In schizophrenia, deficits in natural IgM isotype antibodies including those directed to malondialdehyde and azelaic acid strongly predict negative symptoms, neurocognitive impairments and the deficit syndrome.

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Abstract

Schizophrenia is characterized by an interrelated activation of the immune-inflammatory response system (IRS) and the compensatory immune-regulatory reflex system (CIRS), which downregulates the IRS. Deficit schizophrenia is characterized by a deficit in natural regulatory autoimmune responses to tryptophan catabolites. The presence and correlates of IgM isotype antibodies to oxidative specific epitopes (OSEs), nitroso (NO) and nitro (NO_2) adducts in schizophrenia remain unknown.

This study measured IgM antibodies to malondialdehyde (MDA), azelaic acid, phosphatidylinositol, oleic acid, NO-tryptophan, NO-albumin, NO-cysteinyl and NO_2-tyrosine in a sample of 80 schizophrenia patients, divided into those with and those without deficit schizophrenia, and 38 healthy controls.

Deficit schizophrenia was characterized by significantly lower IgM antibody levels to all OSEs as compared with non-deficit schizophrenia and controls. Lowered IgM antibodies to MDA coupled with increased IgM levels to NO-cysteinyl and NO_2-tyrosine strongly predict deficit schizophrenia versus non-deficit schizophrenia with an area under the ROC curve of 0.913. A large part of the variance (21.2 – 42.2 %) in the negative symptoms of schizophrenia and excitation is explained by IgM antibody titers to MDA (inversely) and NO-cysteinyl and/or NO_2-tyrosine (both positively). Lower IgM antibodies to MDA are significantly associated with impairments in episodic memory including direct and delayed recall.

These findings further indicate that deficit schizophrenia is a distinct phenotype of schizophrenia, which is characterized by lower natural IgM antibody levels to OSEs and relative increments in nitrosylation and nitration of proteins. It is concluded that deficits in natural autoreactive

antibodies of the IgM isotype attenuate CIRS functions and that this impairment may drive negative symptoms and impairments in episodic memory and thus deficit schizophrenia.

Key words: immune, inflammation, natural IgM autoimmune, oxidative stress, kynurenine, schizophrenia, psychosis

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Introduction

In 1995, Smith and Maes [1] proposed the monocyte-T lymphocyte theory of schizophrenia, which considered the role of activated immune-inflammatory pathways in the neurodevelopmental pathology of schizophrenia through effects of prenatal infections, causing increased oxidative and nitrosative stress (O&NS), cytokine-induced stimulation of the tryptophan catabolite (TRYCAT) pathway, modulation of glutamate production and microglial activation. Now, more than 2 decades later, there is abundant evidence that the different schizophrenia phenotypes are characterized by activated immune-inflammatory processes in the peripheral blood [2] and brain [3,4].

Thus, the acute (first episode psychosis and acute relapses) and more chronic phases of schizophrenia (chronic, treatment resistant and stable-phase schizophrenia) are accompanied by activation of the immune-inflammatory response system (IRS) as indicated by increased plasma concentrations of acute phase proteins (APPs), complement factors and pro-inflammatory cytokines and chemokines [2]. Those different schizophrenia phenotypes are characterized by elevated levels of interleukin (IL-1)-1β, IL-6, IL-2, IL-12, IL-17, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, indicating activation of M1 macrophagic, T helper (Th)-1 and Th-17 immune cell phenotypes [2]. In accordance with M1 macrophagic activation, there are also data that schizophrenia is accompanied by increased production of nitric oxide (NO) and nitro-tyrosine coupled with lipid peroxidation and consequent aldehyde production, including malondialdehyde (MDA) [5].

Nevertheless, the same patients also show increased levels of IL-4, IL-5, IL-13, IL-10 and transforming growth factor (TGF)-β1, indicating activated Th-2 and T regulatory (Treg) immune phenotypes, which are generally anti-inflammatory [2]. Moreover, the same patients also show

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increased levels of soluble IL-2 receptor (sIL-2R), sIL-1R antagonist (sIL-1RA), sTNF-R1 and sTNF-R2, findings which not only indicate IRS activation but also concomitant immune-regulatory effects on IL-1, IL-2 and TNF-α pro-inflammatory signaling [2]. Those Th-2 and Treg cytokines, soluble cytokine receptors and APPs (including haptoglobin) exert multiple negative feedback signals on the IRS thereby attenuating the primary IRS response [2]. This system therefore was named the compensatory immune-regulatory system (CIRS) [2,6,7]. Interestingly, in schizophrenia both activation of the IRS and CIRS are strongly interrelated phenomena, while first-episode psychosis is accompanied by a significantly increased IRS / CIRS ratio [2,8]. Immune mediators produced by M1 cells (e.g. IL-1, IL-6 and TNF-α), and Th-1 (e.g. IL-2 and IFN-γ), Th-17 (e.g. IL-17) and Th-2 (e.g. IL-4, IL-5, IL-13 and CCL3 or eotaxin) cells coupled with activated oxidative and nitrosative stress (O&NS) pathways, may exert neurotoxic effects and hence cause neuroprogressive processes [2,8,9,10,11].

Most importantly, deficits in the CIRS were observed in different schizophrenia subtypes. For example, FEP is accompanied by a relative lack of plasma sIL-2R, sTNF-R1, sTNF-R2 and sIL-1RA responses, which may increase the vulnerability to develop more prominent IRS responses after immune injuries **[**8**].** Plasma levels of CC16 or uteroglobulin, an endogenous anti-cytokine, are significantly lowered in patients with schizophrenia versus healthy controls **[**12,13**].** Deficit schizophrenia is characterized by a highly significant deficit in IgM antibody levels directed against tryptophan catabolites **(**TRYCATs**) [**14**].** Since natural IgM antibodies directed against endogenous antigens are generally immune-regulatory, such findings may point towards a deficit in the CIRS functions in deficit schizophrenia **[**14**].** Importantly, the deficit in IgM isotype antibody responses to TRYCATs was highly significantly associated with the negative symptoms of schizophrenia and neurocognitive impairments in semantic and episodic memory **[**14**].**

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Another component of the CIRS consists of IgM antibodies directed against oxidative specific epitopes **(**OSEs**)**, including MDA and azelaic acid **[**15**].** For example, in women with perinatal depression, IgM isotype-mediated responses directed to MDA are inversely associated with multiple signs of nitro-oxidative stress and depressive symptoms as well [15]. IgM antibodies to MDA protect against cardio-vascular disorder, are a first line defense against micro-organisms, have anti-inflammatory activities and eliminate apoptotic cells thereby promoting tissue-homeostasis [16,17]. Nevertheless, no studies have examined IgM antibodies to OSEs (including MDA and azelaic acid) in deficit and non-deficit schizophrenia. Since nitric oxide (NO) production may be enhanced in schizophrenia [18,19], it is plausible that increased nitrosylation (with consequent formation of nitroso-adducts) and nitration (with consequent formation of NO_2-adducts) is present in schizophrenia. Nevertheless, no studies have examined IgM responses to NO- and NO_2-adducts in schizophrenia.

