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Upregulation of the intestinal paracellular pathway with breakdown of tight and adherens junctions in deficit schizophrenia.

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Abstract

In 2001, the first author of this paper reported that schizophrenia is associated with an increased frequency of the haptoglobin (Hp)-2 gene. The precursor of Hp-2 is zonulin, a molecule that affects intercellular tight junction integrity. Recently, we reported increased plasma IgA/IgM responses to Gram-negative bacteria in deficit schizophrenia indicating leaky gut and gut dysbiosis. The current study was performed to examine the integrity of the paracellular (tight and adherens junctions) and transcellular (cytoskeletal proteins) pathways in deficit versus non-deficit schizophrenia. We measured IgM responses to zonulin, occludin, E-cadherin, talin, actin and vinculin in association with IgA responses to Gram-negative bacteria, CCL-11, IgA responses to tryptophan catabolites (TRYCATs), immune activation and IgM to malondialdehyde (MDA) and NO-cysteinyl in 78 schizophrenia patients and 40 controls. We found that the ratio of IgM to zonulin + occudin / talin + actin + viculin (PARA/TRANS) was significantly greater in deficit than in non-deficit schizophrenia and higher in schizophrenia than controls and was significantly associated with increased IgA responses to Gram-negative bacteria. IgM responses to zonulin were positively associated with schizophrenia (versus controls), while IgM to occludin was significantly associated with deficit schizophrenia (versus non-deficit schizophrenia and controls). A large part of the variance (90.8%) in negative and PHEM (psychosis, hostility, excitation and mannerism) symptoms was explained by PARA/TRANS ratio, IgA to Gram-negative bacteria, IgM to E-cadherin and malondialdehyde (MDA) and memory dysfunctions, while 53.3% of the variance in the latter was explained by PARA/TRANS ratio, IgA to Gram-negative bacteria, CCL-11, TRYCATs and immune activation. The results show an upregulated paracellular pathway with breakdown of the tight and ahherens junctions and increased bacterial translocation in deficit schizophrenia. These

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dysfunctions in the intestinal paracellular route together with lowered natural IgM, immune activation and production of CCL-11 and TRYCATs contribute to the phenomenology of deficit schizophrenia.

Key words: schizophrenia, leaky gut, neuro-immune, inflammation, oxidative stress, TRYCATs, cytokines

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Introduction

In 2001, the first author of this paper reported that the Hp-2 gene frequency was significantly higher in schizophrenia patients as compared with the observed frequency in the population, indicating that genetic variation on chromosome 16 is associated with schizophrenia [1]. Hp is an acute phase protein, which is increased together with other acute phase reactants and complement factors, including C3C, during immune-inflammatory responses [1]. Hp shows a polymorphism with three common phenotypes, namely Hp 1-1, Hp 2-1 and Hp 2-2, which are determined by two autosomal codominant alleles, i.e. Hp-1 and Hp-2 [2]. This is important as the Hp 2-2 phenotype is significantly associated with increased oxidative burden, low grade inflammation and increased levels of pro-inflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α [3-5]. Increased Hp 2-2 genotypic distribution not only plays a role in schizophrenia [1], but also in other medical illness, including Crohn's disease, celiac disease and chronic kidney failure [6,7]. We have argued that the increased frequency of the Hp 2-2 phenotype could play a role in the immune-inflammatory pathophysiology of schizophrenia [1].

There is now indeed evidence that schizophrenia and its different phenotypes, including first episode psychosis, chronic schizophrenia, treatment resistant schizophrenia, and deficit schizophrenia are accompanied by activation of the immune-inflammatory response system (IRS) with an acute phase response, activated macrophagic M1, T helper (Th)-1 and Th-17 phenotypes [8-10]. Moreover, the different schizophrenia phenotypes and manifestations are accompanied by simultaneous signs of immune regulatory or anti-inflammatory processes, including activated Th-2 and Tregulatory (Treg) phenotypes and increased levels of cytokine receptors such as soluble IL-2 receptor, sTNF-R1 and sTNFR2 and sIL-1R antagonist (sIL-1RA)

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[10]. As such, the different phenotypes of schizophrenia are accompanied by activated IRS pathways and an activated compensatory immune-regulatory system (CIRS) which tends to downregulate the primary IRS [10,11]. Different IRS and CIRS products have neurotoxic and excitotoxic effects and may deleteriously impact neuroplastic mechanisms and neurogenesis thereby causing neurocognitive impairments and symptoms of schizophrenia, including negative symptoms, psychosis, hostility, excitation and mannerism (PHEM) [9,10,12-16]. These neurotoxic substances belong to the IRS as well as CIRS and include increased levels of IL-1β, IL-6, TNF-α, interferon-γ, IL-4, IL-13, CCL-11 (eotaxin), and cytokine-induced production of neurotoxic tryptophan catabolites (TRYCATs) including xanthurenic acid (XA), picolinic acid (PA) and 3-OH-kynurenine (3HK) [9,10,14-16].

Recently, we discovered a deficit in the CIRS that is strongly associated with deficit schizophrenia, namely lowered IgM antibody levels to conjugated oxidative specific epitopes (OSEs) including malondialdehyde (MDA) [17]. Moreover, lowered levels of IgM to MDA were highly significantly associated with the negative symptoms of schizophrenia and excitation and impairments in semantic and episodic memory including direct and delayed recall [17]. Those antibodies directed against conjugated OSEs are part of the innate immune system and may be present even without antigenic contact [18-22]. These natural antibodies are produced by B1 cells and have housekeeping, immune-regulatory, anti-inflammatory and anti-oxidant properties thereby protecting against uncontrolled inflammation [23,24].

Importantly, we discovered that deficit schizophrenia is accompanied by increased IgA responses to gut commensal bacteria including *Hafnia alvei, Pseudomonas aeruginosa, Morganella morganii*, and *Klebsiella pneumoniae* as compared with non-deficit schizophrenia and that increased IgA directed to *Pseudomonas putida*, IgM responses to all abovementioned

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Gram-negative bacteria coupled with lowered natural IgM to OSEs predict deficit schizophrenia with a bootstrapped area under the ROC curve of 0.960 [25]. Natural IgM to OSEs are an integral pre-existing part of the innate first-line defense system protecting against microorganisms, including Gram-negative bacteria, through early recognition and neutralization of the invading pathogens 'in the lag time for adaptive antibody production' [18,1920,23,26]. The LPS of translocated bacteria, in turn may activate receptors of the innate immune system, including the Toll Like Receptor (TLR)4 complex thereby activating intracellular signalling networks (e.g. nuclear factor (NF)-κB and MAPK) and stimulating the production of inducible nitric oxide (NO) synthase, reactive oxygen species and pro-inflammatory cytokines [27]. Moreover, LPS and other bacterial antigens may exert profound neurotoxic effects [28] by accessing the brain via different routes, including the blood-brain-barrier, the circumventricular organs and area postrema, or via outer membrane vesicles [28-33]. Based on this knowledge, we concluded that schizophrenia patients with lowered natural IgM are more prone to the detrimental effects of LPS and other bacterial neurotoxic antigens, which in turn may drive schizophrenia symptoms including memory impairments [25].

The Hp-2 gene and increased bacterial translocation are both related to pre-Hp-2, namely zonulin [6]. The latter increases the permeability of the tight junctions (TJs) composed of occludin and claudin, which in the gut join epithelial cells together thereby protecting against entrance of unwanted enterobacteria and food molecules [6]. Besides TJs also E-cadherin binds epithelial cells together via adherens junctions (AJs) thereby increasing stability of the gut barrier [34]. Gut dysbiosis and gliadin may cause loosening of the TJ barrier and therefore increased translocation of bacterial and other antigens (e.g. food) into the blood stream via the paracellular route [34-36]. Loss of intracellular cytoskeletal components, including actin,

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vinculin and talin, may allow entrance of antigens via the transcellular route [34]. However, there are no data whether (deficit) schizophrenia is accompanied by upregulated paracellular or transcellular pathways leading to increased translocation of gut commensal Gram-negative bacteria.

Hence, the current study examined whether (deficit) schizophrenia and its phenomenological characteristics (i.e. negative and PHEM symptoms and memory deficits) are accompanied by dysfunctions in the paracellular or transcellular pathways as measured using IgM responses to zonulin, occludin, E-cadherin, talin, actin and vinculin.

Subjects and Methods

Participants

The current study recruited 79 patients with schizophrenia and 40 healthy controls. All participants were recruited from the same catchment area, namely province Bangkok, Thailand. Patients attended the Department of Psychiatry, King Chulalongkorn Memorial Hospital, Bangkok, Thailand, and fulfilled all DSM-IV-TR diagnostic criteria. They were stabilized patients who did not suffer from acute psychotic episodes the year prior to the study. Patients were divided into two groups, namely those with and without deficit schizophrenia [37]. Exclusion criteria for patients were: axis-1 DSM-IV-TR disorders other than schizophrenia, including bipolar disorder, schizoaffective disorder, major depression, substance use disorders and psycho-organic disorders. Exclusion criteria for healthy controls were: lifetime and current diagnoses of axis I, DSM-IV-TR diagnoses and a positive family history of schizophrenia. Exclusion criteria for both patients and controls were: a) medical illnesses such as inflammatory bowel disease, psoriasis, COPD, rheumatoid arthritis, lupus erythematosus and diabetes type 1

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and 2; b) neuro-inflammatory and neurodegenerative disorders, including multiple sclerosis, stroke, Parkinson's disease and Alzheimer's disease; c) use of medications known to suppress immune functions, such as glucocorticoids or immunosuppresiva, and d) use of supplements with ω3-polyunsaturated fatty acids or antioxidants the months prior to the study. All patients and controls as well as the guardians (parents or close family members) gave written informed consent prior to participation in our 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.

