Full-length Article

Profiling antibody signature of schizophrenia by *Escherichia coli* proteome microarrays

Bao-Yu Chen a,1, Chu-Chun Hsu b,1, You-Zuo Chen c, Jin-Jia Lin d, Huai-Hsuan Tseng e,g, Fong-Lin Jang d, Po-See Chen e,i,g, Wan-Ni Chen i, Chien-Sheng Chen h,c,1, Sheng-Hsiang Lin a,h,i,*

a Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
b Department of Food Safety/Hygiene and Risk Management, College of Medicine, National Cheng Kung University, Tainan, Taiwan
c Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan
d Department of Psychiatry, Chi Mei Medical Center, Tainan, Taiwan
e Department of Psychiatry, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan
f Department of Behavioral Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
g Department of Public Health, College of Medicine, National Cheng Kung University, Tainan, Taiwan
h Biostatistics Consulting Center, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

ABSTRACT

Schizophrenia (SZ) is influenced by genetic and environmental factors, and associated with chronic neuro-inflammation. If the symptoms express after adolescence, environmental impacts are more substantial, and the disease is defined as adult-onset schizophrenia (AOS). Effects of environmental factors on antibody responses such as *Escherichia coli* (E. coli) to immunoglobulin G (IgG) and immunoglobulin M (IgM) might increase the severity of symptoms in SZ via the gut-brain axis. The purpose of this study is to reveal antibody profiles of SZ against bacterial protein antigens. We analyzed the IgG and IgM antibodies using *E. coli* proteome microarrays from 80 SZ patients and 40 healthy controls (HC). Using support vector machine to select panels of proteins differentiating between groups and conducted enrichment analysis for those proteins. We identified that the groL, pldA, yjjU, livG, and ftsE can classify IgGs in AOS vs HC achieved accuracy of 0.7. The protein yjjU, livG and ftsE can form the best combination panel to classify IgG in AOS vs HC with accuracy of 0.8. The enrichment results are highly related to ABC (ATP binding cassette) transporter in the protein domain and cellular component. We further found that the human ATP binding cassette subfamily b member 1 (ABCB1) autoantibody level in AOS is significantly higher than in HC. The findings suggest that AOS had different immunoglobulin production compared to early-onset schizophrenia (EOS) and HC. We also identified potential antibody biomarkers of AOS and found their antigens are enriched in ABC transporter related domains, including human ABCB1 protein.

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Abbreviations: SZ, schizophrenia; AOS, adult-onset schizophrenia; GMB, gut microbiota; CNS, central nervous system; GI, gastrointestinal; E. coli, *Escherichia coli*; rMFG, right middle frontal gyrus; EOS, early-onset of schizophrenia; IgG, immunoglobulin G; IgM, Immunoglobulin M; SVM, support vector machine; ABCB1, human ATP binding cassette subfamily b member 1; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; HC, healthy control; EDTA, ethylenediaminetetraacetic acid; PBST, thawed using phosphate-buffered saline with 0.05% tween 20; BSA, bovine serum albumin; PBS, phosphate-buffered saline; BMI, body mass index; AUC, area under curve; BBB, blood-brain barrier; NMDAR, N-methyl-D-aspartate receptors; CSF, cerebrospinal fluid.

* Corresponding authors at: Institute of Clinical Medicine or Department of Food Safety/Hygiene and Risk Management, College of Medicine, National Cheng Kung University, Tainan, Taiwan.

E-mail addresses: cchen@gs.ncku.edu.tw (C.-S. Chen), shlin922@mail.ncku.edu.tw (S.-H. Lin).

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1. Introduction

Schizophrenia (SZ) is a psychiatric disorder with poorly defined molecular mechanisms (Chen et al., 2021) that is influenced by genetic and environmental factors (Liu et al., 2020; Owen et al., 2011). It is commonly accepted that many phenotypes of schizophrenia have neurodevelopmental underpinnings (Chen et al., 2019) associated with chronic neuroinflammation, metabolic dysfunction, and oxidative stress (Comer et al., 2020; Marques et al., 2019). If the symptoms express after adolescence, the condition is considered primarily driven by social and environmental impacts and defined as adult-onset schizophrenia (AOS) (Frazier et al., 2012; Sikich et al., 2008). Schizophrenia and its different phenotypes are accompanied by activation of the immune-inflammatory response system (IRS) (Noto et al., 2019; Roomruangwong et al., 2020; Smith and Maes, 1995). In study of schizophrenia with negative symptoms and neurocognitive deficits also have cumulative effects on elevated Immunoglobulin M (IgM) and/or Immunoglobulin A (IgA) levels to Gram-negative bacteria (Maes et al., 2019a). On the other hand, the significant increase in Immunoglobulin G (IgG) permeability in male patients of schizophrenia with impaired blood–brain barrier (BBB) function might be associated with hippocampal volume reduction pathology (Goldwaser et al., 2022). However, these studies only examined the change in overall IgM, IgG and/or IgA, their antigens were not analyzed for profiling antibody signature.