Hence, the current study was carried out to examine 1) whether deficit schizophrenia is accompanied by a deficit in IgM antibody levels to OSEs as compared with non-deficit schizophrenia and healthy controls; and whether these antibodies are inversely associated with negative symptoms and neurocognitive deficits; and 2) whether IgM isotype antibody levels to NO- and NO_2-adducts are increased in schizophrenia.

Methods

Participants

This study recruited 118 participants, including 38 healthy controls and 80 participants with schizophrenia who attended the Policlinic of the Department of Psychiatry at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. All patients were in a stabilized phase of

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schizophrenia without any acute episodes for at least one year. They all fulfilled the diagnostic criteria for schizophrenia according to DSM-IV-TR criteria. Moreover, patients were divided into those with and without deficit schizophrenia according to the Schedule for Deficit syndrome SDS [20]. Healthy controls were recruited by word of mouth from the same catchment area as the patients, namely Bangkok, Thailand. Controls were excluded when they had suffered from lifetime or current diagnoses of axis I diagnoses according to DSM-IV-TR criteria and when they had a positive family history of schizophrenia. We employed the following exclusion criteria for schizophrenia patients: a) acute psychotic episodes the year prior to inclusion; b) axis-1 DSM-IV-TR disorders other than schizophrenia, including bipolar disorder, major depression, schizoaffective disorder, psycho-organic disorders, and substance use disorders; c) neurological disorders including Parkinson's disease, stroke, Alzheimer's disease and multiple sclerosis; d) use of any medication that could interfere with immune functions, including immunomodulatory drugs, antioxidant supplements and supplements with ω3-polyunsaturated fatty acids; and e) medical illness including rheumatoid arthritis, psoriasis, diabetes (type 1 and 2), COPD, and inflammatory bowel disease.

All controls and patients as well as the guardians of patients, namely parents or other close family members, provided written informed consent prior to participation in this study. The study was conducted according to International and Thai ethics and privacy laws. Approval for the study (298/57) was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, which is in compliance with the International Guidelines for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

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Measurements

Clinical assessments

All socio-demographic and clinical data in all subjects were assessed using a semi-structured interview by one and the same senior psychiatrist, specialized in the treatment of schizophrenia (BK). The latter also scored the SDS [20] and the Scale for the Assessments of Negative Symptoms [21]. We also used the Positive and Negative Syndrome Scale (PANSS) to assess negative (PANSSneg) and positive (PANSSpos) symptoms [22]. The DSM-IV-TR diagnostic criteria of schizophrenia were made using the Mini-International Neuropsychiatric Interview (M.I.N.I.) in a validated Thai translation [23]. Based on the items of the PANSS and the Brief Psychiatric Rating Scale [24] we computed four z-unit weighted composite scores reflecting four different symptom dimension scores, namely psychotic symptoms, hostility, excitation and mannerism [25]. Psychotic symptoms were assessed as the sum of the z score of PANSS P1 (delusion) (zP1) + zP3 (hallucinations) + zP6 (suspiciousness) + zBPRS11 (suspiciousness) + zBPRS12 (hallucinatory behavior) + BPRS15 (unusual thought content). Hostility was computed as the sum of zP7 (hostility) + zPANSS general14 (zG14, poor impulse control) + zBPRS10 (hostility) + zBPRS14 (uncooperativeness). The excitement subscore was computed as zP14 (excitement) + zP5 (grandiosity) + zBPRS8 (grandiosity) + zBPRS17 (excitement); and mannerism was computed as zG5 + zBPRS7 (both mannerism and posturing). The diagnosis of Tobacco Use Disorder (TUD) was made using DSM-IV-TR criteria. Body mass index (BMI) was assessed the same day as the clinical interview and rating scale scoring as body weight (kg) / length (m^2).

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In addition, a well-trained research assistant, master in mental health and blinded to the clinical diagnosis, measured four CERAD (Consortium to Establish a Registry for Alzheimer's disease)-Neuropsychological [26] and three CANTAB (Cambridge Neuropsychological Test Automated Battery) tests [27], which were performed the same day the semistructured interview and clinical scoring were completed. The four CERAD tests are: a) the Mini-Mental State Examination (MMSE), which probes different functions including orientation, naming, concentration, constructional praxis and memory; b) Verbal Fluency Test (VFT) to probe semantic memory and fluency; c) Word List Memory (WLM) to assess verbal episodic memory and learning ability; and d) Word List Recall, true recall (True Recall) to assess be verbal episodic memory recall. In addition, we used an Episodic Memory principal component (PC) extracted from CERAD episodic memory tests [28]. We have also used a latent vector extracted from three CANTAB tests reflecting severity of executive functions [29], namely a) Spatial working memory between errors (SWM_BE) and Strategy (SWM_STR) to probe executive working memory ability and task strategy used by the central executive; and b) One touch stockings of Cambridge, probability solved on first choice (OTS_PSOFC), to probe spatial planning.

Assays

In patients and controls, fasting blood was sampled at 8.00 a.m. for the assay of IgM-mediated autoimmune responses directed against OSEs, NO-adducts and NO_2-tyrosine. An enzyme-linked immunosorbent assay (ELISA) was used to measure IgM levels directed against conjugated azelaic acid, MDA, phosphatidylinositol (Pi) and oleic acid [30-33]. Azelaic acid, MDA, PI and oleic acid were linked to fatty acid free-BSA according to previously described methods [30-33]. Synthesis of the conjugates to delipidated BSA was performed as described