Measurements

Clinical assessments

A senior psychiatrist (BK) specialized in schizophrenia made the diagnosis of schizophrenia using DSM-IV-TR diagnostic criteria and the Mini-International Neuropsychiatric Interview (MINI) in a validated Thai translation [38]. On the same day, the same senior psychiatrist used a semi-structured interview to assess clinical and socio-demographic data in patients and controls. Patients were divided into those with and without primary deficit schizophrenia using the Schedule for the Deficit Syndrome [37]. We also made the diagnosis of first episode of psychosis versus multiple psychotic episodes using DSM-5 criteria. On the same day, BK assessed the Positive and Negative Syndrome Scale (PANSS) [39], the Scale for the Assessment of Negative Symptoms (SANS) [40], the Brief Psychiatric Rating Scale (BPRS)

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[41], and the Hamilton Depression (HAM-D) and Anxiety (HAM-A) Rating Scales [42,43] and the Fibromyalgia and Chronic Fatigue Syndrome Rating scale (FF) [44].

Consequently, we have computed indices reflecting different symptoms clusters of schizophrenia pathology as explained previously [15,16,17,25,46]. A psychomotor retardation index (PMRI) was constructed based on items of the BPRS, HDRS and PANSS as: z-score of HDRS item 8 (HDRS8: psychomotor retardation: slowness of thought and speech, decreased motor activity, impaired inability to concentrate) *plus* z-score of the general psychopathology scale of the PANSS, item G7 (zPANSSG7; reduction in motor activity as reflected in slowing or lessening of movements and speech, diminished responsiveness to stimuli and reduced body tone) *plus* z score of item 13 of the BPRS (zBPRS13; reduction in energy level evidenced in slowed movements). Formal thought disorders (FTD) was computed as z value of PANNS P2 (item P2 of the PANNS scale or conceptual disorganization, zP2) *plus* zN5 (item N5 of the PANNS or difficulty in abstract thinking) *plus* zBPRS4 (item 4 of the BPRS or conceptual disorganization). The severity of psychotic symptoms was computed as sum of zPANSS (positive subscale item 1) P1 (delusion) *plus* zPANSSP3 (hallucinations) + zPANNSP6 (suspiciousness) *plus* zBPRS11 (suspiciousness) *plus* zBPRS12 (hallucinatory behavior) *plus* zBPRS15 (unusual thought content). The severity of hostility was computed as sum of zPANSS7 (hostility) *plus* zPANSSG14 (poor impulse control) *plus* zBPRS10 (hostility) *plus* zBPRS14 (uncooperativeness). The excitement dimension score was computed as zP14 (excitement) *plus* zP5 (grandiosity) *plus* zBPRS8 (grandiosity) *plus* zBPRS17 (excitement). Mannerism was computed as zG5 *plus* zBPRS7 (both mannerism and posturing). Severity of gastro-intestinal symptoms (GIS) was computed as: HAM-A11 *plus* FF10 (both gastro-intestinal symptoms).

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On the same day, a well-trained research assistant (a MSc in Mental Health) assessed neuropsychological functions in all participants using the Consortium to Establish a Registry for Alzheimer's disease (CERAD)-Neuropsychological battery [47]. Here we assessed the Verbal Fluency Test (VFT) to probe semantic memory and fluency; b) Word List Memory (WLM) to test verbal episodic memory and learning ability, and c) Word List Recall, true recall (True Recall) to test verbal episodic memory recall. In addition, we employed DSM-IV-TR criteria to make the diagnosis of Tobacco Use Disorder (TUD), while we computed the body mass index (BMI) using body weight (kg) / length (m^2).

Assays

In patients and controls, fasting blood was sampled at 8.00 a.m. for the assay of IgM responses to intestinal barrier, TJ (zonulin and occludin), AJ (E-Cadherin) and cytoskeletal-related proteins (talin, actin and vinculin), IgM antibody levels to MDA and NO-cysteinyl, IgA responses to TRYCATs, and IL-10, sIL-1RA, macrophage inflammatory protein (MIP)-1α and CCL-11 (eotaxin) levels.

Zonulin, occludin, E-cadherin, talin and vinculin were purchased from Bio-Synthesis® (Lewisville, TX, USA) and Abcam® (Cambridge, MA, USA), while actin was obtained from Sigma-Aldrich® (St. Louis, MO, USA). Proteins and peptides at a concentration of 1 mg/mL were dissolved in 0.01 M Tris buffer and diluted 1:50 in 0.1 M carbonate buffer at pH 9.5. 100 µL of each diluted antigen was added to each well of the microtiter plate. Plates were incubated overnight at 4°C and then washed three times with 200 µL 0.01 M PBS containing 0.05% Tween 20 at a pH of 7.4. After washing, 200 µL of 2% bovine serum albumin (BSA) was added to each well to prevent non-specific binding of the antibody to the plate. Plates were washed, and then

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100 µL of serum diluted 1:100 for IgM detection in serum diluent were added to duplicate wells coated with each antigen. Plates were incubated for an additional 1 hour at room temperature. The plates were then washed five times with Tris-buffered saline (TBS)-Tween. Alkaline phosphatase-labeled IgM at dilutions of 1:400 for IgM were then added to all wells and incubated again for 1 hour at room temperature. The enzyme reaction was started by adding 100 mL of paranitrophenylphosphate at a concentration of 1 mg/mL in diethanolamine buffer containing 1mN MgCl2 and sodium azide at a pH of 9.8. The reaction was stopped 45 minutes later with 75 µL of 1 N NaOH, and the samples were read by an ELISA reader; the optical densities were recorded. Several wells were coated with non-specific proteins such as human serum albumin (HAS) and rabbit serum albumin, which were used as controls for detecting the ELISA background. Sera from healthy subjects were used as negative controls, and sera from patients with intestinal autoimmunity with moderate and high titers of IgM antibodies were used as calibrator and positive controls and for the calculation of ELISA indices using the following formula: antibody ELISA index = OD of tested specimen - OD blank / OD of calibrator - OD of blank. All OD values were then converted into z scores which were used in the statistical analyses. Consequently, we computed z unit weighted composite scores to obtain indices of parracellular and transcellular leaky gut. For paracellular TJ leaky gut we computed the sum of the z scores of zonulin and occludin as z zonulin *plus* z occludin (PARA). Furthermore, we also computed z zonulin *plus* z occludin *plus* z E-cadherin as an index of the total paracellular pathway (PARA2). For the transcellular route we computed the composite score of z talin *plus* z actin *plus* z vinculin (TRANS). In order to estimate which route prevails in schizophrenia, we have computed the ratio between PARA versus TRANS as: z score of (z zonulin *plus* z occludin) - z score of (z talin *plus* z actin *plus* z vinculin) (PARA/TRANS). As an estimate of total leaky

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gut we computed the sum of the z scores of all 6 proteins, namely z zonulin *plus* z occludin *plus* z talin *plus* z actin *plus* z vinculin *plus* z E-cadherin (named "overall LEAKY index").

The assays of IgA responses to different conjugated TRYCATs were performed as described previously [48-50]. The 6 TRYCATs were dissolved in 200 μL dimethylsulfoxide (DMSO) (Acros). BSA (ID Bio) was dissolved in 3 mL 2-morpholino-ethanesulfonic acid monohydrate (MES) buffer 10^{-1} M at pH = 6.3 (Acros). The TRYCATs were then mixed with the BSA solution and supplemented with 15 mg N-hydroxysuccinimide (Sigma) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Acros) as coupling agents. The conjugates were synthesized by linking 3-OH-kynurenine (3HK, Sigma), kynurenic acid (KA, Acros), quinolinic acid (QA, Acros), anthranilic acid (AA, Acros), xanthurenic acid (XA, Akros) and picolinic acid (PA, Akros) to 20 mg BSA. The coupling reaction proceeded at 37°C for 1 hour in the dark. The coupling was stopped by adding 100 mg hydroxylamine (Sigma-Aldrich) per conjugate. Protein conjugates were dialyzed with 10^{-1} M NaCl solution for 72 hours and the bath solution was changed at least four times per day. The conjugated TRYCATs and BSA concentrations were evaluated by spectrophotometry. The coupling ratio of each conjugate was determined by measuring the concentration of TRYCATs and BSA at 310-330 nm and 280 nm, respectively. ELISA tests were used to determine plasma titers of immunoglobulins (Ig) A (IgA). Towards this end, polystyrene 96-well plates (NUNC) were coated with 200 μL solution containing 10–50 μg/mL TRYCAT conjugates in 0.05 M carbonate buffer (pH = 9.6). Well plates were incubated under agitation at 4°C for 16 hours. Then, 200 μL blocking buffer A (Phosphate Buffered Saline, PBS, 2.5 g/L BSA) was applied and all samples were incubated at 37◦C for 1 hour. Well plates were washed with PBS solution and filled up with 100 μL serum diluted 1:130 in blocking buffer and incubated at 37°C for 1 hour and 45 minutes. Well plates were washed 3 times with PBS,

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0.05% Tween 20, incubated with peroxidase-labeled goat anti-human IgA (SouthernBiotech) antibodies at 37°C for 1 hour. The goat anti-human IgA antibody was diluted at 1:10.000 in blocking buffer (PBS, 2.5 g/L BSA). Plates were then washed three times with PBS, 0.05% Tween 20. Fifty microlitre of TMB substrate (3,3',5,5'-Tetramethylbenzidine, SouthernBiotech) was added and incubated for 10 minutes in the dark. The reaction was stopped using 50 µL of TMB stop solution (SouthernBiotech). Optical densities (ODs) were measured at 450 nm using Varioskan Flash (Thermo Scientific). All assays were carried out in one and the same run by the same operator who was blind to all clinical results. All assays were carried out in duplicate. The analytical intra-assays CV values were < 7%. We computed a noxious / protective TRYCAT ratio as z score of PA (zPA) *plus* zXA *plus* zOHK *minus* zAA *minus* zKA (NOX/PRO TRYCAT ratio) [51].

