed. 50 µl of assay diluent was added to each well, followed by 200 µl of standard, control, or sample. These were covered with a plate sealer and then incubated at room temperature for two hours.

After each well was aspirated and washed, the process was repeated twice for a total of three washes. 200 µl of sTNFR1 conjugate was added to each well. The plate was covered with a new plate sealer and incubated at room temperature for two hours. Each well was aspirated and washed three times. 200 µl of substrate solution was added to each well, and the plate was incubated at room temperature for 20 min and protected from light. 50 µl of stop solution was then added to each well. If the color in the well was green or the color change did not appear uniform, the plate was gently tapped to ensure thorough mixing. Within 30 min, the absorbance at 450 nm was read. The wavelength correction was set to 540 nm or 570 nm. The detection limit was 10 pg/ml.

Serum adiponectin levels were measured using a latex particle-enhanced turbidimetric immunoassay (LTIA) (Nishimura and Sawai, 2006). As reagents, polystyrene latex particles (JSR Corporation, Tokyo, Japan), bovine serum albumin (Sigma, St. Louis, MO), and anti-human adiponectin antiserum (Otsuka Pharmaceutical Corporation, Osaka, Japan) were prepared. Next, 2 µl serum and 90 µl of 0.1 mol/l Tris-HCl buffer containing 0.9% NaCl were injected into the reaction cuvette. After incubation for 5 min at 37°C, 90 µl of the antibody-immobilized latex bead suspension in 0.01 mol/l Tris-HCl buffer was added to the cuvette to start the turbidimetric immunoreaction. The adiponectin concentration was then calculated from the difference in absorbance values

at 5 and 10 min with dual wavelength measurement (570 nm and 800 nm). The detection limit was 0.2 μg/ml.

Serum pigment epithelium-derived factor (PEDF) levels were measured using competitive ELISA (Fukami et al., 2010). A 96-well microtiter plate was coated with 5 μg/ml anti-PEDF monoclonal antibody (Transgenic, Kumamoto, Japan) and incubated overnight at 4°C. 50 μl of serum was pretreated with 200 μl of 8 M urea at 4°C for one hour. Then, each sample was diluted 50-fold with 10 mM phosphate buffer saline. After washing the microplate, 100 μl aliquots of standard recombinant human PEDF proteins (Chemicon International, Temecula, CA) or 50-fold diluted serum were added to the wells and the plate was incubated at room temperature for two hours. After the well was washed four times, 50 μl of biotinylated anti-human PEDF polyclonal antibody (R&D Systems, Minneapolis, MN) was added to each well. After incubation at room temperature for two hours, the plate was incubated with 100 μl of horseradish peroxidase-conjugated streptavidin solution (Zymed, South San Francisco, CA) at room temperature for 30 min. After the well was washed again, 50 μl of chromogenic substrate solution (Dako, Tokyo, Japan) was added to each well and the plate was incubated to shake at room temperature for 15 min. After the color developed, 50 μl of reaction stopper was added. The plate was read at 450 nm using a microplate reader. The detection limit was 0.045 ng/ml.

Supplemental References

Fukami, K., Yamagishi, S.I., Okuda, S., 2010. Development of enzyme-linked immunosorbent assay system for PEDF and its clinical utility. Curr. Mol. Med. 10(3), 317-320.

Nishimura, A., Sawai, T., 2006. Determination of adiponectin in serum using a latex particle-enhanced turbidimetric immunoassay with an automated analyzer. Clin. Chim. Acta 371(1-2), 163-168.