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before [32]. In order to mimic nitrosylation and nitration processes, NO-tryptophan (NOW), NO-cysteinyl and NO_2-tyrosine were synthesized by linking haptens to BSA (Sigma-Aldrich) using glutaraldehyde [31,35,36]. The synthesis of these conjugates has been described previously [37]. Each hapten conjugate was nitrosylated using sodium nitrite ($NaNO_2$) dissolved in 2 ml of each conjugate, in 0.5 M HCl at 37°C for 2 h, while shaking in the dark. Conjugates were then dialyzed at 4°C for 24 h against a Phosphate Buffered Saline (PBS: 10^{-2} M NaH_2PO_4, $12H_2O$; 0.15M NaCl; pH 7.4) solution. S-nitrosothiol bond formation was determined by spectrophotometry. The S-nitrosothiol compounds possess two absorbance maxima, at 336 and 550 nm, respectively: e_{336} nm= 900 $M^{-1}cm^{-1}$ for the conjugates, e_{550}nm = 4000 $M^{-1}cm^{-1}$ for BSA. Absorbance was evaluated in order to determine NO concentrations linked to the compounds. The detection of IgM autoantibodies to the conjugates was performed by an indirect ELISA tests [33,37]. Briefly, polystyrene 96-well plates (NUNC) were coated with 200 µl solution containing the conjugates or BSA in 0.05 M carbonate buffer at pH 9.6. Well plates were incubated at 4°C for 16 h under agitation. Then, a 200 µl of blocking solution (PBS, 2.5 g/l BSA) was added for 1 h and placed at 37°C. Following three washes with PBS, plates were filled up with 100 µl of sera diluted at 1:1000 in the blocking buffer A (PBS, 0.05% Tween 20, 10% Glycerol, 2.5 g/l BSA, 1 g/l BSA-G) and incubated at 37°C for 2 h. After three washes with PBS-0.05% Tween 20, plates were incubated at 37°C for 1 h with peroxidase-labeled anti-human IgM secondary antibodies diluted respectively at 1: 15,000, in the blocking buffer (PBS, 0.05% Tween 20, 2.5 g/l BSA). They were then washed three times with PBS-0.05% Tween 20, and incubated with the detection solution for 10 min in the dark. Chromogen detection solution was used for the peroxidase assay at 8% in 0.1 M acetate and 0.01 M phosphate buffer (pH 5.0) containing 0.01% H_2O_2. The reaction was stopped with 25

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µl 2-N HCl. ODs were measured at 492 nm using a multiscan spectrophotometer. All assays were carried out in duplicate. The intra-assay coefficients of variation (CV) were < 6%.

Statistical analysis

Analysis of variance (ANOVAs) was employed to assess differences in scale variables between groups, while analysis of contingency tables (X^2-test) was used to check associations between nominal variables. Multinomial regression analysis was used to assess the most important IgM responses predicting deficit versus non-deficit schizophrenia versus normal controls, while binary regression analysis was used to delineate the most significant predictors of deficit schizophrenia versus non-deficit schizophrenia + normal controls. Odds ratios (OR) and 95% confidence intervals were computed in both multinomial and binary regression analysis. Multiple regression analysis was employed to check the most significant IgM responses that predict dependent variables including negative symptoms and cognitive test results. Correlation matrices were assessed employing Pearson's product moment and Spearman's rank order correlation coefficients. We used multivariate general linear model (GLM) analysis to check whether the IgM responses to OSEs and NO/NO2 adducts are predicted by extraneous variables including age, sex, BMI, and drug state of the patients. Consequently, tests for between-subject effects were used to examine the effects of the significant explanatory variables on the IgM responses. Receiver Operating Characteristics (ROC) analysis was used to compute the area under the ROC curve. We used Ln transformations of the OD values in order to normalize the data distribution of the IgM-responses. All Ln OD data were consequently processed in z transformations and we computed a z-unit weighted composite score reflecting total IgM responses to OSEs (sum zOSEs), as zIgM directed to Ln MDA (zLnMDA) + zLn oleic acid + zLn Pi + zLn azelaic acid. Based on the latter

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index we divided our study group (n=118) into two subsamples (n=59 each) using the median-split method. All results of regression analyses were checked for multicollinearity. In addition, we also interpreted the bootstrapped (n=1000) results and report when there are differences between results with and without bootstrapping. Statistical analyses were performed using IBM SPSS windows version 22. Tests were 2-tailed and a p-value of 0.05 was used for statistical significance.

Results.

1. Socio-demographic data

Table 1 shows the socio-demographic and clinical data in two subgroups divided according to the sum zOSE values using the median split method (median = 0.1186). Doing so we have two study groups, one with increased and a second with lowered IgM levels to sum zOSEs. We did not correct the p-values in this table for multiple testing as these data together with the intercorrelation matrices were only used to delineate the variables to be used as explanatory variables in the ultimate regression analysis. Table 1 shows that there were no differences in age, sex, education, marital status, smoking behavior, BMI, number of psychotic episodes, PANSS positive score, psychosis, hostility, VFT, MMSE and a first PC extracted from the three executive tests between both study groups. Subjects allocated to the low sum zOSE group showed significantly higher scores on SDS, PANSS negative, excitement, mannerism, and episodic memory PC as compared with subjects with higher OSE values. Subjects allocated to the low sum zOSE group showed significantly lower values of WLM and true recall as compared with subjects belonging to the group with higher sum zOSE values.

Associations between IgM antibodies and deficit schizophrenia

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Figure 1 shows the measurements of the IgM antibodies to OSEs, NO and NO_2-adducts (all in z transformations of the Ln transformed values). In order to examine the associations between the IgM responses to OSEs and NO/NO_2-adducts we have performed multinomial logistic regression analysis with diagnosis (deficit versus non-deficit schizophrenia versus control) as dependent variables and the 8 IgM responses as explanatory variables. **Table 2** shows the outcome of these multinomial regression analyses. IgM responses to oleic acid, MDA, azelaic acid and Pi were significantly associated with deficit *versus* non-deficit schizophrenia and *versus* normal controls, whilst these IgM levels did not differ between non-deficit schizophrenia and controls. P-correction for FDR (8 IgM measurements) showed that the differences in IgM responses to oleic acid, MDA, Pi and azelaic acid remained significant at the p=0.002 level. The largest impact size was observed for IgM responses to MDA, followed by azelaic acid and Pi. Entering age, sex and education as additional variables showed that sex had a significant effect on diagnosis, but did not change the associations between the IgM antibody levels and diagnosis.

For example, IgM antibodies to MDA (X^2=32.17, df=2, p<0.001) and sex (X^2=9.18, df=2, p=0.010) were significant in the multinomial regression analysis with diagnosis as dependent variable, whilst sex was significant in the differentiation of non-deficit schizophrenia versus controls (Wald=8.33, df=1, p=0.004) but not the two other differentiations. The IgM antibodies to NO/NO_2 adducts did not significantly predict diagnosis when entered alone in the analysis. Nevertheless, entering IgM levels to OSEs and NO / NO_2-adducts in the same regression showed that also the IgM responses to NO/NO_2-adducts were significant. Table 2 (last regression) shows that IgM antibodies to MDA (inversely) and NO-cysteinyl (positively) strongly predict deficit versus non-deficit schizophrenia and normal controls, while there are no significant effects differentiating between non-deficit schizophrenia and controls.