We used an enzyme-linked immunosorbent assay to measure IgM levels directed against conjugated MDA [52-55]. MDA was linked to fatty acid free-BSA, according to previously described methods [52-55]. Synthesis of the conjugates to delipidated BSA was performed as described before [54]. In order to mimic nitrosylation processes, NO-cysteinyl was synthesized by linking haptens to BSA (Sigma-Aldrich) using glutaraldehyde [53,56,57]. The synthesis of these conjugates has been described previously [58]. The 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. The conjugate was 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 compound possesses 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

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NO concentrations linked to the compound. The detection of IgM autoantibodies to the conjugates was performed by indirect ELISA tests [55,58]. 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 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%.

For the assays of cytokines/chemokines, 50 µl of serum (1:2 dilution in calibrator diluents) was mixed with 50 µl of microparticle cocktail containing CCL-11, sIL-1RA, IL-10 and MIP-1α (R&D Systems, Inc, Minneapolis, MN, USA) per well of a 96-well plate provided by manufacturer and incubated for 2 hours at room temperature on a shaker at 800 rpm. The mixture was then washed 3 times with wash buffer and 50 µl diluted Biotin Antibody cocktail was added and then incubated for 1 hour. Wells were washed 3 times before another 50 µl of diluted Streptavidin-PE was added and further incubated for 30 minutes. Finally, wells were washed 3 times and 100 µl of wash buffer was added and left at room temperature for 2 minutes

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before being read with Bio-Plex® 200 System (Bio-Rad Laboratories, Inc.). The intra-assay CV values were <7.0%. The least detectable dose was 1.82 pg/mL for eotaxin, 1.58 pg/mL for MIP-1, 5.98 pg/mL for IL-1RA and 0.4 pg/mL for IL-10. We computed an immune activation index as z score of interleukin-10 (zIL-10) *plus* zMIP-1α *plus* zsIL-1RA [15,16].

Statistical analysis

Analysis of variance (ANOVA) was used to assess differences in scale variables among categories and analysis of contingency tables (X^2 tests) was employed to assess associations between nominal variables. Correlation matrices were assessed to check associations between sets of scale variables using Pearson's product moment and Spearman's rank order correlation coefficients. Multiple regression analysis was employed to delineate the significant biomarkers predicting symptoms and cognitive scores. Binary and multinomial logistic regression analyses were used to delineate the significant predictors of diagnostic groups (either binary, e.g. deficit versus non-deficit SCZ or multiple groups, e.g. controls versus SCZ with or without deficit). Multivariate GLM analysis was used to examine the effects of explanatory variables (e.g. drug state, age and sex) on the 6 PARA-TRANS proteins. Results of regression analyses were checked for multicollinearity. Moreover, all analyses were bootstrapped (1000 bootstraps) and we report the bootstrapped results if there are differences between analyses with and without bootstrapping. IgM to MDA and CCL-11 were processed in Ln transformations in order to normalize the data distribution of these variables. Tests were 2-tailed and a p-value of 0.05 was used for statistical significance. All abovementioned statistical analyses were performed using IBM SPSS windows version 25.

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We used partial Least Squares (PLS) path modeling to analyze causal associations [59] between input variables, namely biomarkers including PARA/TRANS, E-cadherin, IgA to Gram-negative bacteria, the immune activation index, CCL-11, NOX/PRO TRYCAT ratio, IgM to MDA and NO-Cysteinyl, and symptom dimensions, including negative (the 6 symptoms of the SDS) and PHEM symptoms, which were used as output variables. Based on our new cognitive theory of schizophrenia [15,16], we considered that memory dysfunctions may mediate the association between biomarkers and symptoms. We entered data in the PLS analysis as indicator variables, i.e. CCL-11, TRYCATs, PARA/TRANS, E-cadherin, IgM MDA, immune activation index or as latent vectors (LV), namely a LV extracted from Gram-negative bacteria ("Gram-negative LV"), a LV extracted from negative (the 6 SDS symptoms) and PHEM indicators ("symptom LV") and a LV extracted from memory indicators (VFT, WLM and WL True Recall) and FTD values ("memory LV"). Based on our novel neurocognitive theory, we entered the memory LV as a predictor variable for the symptom LV, while the biomarkers predicted both the memory and symptom LVs. As such, we used a multistep path model with multiple mediators [60] including Gram-negative LV, CCL-11, NOX/PRO TRYCAT ratio and memory LV. PLS path modeling was carried out only if the LVs and overall model quality data complied with specific criteria, namely a) all indicators in the outer model have factor loadings > 0.400 (all $p<0.001$), b) model SRMR < 0.08; and c) all LVs have adequate reliability as indicated by Cronbach's alpha > 0.7, composite reliability > 0.8 and average variance extracted (AVE) > 0.5. We then applied complete, consistent bootstrapping (2000 bootstraps) to compute path coefficients with exact p-values, total effects and total indirect and specific indirect effects.

Results

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Biomarkers and schizophrenia categories

Table 1 shows the outcome of different multinomial logistic regression analyses with the diagnosis, namely controls, deficit schizophrenia (SCZ) and non-deficit SCZ, as dependent variables and the biomarkers as explanatory variables. Regression #1 shows a strong association between PARA/TRANS ratio and diagnosis with an effect size of 0.577 for the total model (including age, sex and IgM MDA), 0.343 for PARA/TRANS alone, 0.268 for IgM MDA alone, 0.330 for age, sex and IgM MDA combined and 0.111 for age and sex. PARA/TRANS was significantly and positively associated with deficit SCZ as compared with non-deficit SCZ and controls, and with non-deficit schizophrenia as compared with controls. Also, ANOVA showed that the PARA/TRANS ratio was significantly different ($F=24.41$, $df=2/114$, $p<0.001$) between the three study groups and increased from controls (mean z score ±SE = -0.698 ±0.804) → non-deficit SCZ (0.008 ±0.817) → deficit SCZ (0.654 ±0.903). **Figure 1** shows the dot plot of the PARA/TRANS ratio in the three study groups.

There were significant and positive correlations between IgM to MDA and IgM to zonulin ($r=0.624$, $p<0.001$), occludin ($r=0.670$, $p<0.001$), E-cadherin ($r=0.672$, $p<0.001$), talin ($r=0.660$, $p<0.001$) and actin ($r=0.712$, $p<0.001$), but not vinculin ($r=0.096$, $p=0.302$, all $n=117$). Therefore, all regressions shown in Table 1 examine whether the IgM levels to the proteins contribute to the prediction of deficit SCZ after considering the highly significant association of IgM MDA and deficit SCZ [17], while controlling for the effects of age and sex (see below). Firstly, we have examined the effect of the LEAKY, PARA, PARA2 and PARA/TRANS indices and the effects of the separate IgM values.

Regression #2 and #3 show that the LEAKY and PARA indices had a significant impact, and were significantly associated with deficit SCZ as compared with non-deficit SCZ and

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controls, while there were no significant differences between controls and non-deficit SCZ. Occludin (regression #4) yielded a higher effect size and showed that increased levels were significantly associated with deficit SCZ versus controls and non-deficit SCZ. Increased IgM to zonulin (regression #5) was significantly associated with both SCZ categories versus controls, without significant differences between the SCZ subgroups. Regression #6 shows that the TRANS proteins were associated with deficit versus non-deficit SCZ albeit with very low impact. There were no significant associations between vinculin and the diagnostic groups, whilst both increased talin (Wald=8.59, df=1, p=0.003) and actin (F=11.88, df=1, p=0.001) were associated with deficit *versus* non-deficit SCZ. PARA2 (regression #7) was significantly associated with deficit SCZ versus non-deficit SCZ and controls, while IgM to E-cadherin was significantly associated with deficit SCZ versus non-deficit SCZ (Wald=18.20, df=1, p<0.001 with an Odds ratio = 10.59 and 95% confidence intervals: 3.65 – 32.90). Regression #8 shows the combined effects of increased PARA/TRANS ratio and IgA against Gram-negative bacteria (z composite score of the 5 bacteria): both increased PARA/TRANS and Gram-negative bacteria were associated with deficit SCZ versus non-deficit SCZ and controls, while PARA/TRANS also differentiated non-deficit SCZ from controls.

Characteristics of increased PARA/TRANS ratio

Based on the high impact of the PARA/TRANS ratio we examined the characteristics of SCZ subjects with an increased PARA/TRANS ratio versus patients with a lower ratio and controls. **Table 2** shows the outcome of ANOVAs / X^2 tests performed on socio-demographic, clinical and biomarker data in three study groups, namely controls and SCZ patients with a lower PARA/TRANS (< 0.666 percentile) versus higher PARA/TRANS ratio (≥ 0.666 percentile).