Table 3 examines the best predictors of deficit schizophrenia as dependent variable (versus the combined group of subjects with non-deficit schizophrenia and controls as reference group) and IgM responses, age, sex and education as explanatory variables. Binary logistic regression analysis shows that IgM antibodies directed against MDA (inversely) and NO-albumin (positively) significantly predict deficit schizophrenia; 79.9% of all subjects were correctly classified with a sensitivity of 67.5% and a specificity of 85.9%. The area under the ROC curve was 0.870 (SE=0.033, 95% confidence intervals: 0.805-0.935). The second logistic regression analysis in Table 3 shows that deficit schizophrenia (versus all other subjects) was also significantly associated with IgM responses directed against MDA (inversely) and to NO-cysteinyl (positively) whereby 81.4% of all subjects are correctly classified with a sensitivity of 70.0% and a specificity of 87.2%. The area under the ROC curve was 0.873 (SE=0.034, 95% confidence intervals: 0.806-0.940). Regression #3 shows that IgM responses to MDA (inversely) and NO-cysteinyl and NO_2-tyrosine (both positively) significantly predicted deficit versus non-deficit schizophrenia; 85.0% of the subjects were correctly classified with a sensitivity of 82.5% and a specificity of 87.5%. The area under the ROC curve was 0.913 (±0.35; 95% CI intervals: 0.843-0.982). Age, sex and education were not significant in these regression analysis.

Effects of extraneous variables on the IgM responses

In order to examine possible effects of age, sex, BMI, and education on the IgM levels we performed multivariate GLM analysis with the IgM responses to 4 OSEs and 4 NO/NO_2-adducts as dependent variables. There were no significant effects of sex (F=1.62, df=8/99, p=0.128), BMI (F=0.98, df=8/99, p=0.468), and education (F=1.14, df=8/99, p=0.344) on the IgM antibodies. There was a significant association between the IgM levels and age (F=4.05, df=4.05, df=8/99,

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p<0.001), although none of the tests for between-subjects effects was significant. In any case, the regression analyses used in this study were adjusted for possible effects of age, sex and education by entering those variables as additional explanatory variables in the regression analyses. In addition, there were no significant effect of smoking (yes or no) on the 8 IgM levels (F=1.04, df=8/98, p=0.415). We have also examined possible effects of the drug state, namely use of risperidone (n=33), clozapine (n=10), haloperidol (n=8), perphenazine (n=20), antidepressants (n=26), mood stabilizers (n=12) and anxiolytics/hypnotics (n=27). Multivariate GLM analysis showed no significant effects of risperidone (F=1.47, df=8/89, p=0.179), clozapine (F=1.89, df=8/89, p=0.072), perphenazine (F=0.31, df=8/89, p=0.960), antidepressants (F=1.74, df=8/89, p0.101), mood stabilizers (F=0.59, df=8/89, p=0.783) and anxiolytics/hypnotics (F=0.99, df=8/89, p=0.446). Without p-correction there was a significant effect of haloperidol on the IgM values (F=2.15, df=8/89, p=0.039). After p-correction for FDR these differences were no longer significant (p=0.236). Tests for between-subject effects showed significant effects (without p-correction) of haloperidol on IgM to Pi (p=0.042) and NO-albumin (p=0.011). Nevertheless, these differences were no longer significant after p-correction for FDR, namely IgM against Pi (p=0.168) and NO-albumin (p=0.088).

Associations between IgM antibodies and schizophrenia phenomenology

In order to examine which IgM values predict schizophrenia symptomatology we have carried out multiple regression analysis in schizophrenia patients with symptoms as dependent variables and the IgM antibodies as explanatory variables. **Table 4** shows that 42.7% of the variance in the SDS score was explained by the regression on IgM levels to MDA and azelaic acid (both inversely) and NO-cysteinyl and NO_2-tryrosine (both positively). 22.7% of the variance in

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restricted affect was explained by IgM responses to MDA (inversely) and NO-albumin (positively). 30.6% of the variance in diminished emotional range was explained by IgM responses to MDA (inversely) and NO-albumin and NO_2-tyrosine (both positively). 27.9% and 28.1% of the variances in poverty of speech and curbing of interest, respectively, were explained by the regressions on IgM responses directed against MDA (inversely) and NO-cysteinyl (positively). 25.9% of the variance in diminished sense of purpose was explained by the regression on IgM levels to MDA and NO_2-tyrosine. 42.2% of the variance in diminished social drive was explained by the regression on IgM levels to MDA and azelaic acid (both inversely) and NO-albumin and NO_2-tyrosine (both positively).

Further analysis showed that positive symptoms as measured with the PANSS were only very moderately (5.8% of the variance) associated with IgM antibodies to NO_2-tyrosine (positively), while a large part (25.8% of the variance) of the negative subscale score of the PANSS was associated with IgM to MDA (inversely) and NO_2-tyrosine (positively). A large part of the variances in excitation (21.2%) was explained by the regression on IgM antibodies to MDA and NO-albumin. There were no significant associations between the IgM responses and either psychotic symptoms, hostility or mannerism.

All above-mentioned analyses were rerun using age, sex and education as additional explanatory variables. Age and sex were not significant in these analyses whereas education was a significant predictor variable that however did not change the association between the schizophrenia symptoms and IgM levels except in the case of excitation. Table 4 shows a second regression with excitation as dependent variable including education as explanatory variable. We found that 28.8% of the variance in excitation was explained by the regression on education and IgM responses to azelaic acid, oleic acid (both negatively) and NO-albumin (positively).

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Associations among IgM antibodies and cognitive probes

We have also examined associations between the IgM antibodies to OSEs and NO/NO_2-adducts and neurocognitive tests (see **Table 5**) using multiple regression analysis with the cognitive tests as dependent variables and the IgM responses, age, sex and education as explanatory variables. There were no significant associations between VFT and the IgM levels. Education and IgM antibodies directed against Pi explained 38.6% of the variance in MMSE, whereby IgM responses to Pi had a weak albeit significant effect (3.7% of the variance) on MMSE scores. Education and IgM antibodies to MDA explained 21.9% of the variance in the episodic memory PC with MDA alone explaining around 12.7%. Education and IgM levels to MDA explained 28.6% of the variance in WLM with the IgM responses to MDA explaining around 8.8%. Education and IgM responses to MDA explained 28.4% of the variance in true recall with IgM responses explaining around 12.9% of the variance.

Discussion

The first major finding of this study is that the IgM isotype antibodies to different OSEs were significantly lower in patients with deficit schizophrenia as compared with non-deficit schizophrenia and controls. The decrease in these IgM levels to OSEs is highly sensitive and specific for deficit schizophrenia versus non-deficit schizophrenia. MDA and azelaic acid are both products of oxidative damage to membrane polyunsaturated fatty acids (PUFAs), which make up a large part of membrane phospholipids and are highly susceptible to oxidative damage by reactive oxygen species (ROS). The latter may induce lipid peroxidation resulting in higher levels of peroxyl radicals [38,39], which may damage more PUFAs thereby propagating lipid peroxidation

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and causing the production of reactive aldehydes including MDA [40,41]. Aldehydes such as MDA may in turn react with DNA thereby forming mutagenic DNA-adducts and promote toxic stress in cells which ultimately may lead to cell death [40,41]. Following aldehyde formation these neoepitopes such as MDA may be expressed on the surface of dying and apoptotic cells and oxidized LDL cholesterol particles as well as on circulating microparticles [42,43]. Expressed on these surfaces, the neoepitopes are recognized by immunocytes and consequently autoimmune, including adaptive IgM responses may be generated directed against these neoepitopes [44-46]. In addition, natural IgM antibodies, which have specificity for many OSEs, including MDA, are present without antigenic contact and in fact are part of the innate first-line defense against microorganisms [16,17]. Increased MDA levels are frequently, but not always, observed in schizophrenia [47,48], while mood disorders including major depression and bipolar depression are characterized by increased MDA levels or increased IgM responses to MDA [49-51, in preparation]. Thus, the findings in depression and bipolar disorder type 1 reporting increased IgM isotype-mediated responses to MDA, Pi and azelaic acid contrast the findings in deficit schizophrenia which is characterized by a highly significant decrease in IgM isotype antibodies to MDA. To the best of our knowledge there are no reports on MDA in the deficit phenotype of schizophrenia while we were unable to find significant associations between chronic schizophrenia and oxidative stress measurements including lipid peroxides and advanced oxidation protein products (AOPPs) [52].

Most importantly, IgM-mediated antibodies to MDA regulate immune-inflammatory responses by clearing inflammatory debris, including apoptotic and dying cells [42,44]. These IgM antibodies participate in first line defense through early recognition and elimination of invading infectious particles and they may suppress pathogenic IgG autoimmune responses [53]. As

reviewed in the introduction, in prenatal depression there are inverse associations between IgM isotype-mediated autoimmune responses directed to MDA and indicants of oxidative stress and depressive symptoms, findings which indicate that these IgM antibodies have protective functions [15]. Therefore, we proposed that this type of IgM antibodies are part of the CIRS [7,15].

Azelaic acid (or nonanedioic acid) is produced following oxidative damage to linoleic acid through formation of oxo-nonanoic acid or alternatively by oxidation of oleic acid at the 9 carbon with consequent degradation to azelaic acid [54]. Interestingly, azelaic acid has anti-inflammatory and anti-oxidant effects and therefore should be regarded as another component of the CIRS. For example, besides its anti-inflammatory functions, azelaic acid is a strong antioxidant which may inhibit the production of O_2-, OH and H_2O_2 and the peroxidation of arachidonic acid by reactive hydroxyl ions [55-57]. Moreover, azelaic acid may inhibit neutrophil functions including the production of ROS produced by neutrophils [58]. Here we observed that deficit schizophrenia is accompanied by highly significant decreases in IgM antibodies to azelaic acid, which are not detected in non-deficit schizophrenia, while depression and bipolar depression are accompanied by increased IgM responses to azelaic acid [49, in preparation]. IgM antibodies to OSEs are, in general, immune-regulatory and play a protective role against immune-inflammatory disorders including cardio-vascular disorder [59-61]. All in all, our results indicate that lower IgM levels to azelaic acid increase risk against deficit schizophrenia probably via lowered immune-regulation.

Also the IgM isotype antibodies directed against two other membrane components, namely oleic acid and Pi, were significantly lowered in deficit schizophrenia. Oleic acid is a monounsaturated omega 9 fatty acid, which plays a key role in membrane fluidity and additionally acts as a neurotrophic factor inducing neuronal differentiation [62]. Pi is another important constituent of membranes that is additionally involved in intracellular signalling cascades that

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regulate cell survival, proliferation, calcium levels and polarization [63]. After oxidative disruption of lipid membranes and oxidative modifications both oleic acid and Pi may be recognized by the immune system, which consequently mounts an IgM-mediate autoimmune response [45,64]. Depression, for example, is accompanied by increased IgM responses to both oleic acid and Pi indicating oxidative damage to lipid biomembranes [45,50]. Increased circulating levels of IgM antibody titers to Pi are observed in other inflammatory disorders including multiple sclerosis in association with the inflammatory responses during acute relapses [65]. Our findings show that lowered IgM antibody titers directed against both oleic acid and Pi are specific for the deficit phenotype as compared with the nondeficit phenotype and therefore that these natural IgM antibody titers confer protection against the deficit phenotype.

It is important to note that deficit schizophrenia is also accompanied by lowered IgM antibody titers to TRYCATs, while the IgA responses to TRYCATs are increased [14,28]. Thus, our studies show that the deficit phenotype is characterized by more general deficits in natural IgM isotype antibody titers to OSEs and TRYCATs and that such deficits do not occur in nondeficit schizophrenia. This deficit in CIRS functions may result in attenuated regulation of neuro-immune responses and increased responsivity of immune-inflammatory and TRYCAT pathways.

The second major finding of this study is that after considering the effects of IgM titers to OSEs, the IgM responses to NO and NO_2-adducts significantly and positively predicted deficit schizophrenia. Increased IgM responses to NO-adducts, including NOW, albumin and cysteinyl, and NO_2-adducts (NO_2-tyrosine) indicate increased nitrosylation and nitration of proteins, respectively. Interestingly, the IgM antibody titers to OSEs and NO-adducts were significantly correlated ($r=0.763$, $p<0.001$, $n=118$) and, therefore, the relative increments in IgM isotype responses to NO/NO_2 adducts in deficit schizophrenia probably reflect two divergent mechanisms,

namely a more general decrease in natural IgM isotype levels as well as a relative increase in nitrosylation and nitration. Interestingly, schizophrenia is accompanied by increased nitro-tyrosine production although the production of nitric oxide is not always increased [5,18,19], while hypernitrosylation is a hallmark of mood disorders, either depression or bipolar disorder [66; Maes et al., in preparation].