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There were no significant differences in age, TUD and BMI between the three study groups. There were somewhat more females in the lower PARA/TRANS group than in controls, while years of education was somewhat lower in SCZ patients with an increased PARA/TRANS ratio. The number of psychotic episodes was significantly lower in those with an increased PARA/TRANS ratio, while the latter showed increased SDS, SANS, PANSS negative, psychosis, excitements and PMRI scores as compared with those with a lowered PARA/TRANS ratio. There were, however, no significant differences in PANSS positive symptoms, hostility, mannerism, FTD, GIS symptoms, between both SCZ subgroups. There was a significant association between the PARA/TRANS groups and deficit SCZ. IgA levels to Gram-negative bacteria and IgM to NO-cysteinyl were significantly greater in SCZ patients allocated to the high PARA/TRANS group. The other biomarkers and VFT, WLM and WL True Recall did not differ significantly between both PARA/TRANS groups.

Multivariate biomarker prediction of SCZ and deficit SCZ.

Table 3 examines the best prediction of deficit versus non-deficit SCZ and SCZ versus healthy controls using logistic regression analyses with PARA/TRANS, IgA to Gram-negative bacteria, IgM to NO-cysteinyl and MDA, CCL-11 and NOX/PRO TRYCAT ratio as explanatory variables. Logistic regression #1 shows that deficit SCZ (versus non-deficit SCZ) was highly predicted (effect size=0.773) by IgA Gram-negative bacteria, PARA/TRANS ratio, and IgM to NO-cysteinyl (all positively) and IgM to MDA (inversely); 88.6% of all cases were correctly classified with a sensitivity of 87.2% and a specificity of 90.0%. We found that zonulin, occludin, IgA to Gram-negative bacteria, IgM to NO-cysteinyl and CCL-11 (all positively) and IgM to MDA (inversely) significantly discriminated deficit from non-deficit SCZ (X^2=109.50,

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df=6, p<0.001, Nagelkerke=1) with a sensitivity and specificity of 100%. Regression #2 shows that SCZ (versus controls) was highly predicted by CCL-11, the immune activation index, the NOX/PRO TRYCAT ratio, PARA/TRANS (all positively) and age (inversely); 92.3% of all cases were correctly classified with a sensitivity of 94.9% and a specificity of 86.8%. A second logistic regression analysis showed that PARA/TRANS could be replaced by zonulin levels (Wald=6.73, df=1, p=0.009) and that zonulin together with the variables shown in Table 3, regression #2, yielded an effect size of 0.829; 93.2% of the cases were correctly classified with a sensitivity of 94.9% and a specificity of 89.5%.

Effects of extraneous variables

We have also examined possible effects of the drug state of the patients on the IgM responses to paracellular and transcellular proteins. Multivariate GLM analysis did not show a significant effect of use of risperidone (F=1.59, df=6/96, p=0.158; n=34), clozapine (F=1.16, df=6/96, p=0.335; n=9), haloperidol (F=0.69, df=6/96, p=0.661; n=11), perphenazine (F=1.25, df=6/96, p=0.290; n=21), antidepressants (F=0.40, df=6/96, p=0.880; n=26), mood stabilizers (F=0.37, df=6/96, p=0.899; n=13) and anxiolytics / hyponotics (F=1.04, df=6/96, p=0.406; n=29). Previously, we have shown that the same drugs have no effect on CCL-11, TRYCATs, IgM to MDA and NO-cysteinyl, IgA/IgM to Gram-negative bacteria and the clinical assessments [14-17,25,46]. There was however a significant effect of age (F=4.58, df=6/106, p<0.001), but not sex (F=2.05, df=6/106, p=0.065). Parameter estimates showed significant inverse associations between age and IgM to E-cadherin (F=15.28, df=1/111, p<0.001) and vinculin (F=10.62, df=1/111, p=0.001). In any case, all regression analyses were controlled for possible

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effects of age and sex. There were no significant effects of TUD (F=0.26, df=6/105, p=0.953) and BMI (F=1.05, df=6/100, p=0.400) on the IgM levels to PARA-TRANS proteins.

Prediction of SCZ symptomatology using biomarkers.

In SCZ patients we have examined the effects of PARA/TRANS, E-cadherin, IgM to Gram-negative bacteria, IgA to *K. pneumonia* or *M. Morganii* on negative and PHEM symptoms while also allowing for the effects of age, sex and education. **Table 4**, regression #1 shows that 58.6% of the variance in the total SDS score was explained by the regression on PARA/TRANS, IgA to *K. pneumonia* and E-cadherin (all positively) and IgM to MDA and education (inversely). Regressions #2-7 show that a large part of the variance (35.3%-53.3%) in the items of the SDS was predicted by PARA/TRANS and IgM to MDA with or without Gram-negative bacteria, E-cadherin and education. There were no associations between the explanatory variables used here and PANSS positive symptoms (regression #8), while a large part of the variance in PANSS negative symptoms (regression #9) was explained by the regression on PARA/TRANS, IgA to *K. pneumonia* and E-Cadherin (all positively) and IgM to MDA (inversely). The same 4 variables together with education also predicted the SANS total score with an explained variance of 50.8% (regression #10). Regression #11 shows that 46.8% of the variance in psychomotor retardation was explained by PARA/TRANS, IgA to *K. pneumoniae*, E-cadherin (all positively) and IgM MDA (inversely). We found that 23.3% of the variance in psychotic symptoms (regression #12) was explained by 2 explanatory variables, namely PARA/TRANS and IgA to *K. pneumoniae*, while 33.2% of the variance in excitation (regression #13) was explained by PARA/TRANS, IgA to *K. pneumonia* (both positively) and education and IgM to MDA (both inversely).

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Prediction of SCZ symptomatology using all biomarkers.

Table 5 shows the prediction of key symptoms using the variables shown in Table 4 combined with CCL-11, NOX/PRO TRYCAT ratio, IgM NO-cysteinyl and immune activation index, while allowing for possible effects of age, sex and education. We used multiple regression analyses performed in the total study group to examine these associations. Regression #1 shows that 18.4% of the variance in GIS symptoms was explained by the immune activation index and zonulin levels. We found that (regression #2) 37.9% of the variance in WLM was explained by immune activation and PARA/TRANS (both inversely) and education and IgM MDA (both positively). 50.1% of the variance in PMRI (regression #3) was explained by the multiple regression on PARA/TRANS, IgA to *K. pneumoniae*, CCL-11, E-cadherin, and the immune activation index (all positively) and IgM MDA (inversely). FTD (regression #4) was best predicted by IgA NOX/PRO, PARA/TRANS and male sex (26.9% of the variance). A large part of the variance in SDS score (57.1%, regression #5) was explained by PARA/TRANS, IgA to *K. pneumoniae*, CCL-11, IgA NOX/PRO, E-cadherin and the immune activation index (all positively) and IgM MDA (inversely). A large part of the variance in psychosis (regression #6) was explained by PARA/TRANS, IgA to *K. pneumoniae*, IgA to NOX/PRO and male sex. A relatively smaller part of the variances in hostility and mannerism (regressions # 7 and 9) were explained by PARA/TRANS and male sex and immune activation index. We found that PARA/TRANS, IgA to *K. pneumoniae*, IgA to NOX/PRO and CCL-11 (all positively) and IgM MDA (inversely) were significantly associated with excitation (regression #8).

Results of SmartPLS analysis.

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Figure 2 shows the results of a PLS analysis with as final outcome variable the symptom LV and as direct explanatory variable the memory LV. The biomarkers were entered as input variables, including the Gram-negative LV (two Gram-negative bacteria did not load significantly and therefore were deleted from the model). Figure 2 shows the multistep path model with multiple mediators including the Gram-negative LV, CCL-11, TRYCATs and memory LV. We found that the overall model fit was excellent, namely SRMR=0.047, the construct reliability and discriminant validity of all LVs were good to excellent, namely all Cronbach's alpha > 0.787, composite reliability > 0.865 and average variance extracted > 0.618 and all outer loadings of the 3 LVs were > 0.701.

A large part of the variance in the symptoms LV (90.8%) was explained by the regression on memory LV, the Gram-negative LV, PARA/TRANS, E-cadherin (all positively) and IgM MDA (inversely); 53.3% of the variance in the memory LV was explained by Gram-negative LV, PARA/TRANS, CCL-11, TRYCATs and the immune activation index. The Gram-negative LV was predicted by PARA/TRANS, whilst CCL-11 and TRYCATs were both predicted by the immune activation index (both positively), which in turn was predicted by IgM MDA (inversely). We found significant total effects of E-cadherin ($t=3.59$, $p<0.001$), CCL-11 ($t=4.68$, $p<0.001$), Gram-negative bacteria LV ($t=2.07$, $p<0.0001$), IgM MDA ($t=-4.90$, $p<0.001$), immune activation index ($t=3.85$, $p<0.001$), PARA/TRANS ($t=4.75$, $p<0.001$) and NOX/PRO TRYCAT ratio ($t=4.75$, $p<0.001$) on the symptom LV. There were significant total effects of CCL-11 ($t=5.33$, $p<0.001$), Gram-negative bacteria LV ($t=2.25$, $p=0.024$), immune activation index ($t=4.39$, $p<0.001$), PARA/TRANS ($t=2.77$, $p=0.006$) and the NOX/PRO TRYCAT ratio ($t=2.52$, $p=0.012$) on the memory LV. There were significant specific indirect effects of PARA/TRANS ($t=2.94$, $p=0.003$), Gram-negative bacteria LV ($t=2.27$, $p=0.023$), CCL-11

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(t=4.68, p<0.001), TRYCATs (t=2.50, p=0.013), and immune activation index (t=3.85, p<0.001) on the symptoms LV which were all mediated by the memory LV.