Recently, we have reviewed that mild nitrosylation when occurring in physiologic levels has immune-regulatory and neuroprotective effects [67,68]. For example, mild nitrosylation regulates cellular processes, has DNA repairing properties, mediates synaptic plasticity and neuronal survival [67,68]. Nevertheless, hypernitrosylation as a consequence of chronically activated nitro-oxidative and immune-inflammatory processes has many detrimental effects, including inactivation of proteins involved in autophagy, apoptosis and proteomic degradation, which may negatively affect neural functions and cell survival. In addition, hypernitrosylation, may adversely affect transcription factor activity and electron transport chain (ETC) enzymes, which may cause decreased mitochondrial function and energy production [68]. Finally, such changes may cause loss of immune tolerance and consequent development of autoimmunity. Increased IgM responses to NO_2-tyrosine indicate increased nitration of tyrosine (incorporation of a nitro-group) as a consequence of increased ROS and $^{\bullet}NO$ formation during immune-inflammatory responses [69,70]. In biomembranes, the formation of NO_2-tyrosine is associated with increased lipid peroxidation through formation of "one-electron oxidation of tyrosine by lipid peroxyl radicals" [69]. These findings may explain the significant associations between IgM isotype antibody titers to OSEs and NO_2-tyrosine ($r=0.680$, $p<0.001$) as detected in the present study. Tyrosine nitration yields immunogenic neoepitopes, which may cause functional changes

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of proteins that may contribute to dysfunctions in cell homeostasis, alter tyrosine-kinase-dependent pathways and facilitate protein degradation [69,70].

Nevertheless, the results of the present study showing that IgM-mediated immune responses to NO-adducts become significant after considering the effects of IgM antibody titers to MDA, do not allow to conclude that deficit schizophrenia is accompanied by hypernitrosylation, but rather that there may be a mild nitrosylation response in deficit schizophrenia. In this respect, we found that at the end of term pregnancy, there are significant inverse associations between prenatal depressive symptoms and IgM isotype-mediated responses to NO-adducts, suggesting that the latter have some immune-regulatory functions [15]. Nevertheless, increased IgM isotype antibody responses directed against SNO-cysteine have neurotoxic effects and are shown to cause demyelination and neurodegeneration [35-37]. Interestingly, recently we reported that increased IgM-mediated responses to NO-cysteinyl are a possible trait marker for major depression [15].

The third major finding of this study is that the negative symptoms of schizophrenia and excitation (but not psychotic symptoms. hostility and mannerism) as well as impairments in episodic memory are strongly associated with IgM antibody titers to OSEs (especially MDA) and IgM responses to NO-adducts (especially with NO-albumin and NO-cysteinyl). Based on the above discussion, we may conclude that these relationships may be explained by three factors. Firstly, a deficit in immune-regulatory IgM antibodies to MDA and azelaic acid may lower the regulatory effects on the immune-inflammatory processes. Recently, we reported that a deficit in IgM antibody titers to TRYCATs was also highly significantly associated with negative symptoms of schizophrenia and neurocognitive impairments [28], further indicating that deficits in the CIRS are extremely important for the negative symptoms of schizophrenia. Secondly, the association with increased IgM isotype antibody responses to NO-albumin may indicate a relatively increased

nitrosylation. Thirdly, it is also possible that the relatively increased levels of IgM isotype-mediated responses directed to NO_2-cyteinyl may contribute to the phenomenology of deficit schizophrenia through its neurotoxic effects. Future translational research should focus on the effects of nitrosylation, nitration and IgM responses to NO-cysteinyl on neurocognitive deficits and negative symptoms as well.

In conclusion, deficit schizophrenia is a distinct phenotype of schizophrenia, characterized by lowered natural IgM isotype antibody titers to OSEs and thus a deficit in the CIRS with lowered immune-regulatory feedback on the IRS. Moreover, deficit schizophrenia is accompanied by signs of increased protein nitration and nitrosylation. It is concluded that specific deficits in the CIRS coupled with increased neurotoxic effects of IgM responses to cysteinyl may drive the hallmarks of deficit schizophrenia, namely negative symptoms and related neurocognitive impairments in episodic memory.

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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. MM and BK designed the study. BK recruited patients and completed diagnostic interviews and rating scale measurements. MM carried out the statistical analyses. All authors (BK, SS, MM and AC) 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

Z transformations of the (Ln transformed) measurements of IgM antibodies to conjugated oleic acid (Lnoleic), malondialdehyde (MDA), azelaic acic, phosphatidylinositol (Pi), nitroso-cysteinyl (NOCys), nitro-tyrosine (NO2Tyr), NO-tryptophan (NOW) and NO-albumin (NOBSA) in healthy controls (HC), nondeficit schizophrenia (NONDEF) and deficit schizophrenia (DEF).

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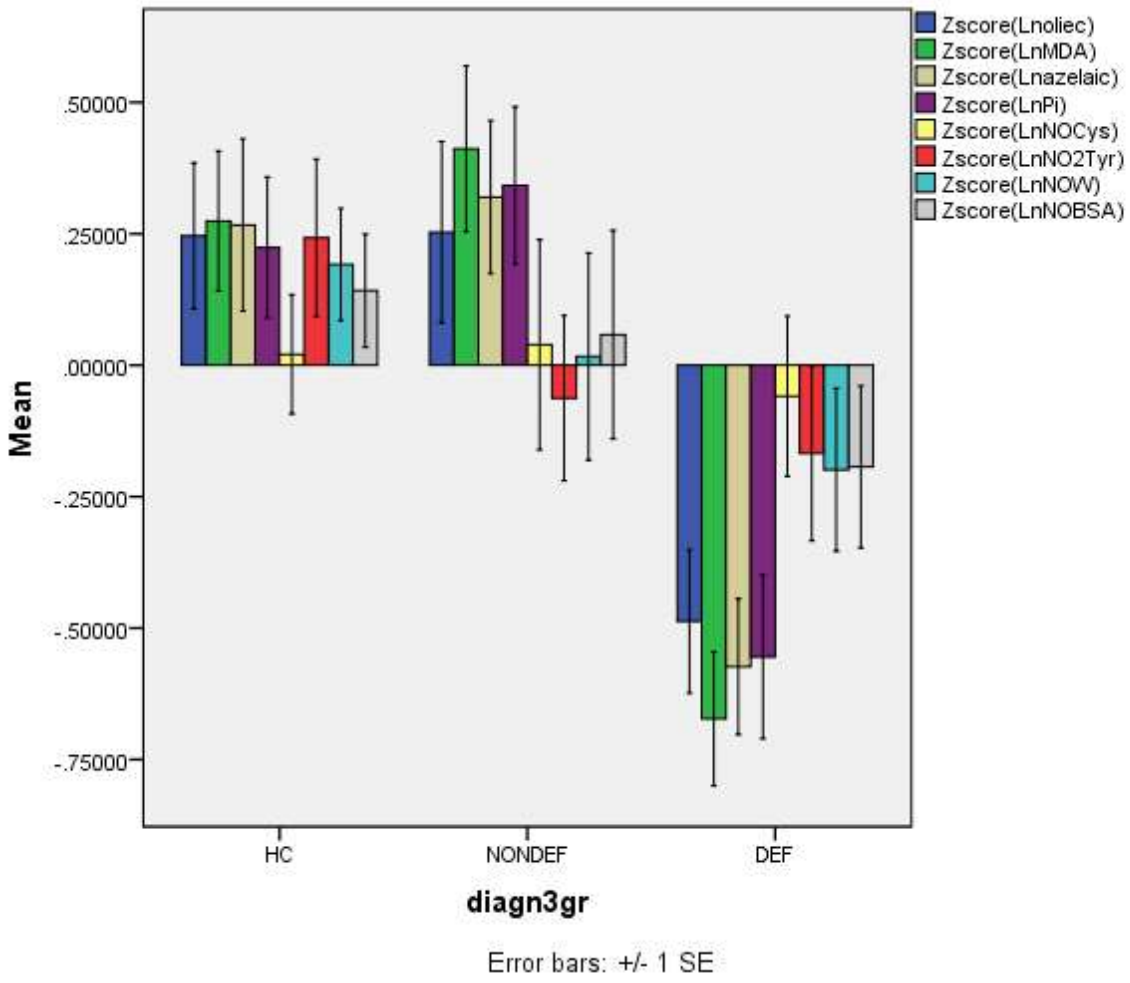

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Table 1. Socio-demographic and clinical data in subjects with lower IgM antibodies oxidative specific epitopes (sum zOSE) as compared to those with higher OSE values.

Variables	Sum zOSE < median (n=59)	Sum zOSE ≥ median (n=59)	F/X^2/Ψ	df	p
Age (years)	41.2 (11.8)	38.7 (11.6)	1.42	1/116	0.235
Gender (M/F)	31/28	21/38	3.44	1	0.064
Education (years)	12.4 (4.5)	13.4 (4.5)	1.28	1/116	0.260
Single / married / separated	41/12/5	41/12/5	0.00	2	1.00
TUD (N/Y)	55/4	56/3	Ψ=-0.036	-	0.697
BMI	24.1 (5.4)	24.4 (4.3)	0.11	1/111	0.740
Number of psychotic episodes	2.6 (2.9)	1.7 (2.4)	1.59	1/71	0.211
SDS total score	6.5 (6.5)	2.6 (4.0)	14.61	1/114	<0.001
PANSS negative	18.2 (11.6)	12.3 (7.9)	10.15	1/115	0.002
PANSS positive	12.2 (6.1)	11.8 (8.0)	0.10	1/115	0.756
Psychosis	0.187 (1.007)	-0.162 (0.976)	3.63	1/115	0.059
Hostility	0.101 (0.973)	-0.082 (1.037)	0.98	1/115	0.324
Excitement	0.226 (1.075)	-0.199 (0.883)	5.53	1/116	0.020
Mannerism	0.216 (1.085)	-0.194 (0.876)	5.04	1/115	0.027
HC / Nondeficit / deficit SCZ	15/14/30	23/26/10	15.28	2	<0.001
VFT	20.1 (7.7)	21.5 (7.3)	0.60	1/116	0.440
MMSE	26.1 (3.7)	27.0 (3.2)	2.06	1/116	0.154
Episodic memory PC	0.275 (1.150)	-0.237 (0.744)	8.11	1/114	0.005
WLM	17.0 (6.0)	19.8 (4.6)	7.84	1/116	0.006
True Recall	6.3 (2.5)	7.3 (1.9)	6.52	1/116	0.012
Executive functions PC	0.166 (1.021)	-0.120 (0.942)	2.48	1/115	0.118

All results are shown as mean (±SD).

Sum zOSE: indicates sum of z values of IgM responses directed to 4 OSEs;

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F/X^2/Ψ: results of analyses of variance (F) or analyses of contingency analyses (X^2) or Ψ coefficient;
TUD: tobacco use disorder; BMI: body mass index; SDS: total score on the Schedule for Deficit Syndrome; PANSS: total score on the Positive and Negative Syndrome Scale;
Psychotic dimension: computed as z score PANSS P1 (delusion) (zP1) + zP3 (hallucinations) + zP6 (suspiciousness) + zBPRS11 (suspiciousness) + zBPRS12 (hallucinatory behavior) + BPRS15 (unusual thought content); Hostility dimension: computed as zP7 (hostility) + zPANSS general14 (zG14, poor impulse control) + zBPRS10 (hostility) + zBPRS14 (uncooperativeness); Excitement-grandiosity dimension: computed as zP14 (excitement) + zP5 (grandiosity) + zBPRS8 (grandiosity) + zBPRS17 (excitement); Mannerism-posturing dimension: computed as zG5 + zBPRS7 (both mannerism and posturing);
HC: healthy controls / non-deficit schizophrenia / deficit schizophrenia;

VFT: verbal fluency test; MMSE: Mini mental State Examination; PC: principal component; WLM: Word List Memory.

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Table 2. Results of multinomial regression analysis with diagnosis (into three groups) as dependent variable and IgM isotype antibody levels to oxidative specific epitopes (OSE), nitro (NO_2) and nitroso (NO) adducts as explanatory variables.

Independent variables	Nagelkerke X^2, df, p	Dichotomies	W	df	p	OR	95% CI intervals
IgM Oleic acid	0.144	Non-Def / HC	0.00	1	0.954	1.00	0.64 – 1.59
	X^2=16.12, df=2, p<0.001	Def / HC	10.25	1	<0.001	0.40	0.23 – 0.70
		Def / Non-Def	10.58	1	0.001	0.40	0.23 – 0.69
IgM MDA	0.274	Non-Def / HC	0.429	1	0.512	0.85	0.52 – 1.38
	X^2=32.90, df=2, p<0.001	Def / HC	17.09	1	<0.001	0.27	0.14 – 0.50
		Def / Non-Def	20.71	1	<0.001	0.23	0.12 – 0.43
IgM Azelaic	0.203	Non-Def / HC	0.06	1	0.810	0.95	0.60 – 1.50
	X^2=23.54, df=2, p<0.001	Def / HC	13.81	1	<0.001	0.32	0.17 – 0.58
		Def / Non-Def	15.20	1	<0.001	0.30	0.17 – 0.55
IgM Pi	0.185	Non-Def / HC	0.33	1	0.568	0.87	0.53 – 1.42
	X^2=21.17, df=2, p<0.001	Def / HC	11.16	1	0.001	0.38	0.21 – 0.67
		Def / Non-Def	14.14	1	<0.001	0.33	0.18 – 0.58

IgM MDA	0.395 X^2=51.01, df=4, p<0.001	Non-Def / HC	0.77	1	0.381	1.39	0.67 – 2.87
		Def / HC	21.41	1	<0.001	0.09	0.03 – 0.25
		Def / Non-Def	25.80	1	<0.001	0.07	0.02 – 0.19
IgM NOcyst		Non-Def / HC	0.36	1	0.548	0.80	0.40 – 1.64
		Def / HC	9.25	1	0.002	4.07	1.65 – 10.04
		Def / Non-Def	11.66	1	0.001	5.06	1.99 – 12.82

Diagnosis: 3 groups are included, namely HC: healthy controls, Non-Def: non-deficit schizophrenia and Def: deficit schizophrenia.