Discussion

The first major finding of this study is that the PARA/TRANS ratio (i.e. increased IgM responses to zonulin *plus* occludin *versus* talin *plus* actin *plus* viculin) and IgM responses to occludin are positively associated with deficit schizophrenia versus non-deficit schizophrenia and controls. **Table 6** summarizes the major findings of this study with regard to diagnoses. The distance in the TPARA/TRANS ratio between deficit schizophrenia and controls was 1.35 standard deviations and between deficit and non-deficit schizophrenia 0.65 standard deviations. The appearance of IgM antibodies to occludin in the peripheral blood of patients with deficit schizophrenia may indicate damage to the tight junctions system and breakdown of occludin TJs leading to an upregulated intestinal paracellular pathway [34]. Moreover, we found that the increased PARA/TRANS ratio was significantly associated with increased IgA responses to Gram-negative bacteria, suggesting that an upregulated paracellular leak pathway allowed ingress of these pathogenic bacteria leading to breakdown of oral tolerance. Occludin is one of the key transmembrane proteins of the TJs that contributes to TJ stability, maintenance of selective paracellular permeability and TJ integrity [61-66]. TJ strands from adjacent epithelial cells associate with strands from the opposing membrane to form a TJ fence and occludin is together with claudin a main functional component of this TJ fence [67]. Increased expression of occludin in cultured Madin-Darby canine kidney (MDCK) cells elevates transepithelial resistance of these cells, whereas introduction of truncated occludin into MDCK cells increases paracellular leakage of small molecules [68,69]. Moreover, mice lacking occludin show a

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complex phenotype characterized by chronic inflammation of the gastric epithelium, growth retardation, male sterility, testicular atrophy and calcifications in the brain [67]. A leaky paracellular pathway is involved in autoimmune and inflammatory disorders, including celiac disease, diabetes mellitus type 1 and inflammatory bowel disease [6,7,70].

Another finding is that IgM antibodies to E-cadherin are significantly and positively associated with deficit versus non-deficit schizophrenia and explain part of the variance in negative symptoms and psychomotor retardation. Cadherin molecules are core components of AJs (or intermediate junctions) that give stability, mediate cell-cell adhesion and regulate the turnover of the actin cytoskeleton through binding of catenins [71-74]. AJs are important for the integrity of the epithelium and tissue morphogenesis, and they mediate transcriptional regulation and intracellular signaling [74]. Moreover, we also detected that there was a weak albeit significant association of IgM antibodies to the cytoskeletal proteins talin and actin and deficit schizophrenia, although the increased PARA/TRANS ratio indicates that the paracellular pathway is significantly more upregulated than the transcellular pathway. Actin is a key component in cellular functions including regulation of transcription and maintenance of cell structure, polarity and shape [75], whilst talin is another cytoskeletal molecule than links integrins to the actin cytoskeleton and functions in the attachment of microfilaments to the plasma membrane [76]. TJs are closely associated with the actin cytoskeleton whilst occludin may regulate the structural maintenance of the actin cytoskeleton in endothelial cells [77,78]. Therefore, it is plausible that the minor changes in the cytoskeleton observed in this study are secondary to degradation of the TJs (and AJs), which in part regulate the cytoskeleton.

The third major finding of this study is that IgM responses to zonulin were significantly and positively associated with schizophrenia (as compared with controls) and with gastro-

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intestinal (GIS) symptoms, indicating that zonulin may play a role in schizophrenia and GIS symptoms in that disorder. The expression of zonulin in plasma is elevated in several autoimmune disorders and neurodegenerative disorders, including autoimmune disorders, diabetes mellitus type 1 and celiac disease [6]. In fact, the latter author concluded that plasma levels of zonulin may be employed as a biomarker of impaired TJ functions for these disorders. Nevertheless, the assay of plasma zonulin levels is not very reproducible, whereas measurements of plasma antibodies directed to zonulin may be more reliable and sensitive to measure zonulin status [34]. Zonulin may displace zona occludens proteins from the TJ complex thereby modulating the permeability of TJs [6] and, therefore, this molecule was called the doorway to inflammation, autoimmunity and cancer [6]. We reported that schizophrenia is accompanied by increased Hp-2 gene frequency, Hp 2-2 phenotype and increased Hp levels [1]. Also, Wan et al. [79] reported increased Hp levels in schizophrenia and a significant association between the Hp gene and schizophrenia. Because pre-Hp-2 is similar to zonulin [6], it is safe to hypothesize that Hp-2 carriers are more prone to develop schizophrenia [1] through effects on zonulin with consequent breakdown of the TJs.

Nevertheless, it should be underscored that zonulin levels are not only affected by the Hp-2 gene, but also by gliadin and other bacterial toxins, which may induce the release of zonulin and consequently displace the zonula occludens thereby opening the paracellular leak pathway which allows pathogens, bacterial antigens and other proteins to be translocated into the plasma [6]. In this respect, it is interesting to note that gluten sensitivity, antigliadin antibodies and celiac disease may play a role in schizophrenia [80-82] and that gut dysbiosis may occur in some patients with schizophrenia [83]. However, increased levels of pro-inflammatory cytokines, including IL-6, IFN-γ and TNF-α, cause gut barrier dysfunctions and disassemble TJs

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and AJs thereby upregulating the paracellular leak pathway [84-87]. Thus, the primary IRS during acute episodes of schizophrenia may affect the apical junction complex (TJs and AJs) especially in Hp-2 carriers and in people with lowered natural IgM defenses against immune-inflammatory injuries. The consequent upregulation of the paracellular leak pathway with increased translocation of Gram-negative bacteria (and possibly undigested food macromolecules and other microbiota) may, in turn, contribute to the ongoing immune-inflammatory response.

The fourth major finding of this study is that the upregulated paracellular leak pathway and increased bacterial translocation coupled with lower IgM to MDA, immune activation, and increased CCL-11 and noxious TRYCAT levels are, together, strong predictors of deficit schizophrenia (versus non-deficit schizophrenia and controls), negative symptoms, psychosis, excitation, psychomotor retardation, formal thought disorders and memory impairments. A large part of the variance (90.8%) in negative and PHEM (psychosis, hostility, excitation and mannerism) symptoms was explained by Gram-negative bacteria, PARA/TRANS ratio, E-cadherin, IgM to malondialdehyde (MDA) and memory dysfunctions, which in turn were predicted (53.3% of the variance) by an increased PARA/TRANS ratio, Gram-negative bacteria, CCL-11, TRYCATs and immune activation. One mechanistic explanation is that breakdown of TJs and AJs with increased bacterial translocation may induce different neuro-immune pathways that ultimately cause neuroprogression. We have reviewed elsewhere the mechanism by which gut microbiota, CCL-11, some pro-inflammatory cytokines and noxious TRYCATs may cause cognitive decline and impairments in long-term memory performance [14-17,25]. Interestingly, based on our new cognitive model of schizophrenia [15-17], we found that the effects of an upregulated paracellular pathway with increased bacterial translocation are at least in part

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mediated by memory deficits. As such, the upregulated paracellular leak pathway in deficit schizophrenia may aggravate the cognitive decline in semantic and episodic memory and, as a consequence, schizophrenia symptomatology. Nevertheless, our PLS analysis showed that different pathways may lead to schizophrenia phenomenology: a) the paracellular path with bacterial translocation; b) immune activation with increased TRYCAT and eotaxin production; and c) lowered natural IgM which aggravates the effects of the first two paths. These findings also suggest that the paracellular leak path may affect other (unknown) pathways that were not assayed in our study comprising other cytokines and microbiota and food hypersensitivities (including gliadin and gluten).

The results of the current study should be interpreted with regard to its limitations. Firstly, this is a cross-sectional study which precludes the establishment of firm causal inferences. Secondly, while the associations of the PARA/TRANS ratio with deficit schizophrenia and its phenomenology are highly significant, the IgM antibody data to PARA/TRANS proteins appear to be useful as external validating criteria when combined with other biomarkers including IgM to MDA. Furthermore, the strong associations between IgM to MDA (an established natural IgM to an oxidation-associated neo-determinant) and IgM to PARA/TRANS proteins suggest that the latter reflects two phenomena, namely natural IgM and induced IgM responses secondary to damage to paracellular proteins. It is well established that IgM antibodies to MDA have housekeeping properties protecting against uncontrolled inflammation [88], while also IgM to various proteins may have protective properties [89], e.g. IgM responses to tubulin (another cytoskeleton protein) in neuroinflammatory disorders [90]. Consequently, in conditions characterized by a relative decrease in natural IgM (such as deficit

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schizophrenia) it may be more difficult to interpret the induced IgM responses in those proteins without considering the effects of other biomarkers.

In conclusion, we found that the paracellular leak pathway was upregulated in deficit schizophrenia and that mainly TJs (but also AJs) contribute to this phenomenon. An increased paracellular pathway and associated translocation of Gram-negative bacteria and lower IgM to MDA coupled with immune activation and increased CCL-11 and TRYCATs predict memory dysfunctions (including formal thought disorders), while the latter coupled with an upregulated paracellular leak pathway, Gram-negative bacteria and lowered natural IgM predict schizophrenia symptomatology comprising negative and PHEM symptoms. The results suggest that breakdown of the TJs and dysfunctions in the intestinal paracellular route contribute to the phenomenology of deficit schizophrenia.