MDA: malondialdehyde; Pi: phosphatidylinositol; NOcyst: NO-cysteinyl; OR: Odds Ratio with 95% confidence intervals (CI).

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Table 3. Results of binary logistic regression analyses with deficit schizophrenia (DEF) as dependent variable and the IgM isotype antibody levels directed against oxidative specific epitopes (OSEs) and nitroso (NO) and nitro (NO_2)-adducts as explanatory variables

Dependent variables	**Nagelkerke Model X^2**	**Significant explanatory variables**	**B (SE)**	**W**	**df**	**p**	**OR**	**95% CI**
#1. DEF/rest	0.489	IgM MDA	-2.92 (0.55)	28.66	1	<0.001	0.05	0.02 – 0.15
	51.46, df=2, <0.001	IgM NO-albumin	1.69 (0.43)	15.16	1	<0.001	5.43	2.32 – 12.71
#2. DEF/rest	0.480	IgM MDA	-2.56 (0.50)	26.80	1	< 0.001	0.08	0.03 – 0.20
	50.22, df=2, <0.001	IgM NO-cysteinyl	1.50 (0.43)	12.18	1	<0.001	4.51	1.94 – 10.49
#3. DEF/NON-DEF	0.633	IgM MDA	-3.64 (0.75)	23.74	1	<0.001	0.026	0.01 – 0.11
	51.56, df=3, <0.001	IgM NO-cysteinyl	1.27 (0.65)	3.86	1	0.049	3.55	1.01 – 12.56
		IgM NO_2-tyrosine	1.34 (0.65)	4.20	1	0.040	3.80	1.06 – 13.63

DEF/rest: the logistic regression analysis are performed with deficit schizophrenia (DEF) as dependent variable and rest (controls + non-deficit schizophrenia) as reference group; MDA: malondialdehyde.

All IgM responses were entered as z values.

OR: Odds ratio, 95% confidence intervals (CI).

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Table 4. Results of stepwise multiple regression analyses with severity of schizophrenia symptoms as dependent variables and IgM antibody titers to oxidative specific epitopes (OSEs), nitroso (NO) and nitro (NO_2)-adducts as explanatory variables.

Dependent Variables	**Explanatory variables**	**BE (SE)**	**t**	**p**	**R2**	**Model F**	**df**	**p**
SDS	IgM MDA	-0.62 (0.16)	-3.85	<0.001	0.427	13.61	4/73	<0.001
	IgM azelaic	-0.37 (0.17)	-2.19	0.032				
	IgM NO-cysteinyl	0.29 (0.13)	+2.31	0.024				
	IgM NO_2-tyrosine	0.36 (0.14)	+2.53	0.014				
Restricted affect	IgM MDA	-0.73 (0.16)	-4.49	<0.001	0.227	11.03	2/75	<0.001
	IgM NO-albumin	0.37 (0.15)	+2.44	0.017				
Diminished emotional range	IgM MDA	-0.89 (0.16)	-5.66	<0.001	0.306	10.88	3/74	<0.001
	IgM NO-albumin	0.36 (0.16)	+2.23	0.029				
	IgM NO_2-tyrosine	0.33 (0.16)	+2.07	0.042				
Poverty of speech	IgM MDA	-0.68 (0.13)	-5.34	<0.001	0.279	14.51	2/75	<0.001
	IgM NO-cysteinyl	0.35 (0.12)	+2.93	0.004				
Curbing of interest	IgM MDA	-0.73 (0.13)	-5.41	<0.001	0.281	14.68	2/75	<0.001
	IgM NO-cysteinyl	0.42 (0.13)	+3.27	0.002				
Diminished sense of purpose	IgM MDA	-0.75 (0.15)	-5.12	<0.001	0.259	13.09	2/75	<0.001

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	IgM NO_2-tyrosine	0.53 (0.15)	+3.43	0.001				
Diminished social drive	IgM MDA IgM azelaic IgM NO-albumin IgM NO_2-tyrosine	-0.82 (0.21) -0.50 (0.21) 0.36 (0.18) 0.59 (0.17)	-3.88 -2.37 +2.06 +3.43	<0.001 0.020 0.043 0.001	0.422	13.34	4/73	<0.001
PANSS positive	IgM NO_2-tyrosine	0.24 (0.11)	+2.18	0.032	0.058	4.77	1/77	0.032
PANSS negative	IgM MDA IgM NO_2-tyrosine	-0.64 (0.13) 0.45 (0.13)	-5.15 +3.44	<0.001 0.001	0.258	13.23	2/76	<0.001
Psychotic symptoms	-							
Hostility	-							
Excitation	IgM MDA IgM NO-albumin	-0.66 (0.15) 0.49 (0.14)	-4.54 +3.55	<0.001 0.001	0.212	10.33	2/77	<0.001
Excitation	IgM azelaic IgM oleic acid IgM NO-albumin Education	-0.39 (0.17) -0.39 (0.18) 0.51 (0.15) -0.07 (0.03)	-2.39 -2.18 +3.36 -2.53	0.019 0.033 0.001 0.014	0.288	7.09	4/70	<0.001
Mannerism	-							

*All dependent and explanatory variables were entered as z-scores (the IgM data were first Ln transformed)

Table 5. Results of multiple regression analyses with Mini Mental State Examination (MMSE), a principal component extracted from episodic memory tests, Word List memory (WLM) and True Recall as dependent variables and IgM levels to oxidative specific epitopes, nitroso and nitro-adducts as primary explanatory variables.

Dependent Variables	**Explanatory variables**	**BE (SE)***	**t**	**p**	**R^2**	**Model F**	**Model df**	**Model p**
MMSE	Education	0.65 (0.10)	+6.74	<0.001	0.386	24.23	2/77	<0.001
	IgM Pi	0.17 (0.09)	+2.06	0.043				
Episodic Memory PC	Education	-0.32 (0.12)	-2.77	0.007	0.219	10.52	2/75	0.001
	IgM MDA	-0.37 (0.10)	-3.82	<0.001				
WLM	Education	0.49 (0.10)	+4.76	<0.001	0.286	15.44	2/77	<0.001
	IgM MDA	0.27 (0.09)	+2.94	0.004				
True Recall	Education	0.38 (0.10)	+3.66	<0.001	0.284	15.30	2/77	<0.001
	IgM MDA	0.39 (0.09)	+4.20	<0.001				

*All dependent and explanatory variables were entered as z-scores (the IgM data were first Ln transformed)

Pi: phosphatidylinositol; MDA: malondialdehyde; education: number of education years.