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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

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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, MM, SS, and AV) 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. Dot plot of the PARA/TRANS ratio in healthy controls (HC) and schizophrenia patients with (DEF) and without (NONDEF) the deficit syndrome. The PARA/TRANS ratio is expressed as z score.

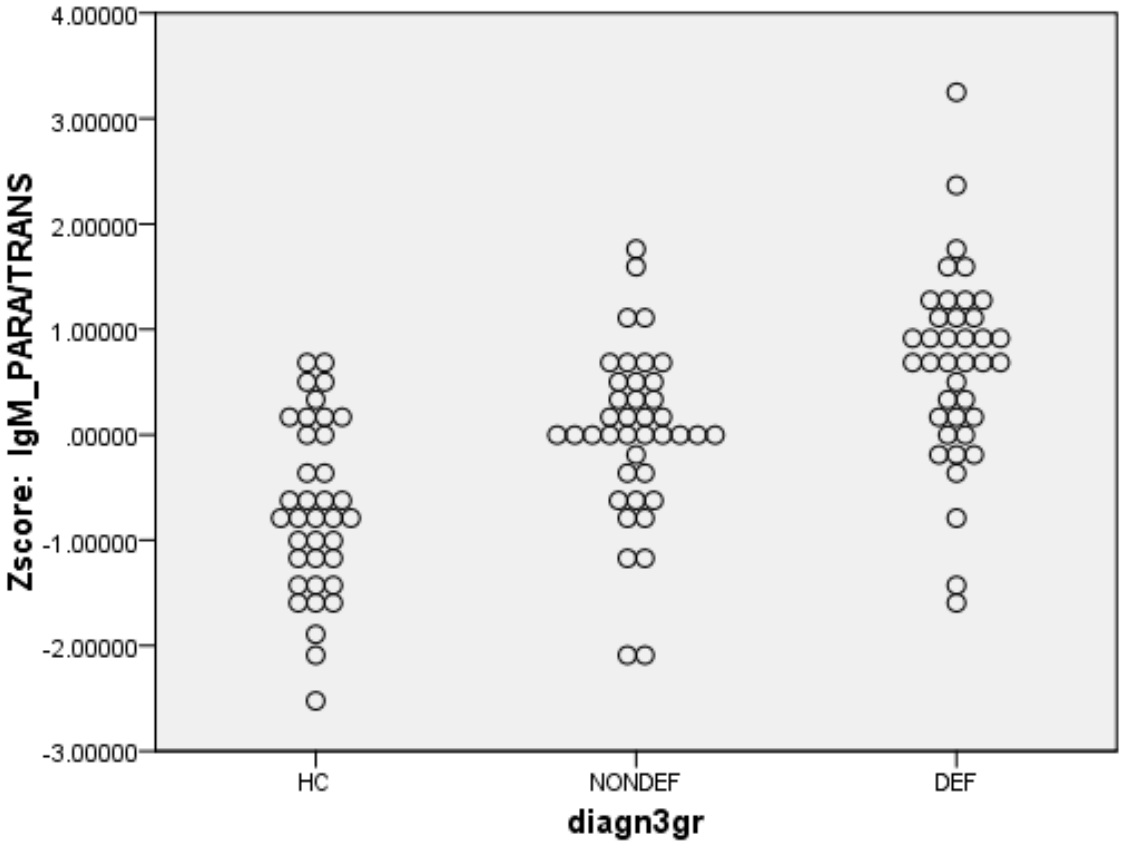

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Figure 2. Results of Partial Least Squares (PLS) path modeling.

This figure shows the results of PLS analysis with as final outcome variable a latent vector (LV) extracted from 6 negative SDS (Schedule of the Deficit Syndrome) and 4 PHEM (psychosis, hostility, excitation, mannerism) symptoms (named: “symptoms LV”) with as direct explanatory variable a LV extracted from memory indicators, namely verbal fluency test (VFT), word list memory (WLM), WL True Recall and formal thought disorders (FTD) (named “memory LV”). Biomarkers are entered as input variables predicting both the symptom and memory LV: a) the ratio between IgM responses to zonulin and occludin versus talin, actin and vinculin (PARA/TRANS) and a LV extracted from IgA to *H. alvei*, *K. pneumoniae* and *M. morganii* (named “Gram-negative LV”), b) IgM to malondialdehyde (MDA), and c) an immune activation indicator (composite score of soluble interleukin-1 receptor antagonist, interleukin-10 and macrophage inflammatory protein-1α), CCL-11 or eotaxin, and the noxious/protective tryptophan catabolite (TRYCAT) ratio. Shown are path coefficients with exact p-values for the inner model and t-values for the indicators in the outer model.

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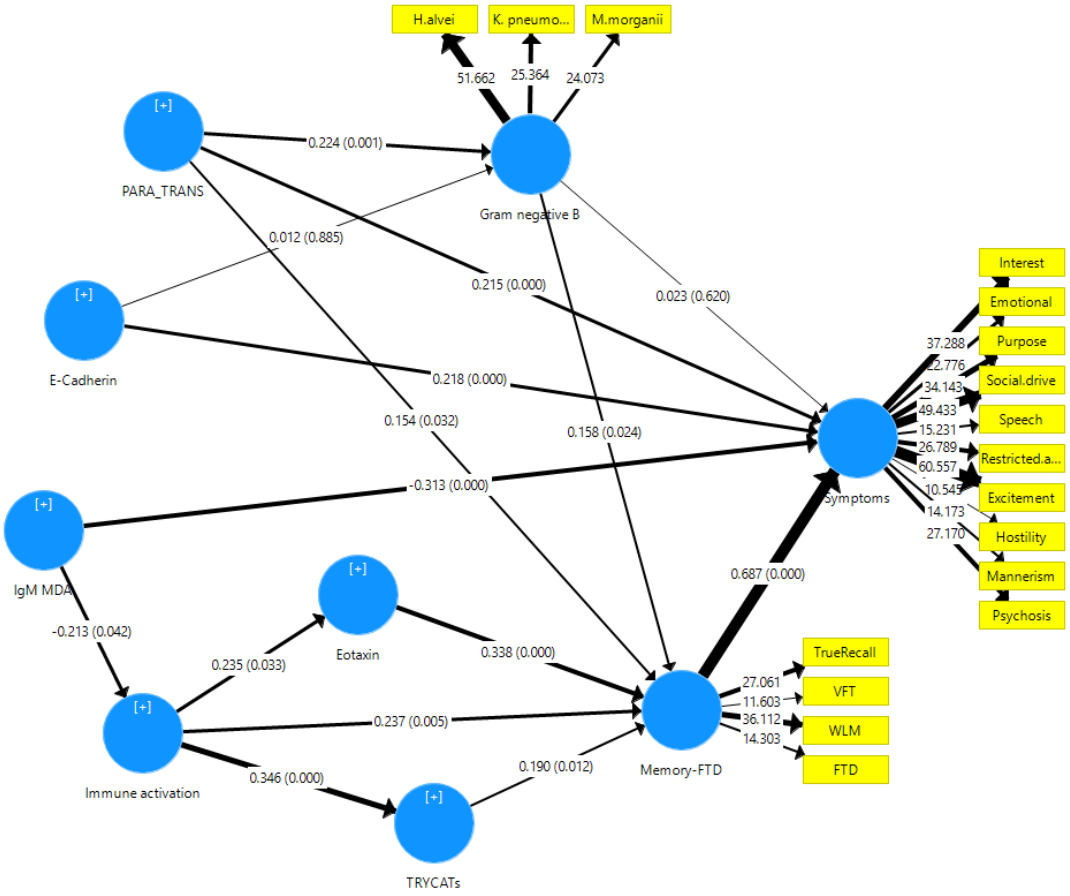

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Table 1. Results of multinomial logistic regression analysis with diagnoses as dependent variablee and IgM responses to tight and adherens junctions and cytoskeletal proteins and Gram-negative bacteria as explanatory variables. Diagnostic groups are: healthy controls (HC) and patients with (Def) and without (Non-Def) deficit schizophrenia.

Independent Variables	Nagelkerke (model) * X^2, df, p (independent)	Dichotomies	Wald	df	P	OR	95% CI intervals
1. PARA/TRANS	0.577 X^2=40.42, df=2, p<0.001	Non-Def / HC Def / HC Def / Non-Def	6.34 21.80 11.92	1 1 1	0.012 <0.001 0.001	2.27 12.91 5.68	1.20 – 4.31 4.41 – 37.77 2.12 – 15.23
2. LEAKY	0.438 X^2=14.09, df=2, p<0.001	Non-Def / HC Def / HC Def / Non-Def	2.11 6.21 12.09	1 1 1	0.146 0.013 <0.001	0.58 2.97 5.14	0.28 – 1.21 1.26 – 7.00 2.04 – 12.92
3. PARA	0.473 X^2=20.24, df=2, p<0.001	Non-Def / HC Def / HC Def / Non-Def	0.07 12.83 13.92	1 1 1	0.786 <0.001 <0.001	0.91 4.91 5.40	0.45 – 1.82 2.06 – 11.71 2.23 – 13.10
4. Occludin	0.559 X^2=36.52, df=2, p<0.001	Non-Def / HC Def / HC Def / Non-Def	9.07 9.74 23.91	1 1 1	0.003 0.002 <0.001	0.27 3.97 14.82	0.11 – 0.63 1.67 – 9.42 5.03 – 43.68
5. Zonulin	0.447 X^2=15.67, df=2, p=0.004	Non-Def / HC Def / HC Def / Non-Def	5.69 12.42 3.28	1 1 1	0.017 <0.001 0.070	2.49 5.10 2.04	1.17 – 5.29 2.06 – 12.60 0.94 – 4.43
6. TRANS	0.391 X^2=6.77, df=2, p=0.034	Non-Def / HC Def / HC Def / Non-Def	1.93 2.18 6.26	1 1 1	0.165 0.140 0.012	0.60 1.84 3.06	0.30 – 1.23 0.82 – 4.15 1.27 – 7.35
7. PARA2	0.488	Non-Def / HC	2.36	1	0.125	0.55	0.26 – 1.18

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	X^2=22.81, df=2, p<0.001	Def / HC Def / Non-Def	10.55 17.22	1 1	0.001 <0.001	4.43 8.01	1.81 – 10.89 3.00 – 21.40
8. PARA/TRANS **IgA Gram-negative bacteria**	0.640 X^2=40.42, df=2, p<0.001 X^2=14.16, df=2, p<0.001	Non-Def / HC Def / HC Def / Non-Def Non-Def / HC Def / HC Def / Non-Def	6.12 18.83 9.79 2.20 4.42 9.64	1 1 1 1 1 1	0.013 <0.001 0.002 0.138 0.035 0.002	2.27 13.82 6.09 0.63 2.90 4.64	1.19 – 4.35 4.22 – 45.27 1.96 – 18.86 0.34 – 1.16 1.08 – 7.83 1.76 – 12.25

OR: Odd's ratio, 95%CI: 95% confidence intervals with upper and lower limits

All regression analyses are adjusted for age, sex and IgM to malondialdehyde

PARA/PARA2: index of paracellular pathway based on zonulin + occludin (PARA) + E-cadherin (PARA2)

PARA/TRANS: paracellular / transcellular pathway ratio

IgA Gram-negative bacteria: composite score computed as sum of all z transformations of the IgA responses to Gram-negative bacteria

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Table 2. Socio-demographic, clinical and biomarker data in schizophrenia (SCZ) subjects with higher versus lower paracellular / transcellular (PARA/TRANS) pathway ratio as compared with healthy controls (HC).

Variables	HC (n=40)	PARA / TRANS < 0.666 % (n=44)	PARA / TRANS ≥ 0.666% (n=35)	F/X^2/Ψ	df	p
Age (years)	37.4 (12.8)	40.8 (10.6)	41.7 (11.7)	1.47	2/116	0.235
Gender (M/F)	10/30 B	24/20 [A]	18/17	8.64	2	0.013
Education (years)	14.3 (4.9) [C]	13.0 (3.5)	11.5 (4.8) [A]	3.56	2/116	0.032
Single / married / separated	23/14/3	30/7/6	29/4/1	Ψ=0.30	-	0.036
TUD (N/Y)	38/2	40/4	34/1	Ψ=0.11	-	0.484
BMI (kg/m^2)	24.0 (4.3)	25.3 (5.1)	23.4 (5.2)	1.52	2/111	0.223
Number of psychotic episodes	-	3.81 (3.18) [C]	2.34 (1.86) [B]	5.39	1/72	0.023
Age at onset (years)	-	25.4 (9.0)	25.1 (11.1)	0.02	1/73	0.898
SDS total score	0.0 (0.0)	4.9 (5.6)	9.1 (5.3)	38.13	2/114	<0.001
SANS	0.5 (1.7) [B,C]	27.8 (23.6) [A,C]	43.8 (21.1) [A,B]	53.35	2/115	<0.001
PANSS negative	7.0 (0.0) [B,C]	15.3 (9.7) [A,C]	24.1 (9.0) [A,B]	45.72	2/115	<0.001
PANSS positive	7.0 (0.0) [B,C]	13.9 (8.6) [A]	15.0 (6.1) [A]	19.17	2/115	<0.001
Psychosis (z score)	-0.820 (0.0) [B,C]	0.415 (1.043) [A,C]	0.746 (0.842) [A,B]	38.32	2/115	<0.001
Hostility (z score)	-0.595 (0.0) [B,C]	0.196 (1.067) [A]	0.430 (1.887) [A]	13.30	2/115	<0.001
Excitement (z score)	-0.809 (0.0) [B,C]	0.140 (1.046) [A,C]	0.694 (0.848) [A,B]	35.55	2/116	<0.001
Mannerism (z score)	-0.734 (0.0) [B,C]	0.271 (1.085) [A]	0.514 (1.007) [A]	23.18	2/115	<0.001
PMRI (z score)	-0.772 (0.143) [B,C]	0.064 (0.976) [A,C]	0.804 (0.950) [A,B]	36.98	2/115	<0.001
FTD (z score)	-0.761 (0.0) [B,C]	0.304 (1.149) [A]	0.459 (0.863) [A]	24.27	2/115	<0.001
GIS symptoms (z score)	-0.400 (0.401) [B,C]	0.166 (1.059) [A]	0.271 (1.268) [A]	5.41	2/115	0.006
Nondeficit / deficit SCZ	-	30/14 [C]	10/24 [B]	12.24	1	<0.001
IgM MDA (z score)	0.275 (0.821)	-0.164 (1.149)	-0.065 (0.942)	2.13	2/114	0.123
IgA Gram-negative (z score)	-0.017 (0.889)	-0.276 (0.894) [C]	0.362 (1.557) [B]	4.15	2/114	0.018
IgM NO-Cysteinyl (z score)	0.021 (0.700)	-0.266 (1.291) [C]	0.336 (0.755) [B]	3.70	2/114	0.028
Immune activation index(z score)	-0.674 (0.857) [B,C]	0.400 (0.971) [A]	0.252 (0.807) [A]	17.42	2/116	<0.001

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Eotaxin (pg/mL) *	129.6 (54.1) [B,C]	215.1 (90.1) [A]	207.3 (114.7) [A]	18.36	2/116	<0.001
IgA NOX/PRO (z score)	-0.695 (0.655) [B,C]	0.229 (1.078) [A]	0.484 (0.801) [A]	19.56	2/116	<0.001
Verbal Fluency Test	26.6 (6.3) [B,C]	18.8 (6.9) [A]	17.9 (6.0) [A]	21.97	2/116	<0.001
Word List (WL) Recall	22.1 (4.4) [B,C]	17.4 (4.9) [A]	16.1 (5.5) [A]	15.54	2/116	<0.001
WL True Recall	8.1 (1.8) [B,C]	6.4 (2.3) [A]	6.0 (2.2) [A]	4.13	1/116	0.045

All results are shown as mean (±SD).
$F/X^2/\Psi$: 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;
PMRI: psychomotor retardation; FTD: formal thought disorders; GIS: gastro-intestinal;
Nondeficit/Deficit SCZ: non-deficit schizophrenia / deficit schizophrenia;
IgM MDA: IgM antibodies to malondialdehyde;
IgA Gram-negative bacteria: composite score computed as sum of all z transformations of the IgA responses to Gram-negative bacteria
Immune activation index: composite score computed as sum of all z transformations of three cytokines;
IgA NOX/PRO: IgA responses to noxious / protective tryptophan catabolites;
VFT: verbal fluency test; MMSE: Mini mental State Examination; WLM: Word List Memory.

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Table 3. Results of binary logistic regression analyses with deficit schizophrenia (SCZ) as dependent variable and the IgM responses to tight and adherens junctions and cytoskeletal proteins and other relevant biomarkers as explanatory variables

Dependent variables	**Nagelkerke Model X^2**	**Significant explanatory variables**	**B (SE)**	**W**	**df**	**p**	**OR**	**95% CI**
#1. Defici t / Non-deficit	0.773	IgA Gram-negative	1.98 (0.71)	7.66	1	0.006	7.20	1.78 – 29.17
	X^2=68.43, df=4, <0.001	PARA/TRANS	1.89 (0.73)	6.66	1	0.010	6.63	1.58 – 27.90
		IgM NO-Cysteinyl	1.47 (0.60)	5.96	1	0.015	4.33	1.33 – 14.03
		IgM MDA	-3.89 (0.97)	16.02	1	<0.001	0.02	0.01 – 0.14
#2. SCZ / HC	0.821	Eotaxin	3.88 (1.13)	11.81	1	0.001	48.76	5.31 – 447
	X^2=103.89, df=5, <0.001	Immune activation	1.76 (0.59)	9.53	1	0.002	5.80	1.90 – 17.71
		IgA NOX/PRO	2.46 (0.68)	12.87	1	<0.001	11.69	3.05 – 44.79
		PARA/TRANS	1.24 (0.49)	6.46	1	0.011	3.45	1.33 – 8.98
		Age	-0.11 (0.05)	5.08	1	0.024	0.89	0.80 – 0.99

OR: Odds ratio, 95% confidence intervals (CI).
Deficit/non-deficit : this logistic regression analysis is performed with deficit SCZ as dependent variable and non-deficit SCZ as reference group;
SCZ/HC: this logistic regression analysis is performed with SCZ as dependent variable and healthy controls (HC) as reference group;
IgA Gram-negative bacteria: composite score computed as sum of all z transformations of the IgA responses to Gram-negative bacteria
PARA/TRANS: paracellular / transcellular pathway ratio
IgM MDA: IgM responses to malondialdehyde
Immune activation index: composite score computed as sum of all z transformations of three cytokines;
IgA NOX/PRO: IgA responses to noxious / protective tryptophan catabolites;

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Table 4. Results of hierchical multiple regression analyses with severity of schizophrenia symptoms as dependent variables and IgM responses to tight and adherens junctions and cytoskeletal proteins and IgA/IgM to Gram-negative bacteria and natural IgM as explanatory variables

Dependent Variables	Explanatory variables	BE (SE)	t	p	R2	Model F	df	p
#1. SDS	IgM MDA	-3.52 (0.52)	-6.81	<0.001	0.586	20.11	5/71	<0.001
	PARA/TRANS	2.10 (0.52)	4.03	<0.001				
	IgA *K. pneumoniae*	1.46 (0.44)	3.33	0.001				
	E-Cadherin	2.03 (0.61)	3.31	0.001				
	Education	-0.24 (0.11)	-2.11	0.038				
#2. Restricted affect	IgM MDA	-0.60 (0.12)	-5.08	<0.001	0.353	13.28	3/73	<0.001
	PARA/TRANS	0.47 (0.12)	3.92	<0.001				
	IgM Gram-negative	0.36 (0.13)	2.72	0.008				
#3. Diminished emotional range	IgM MDA	-0.59 (0.11)	-5.17	<0.001	0.430	13.55	4/72	<0.001
	PARA/TRANS	0.40 (0.11)	3.52	0.001				
	Education	-0.07 (0.02)	-2.90	0.005				
	E-Cadherin	0.36 (0.14)	2.66	0.010				
#4. Poverty of speech	IgM MDA	-0.44 (0.09)	-4.92	<0.001	0.400	11.99	4/72	<0.001
	IgA *M. morganii*	0.29 (0.09)	3.07	0.003				
	PARA/TRANS	0.25 (0.11)	2.21	0.030				
	Education	-0.05 (0.02)	-2.03	0.046				
#5. Curbing of interest	IgA *K. pneumoniae*	0.36 (0.09)	3.99	<0.001	0.457	15.65	3/74	<0.001
	IgM MDA	-0.57 (0.11)	-5.18	<0.001				
	PARA/TRANS	0.31 (0.11)	2.81	0.006				
	E-Cadherin	0.36 (0.13)	2.76	0.007				
#6. Diminished sense of purpose	IgA *K. pneumoniae*	0.35 (0.10)	3.41	0.001	0.382	11.13	4/72	<0.001
	IgM MDA	-0.54 (0.12)	-4.33	<0.001				
	PARA/TRANS	0.32 (0.12)	2.58	0.012				
	E-Cadherin	0.34 (0.15)	2.31	0.024				
#7. Diminished social	IgM MDA	-0.74 (0.12)	-6.17	<0.001	0.533	20.55	4/72	<0.001

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drive	PARA/TRANS IgA *K. Pneumonia* E-Cadherin	0.53 (0.12) 0.34 (0.10) 0.48 (0.14)	4.39 3.49 3.41	<0.001 0.001 0.001				
#8. PANSS positive	-							
#9. PANSS negative	PARA/TRANS IgM MDA IgA *K. pneumoniae* E-Cadherin	0.45 (0.10) 0.29 (0.08) -0.45 (0.10) 0.34 (0.11)	4.54 3.64 -4.65 3.09	<0.001 0.001 <0.001 0.003	0.484	17.09	4/73	<0.001
#10. SANS	IgA *K. pneumoniae* IgM MDA PARA/TRANS Education E-Cadherin	0.22 (0.08) -0.47 (0.09) 0.30 (0.09) -0.06 (0.02) 0.26 (0.10)	2.93 -5.24 3.28 -2.91 2.56	0.005 <0.001 0.002 0.005 0.013	0.508	14.85	5/72	<0.001
#11. PMRI	IgA *K. pneumoniae* IgM MDA PARA/TRANS E-Cadherin	0.34 (0.08) -0.50 (0.10) 0.35 (0.10) 0.33 (0.12)	4.09 -4.96 3.40 2.85	<0.001 <0.001 0.001 0.006	0.468	16.07	4/73	<0.001
#12. Psychotic symptoms	PARA/TRANS IgA *K. pneumoniae*	0.39 (0.12) 0.27 (0.10)	3.30 2.83	0.002 0.006	0.233	7.67	2/76	0.001
#13. Excitation	IgA *K. pneumoniae* PARA/TRANS IgM MDA Education	0.25 (0.09) 0.30 (0.11) -0.25 (0.09) -0.05 (0.02)	2.81 2.68 -2.80 -2.13	0.006 0.009 0.007 0.036	0.332	9.20	4/74	<0.001

*All dependent and explanatory variables were entered as z-scores (the IgM MDA data were first Ln transformed);
SDS: total score on the Schedule for Deficit Syndrome; PANSS: total score on the Positive and Negative Syndrome Scale;
SANS: Scale for the Assessment of negative Symptoms;
PMRI: psychomotor retardation;
IgM MDA: IgM responses to malondialdehyde
PARA/TRANS: paracellular / transcellular pathway ratio
IgM Gram-negative bacteria: composite score computed as sum of all z transformations of the IgA responses to Gram-negative bacteria

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Table 5. Results of multiple regression analyses with symptoms and neurocognitive tests as dependent variables and IgM responses to tight and adherens junctions and cytoskeletal proteins and other relevant biomarkers as explanatory variables.

Dependent Variables	Explanatory variables	BE (SE)**	t	p	Model R^2	F	df	p
#1. GIS symptoms	Immune activation Zonulin	0.38 (0.09) 0.24 (0.09)	4.45 2.76	<0.001 0.007	0.184	12.74	2/113	<0.001
#2. Word List Memory	Education Immune activation IgM MDA PARA/TRANS	0.08 0.02 -0.23 0.08 0.19 0.08 -0.17 0.08	4.92 -2.99 2.49 -2.23	<0.001 0.003 0.014 0.028	0.379	17.08	4/112	<0.001
#3. PMRI	PARA/TRANS IgM MDA IgA K. Pneumoniae Eotaxin E-Cadherin Immune activation	0.31 0.07 -0.44 0.09 0.22 0.07 0.52 0.08 0.33 0.09 0.22 0.07	1.63 -5.04 3.10 3.28 3.54 3.00	<0.001 <0.001 0.002 0.001 0.001 0.004	0.501	18.21	6/109	<0.001
#4. FTD	IgA NOX/PRO PARA/TRANS Male Sex	0.32 0.08 0.26 0.08 0.42 0.16	3.85 3.10 2.58	<0.001 0.002 0.011	0.269	13.75	3/112	<0.001
#5. SDS	PARA/TRANS IgM MDA IgA K. Pneumoniae Eotaxin IgA NOX/PRO E-Cadherin Immune activation	0.30 (0.07) -0.48 (0.08) 0.19 (0.07) 0.21 (0.07) 0.16 (0.07) 0.34 (0.09) 0.21 (0.07)	4.41 -5.89 2.79 2.90 2.34 3.82 2.93	<0.001 <0.001 0.006 0.005 0.021 <0.001 0.004	0.571	20.32	7/107	<0.001
#6. Psychosis	PARA/TRANS IgA K Pneumoniae IgA NOX/PRO Male Sex	0.35 (0.08) 0.18 (0.08) 0.24 (0.08) 0.42 (0.16)	4.36 2.27 2.93 2.70	0.001 0.025 0.004 0.008	0.343	14.52	4/111	<0.001

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#7. Hostility	PARA/TRANS Male Sex	0.24 (0.09) 0.61 (0.18)	2.70 3.48	0.008 0.001	0.163	9.51	2/113	<0.001
#8. Excitation	PARA/TRANS IgA K Pneumoniae IgA NOX/PRO IgM MDA Eotaxin	0.27 (0.08) 0.25 (0.08) 0.19 (0.07) -0.17 (0.08) 0.16 (0.08)	3.49 3.28 2.48 -2.25 2.01	0.001 0.001 0.015 0.027 0.047	0.382	13.71	5/111	<0.001
#9. Mannerism	Male Sex PARA/TRANS Immune activation	0.79 (0.27) 0.40 (0.13) 0.32 (0.14)	-2.91 3.00 2.30	0.004 0.003 0.023	0.228	11.02	3/112	<0.001

*All dependent and explanatory variables are entered as z-scores (the IgM MDA data were first Ln transformed)
GIS: gastro-intestinal symptoms
PMRI: psychomotor retardation
FTD: formal thought disoders
SDS: Schedule for the Deficit Syndrome
Immune activation index: composite score computed as sum of all z transformations of three cytokines;
IgM MDA: IgM responses to malondialdehyde
PARA/TRANS: paracellular / transcellular pathway ratio
IgA NOX/PRO: IgA responses to noxious / protective tryptophan catabolites;

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Table 6. Summary of the findings

Antigens (IgM)	**Classification**	**Non-deficit Schizophrenia *versus* controls**	**Deficit schizophrenia *versus* controls**	**Deficit *versus* non-deficit schizophrenia**
Zonulin	Intestinal barrier, tight junctions	**XX**	**XX**	-
Occludin	Intestinal barrier, tight junctions	-	**XX**	**XXXX**
zonulin + occludin (PARA)	Paracellular route, tight junctions	-	**XXXX**	**XXXX**
E-cadherin	Paracellular route, adherens junctions	-	-	**XXX**
Talin	Cytoskeletal	-	-	**X**
Actin	Cytoskeletal	-	-	**X**
Vinculin	Cytoskeletal	-	-	-
Talin + actin + vinculin (TRANS)	Transcellular route	-	-	**X**
PARA/TRANS ratio	Paracellular / transcellular route	**XX**	**XXXXXX**	**XXXX**

X: positive association