Emerging preclinical and clinical studies have indicated that gut microbiota (GMB) may be influenced by environmental factors (such as diet, physical activity, smoking, substance abuse, etc.), however this is not limited to acute or chronic disorders of the gastrointestinal tract (Kelsen and Wu, 2012; Stavropoulou and Bezirtzoglou, 2019) and may also express as interactions with the central nervous system (CNS) through the gut–brain axis (Cowan et al., 2020). Thus, while different IRS and compensatory immune-regulatory systems (CIRS) are activated, it would ultimately producing diversified neurotoxic and excitotoxic. It could result in adverse impacts of neuroplastic mechanisms and neurogenesis, thereby causing different schizophrenia subgroups (Davis et al., 2016; Davis et al., 2014; Kanchanatawan et al., 2018; Noto et al., 2019; Roomruangwong et al., 2020; Sirivichayakul et al., 2019a, b), such as AOS. Thus, their underlying molecular mechanisms may play an important role (Carabotti et al., 2015; Dinan and Cryan, 2017; Ma et al., 2019; Rea et al., 2016). However, the details have remained unclear (Lavretsky, 2008; Owen et al., 2011).

Many enteric microflora studies of schizophrenia have suggested increased taxa abundance of Proteobacteria (Nguyen et al., 2019; Maes et al., 2019b; Zhang et al., 2020; Zheng et al., 2019). For example, increased Escherichia coli (E. coli) has been found in first-episode drug-naive and chronic antipsychotic-treated schizophrenia patients compared to healthy controls (Ma et al., 2020). Another study of schizophrenia revealed higher plasma IgM and/or IgA responses to Gram-negative bacteria, and is positively associated with deficit schizophrenia compared with non-deficit schizophrenia and healthy controls (Maes et al., 2019b). In the light of the above results, we sought to investigate the relationship between E. coli antibodies and schizophrenia in further details.

Brain-reactive antibodies have been suggested as candidate biomarkers for immune dysregulation in schizophrenia (Borda et al., 2004; Dahm et al., 2014; Delunardo et al., 2016; Jones et al., 2014; Levin et al., 2010; Margari et al., 2013; Tanaka et al., 2003). To improve the accuracy of distinguishing between different types of schizophrenia, we developed a laboratory test to better assist clinical diagnosis. Imbalanced anti-nuclear and anti-cytoplasmic levels of IgG and IgM have been previously reported to be associated with schizophrenia (Morais et al., 2019), suggesting that schizophrenia is an autoimmune disease. Thus, our research team used the entire genome library of E. coli K12, which contains a genome-wide microarray of ~ 4,300 proteins (Chen et al., 2008), to identify several clinical diagnostic biomarkers for diseases for which it is challenging to find diagnostic tools with advanced accuracy. These include inflammatory bowel disease (Chen et al., 2009), pre-eclampsia (Hsu et al., 2018), Kawasaki disease (Kuo et al., 2018) and bipolar disorder (Chen et al., 2015). Antibody in the blood sample is an ideal biomarker due to their high concentration (Chen et al., 2009; Chen et al., 2015; Hsu et al., 2018; Kuo et al., 2018), and may be suitable for detecting schizophrenia. We are not aware of any previous studies of antibodyyme analysis for schizophrenia, and specifically of any related work on schizophrenia with a different onset age.

In this study, we used the E. coli proteome microarray to analyse antibody differences among adult-onset and early-onset schizophrenia patients and compared them to healthy controls. We used random sampling to construct training and validation sets to avoid sampling bias when identifying the proteins that differ the antibodies among those groups, and then used a support vector machine (SVM) to find the best combination panel. Enrichment analysis for the differentiated proteins was also conducted, leading to the discovery of an autoantibody in AOS against the human ATP binding cassette sub-family b member 1 (ABCB1) protein.

2. Materials and methods

2.1. Study design and participants

The study design and framework are shown in Fig. 1A. All schizophrenia patients were recruited from the inpatient wards and outpatient clinics of the Chi Mei Medical Center and National Cheng Kung University Hospital in southern Taiwan. The patients of the present study were diagnosed with schizophrenia according to the Structured Clinical Interview for the Diagnostic and Statistical Manual for Mental Disorders, Fifth Edition (DSM-5) by psychiatrists from the recruited hospitals and considered to be medically stable. Healthy controls (HC) were all without personal and family histories of any psychiatric illnesses and composed of hospital staff and the general community. All participants were of Taiwanese Chinese Han origin and 20–65 years of age. A total of 80 SZ patients and 40 HC were recruited between July 2018 and June 2020. The exclusion criteria were traumatic brain injury or surgery, severe neurological abnormality, somatic symptom disorder, mental retardation, and illegal substance use. All included patients signed an informed consent form. This study was approved by the Institutional Review Board (IRBs) of the two participating hospitals (IRB numbers: 10612–011, B-TR-106–088, B-TR-108–094 and 10901–006). Each participant provided written informed consent after being made fully aware of the description of the study.

2.2. Age at onset of schizophrenia

In our previous studies, we defined the individual’s condition as AOS if their symptoms occurred after they were 20 years old (Chen et al., 2021; Chen et al., 2019; Liu et al., 2020; Tsai et al., 2016). Conversely, if the individual was diagnosed before the age of 20, their condition was classified as early-onset schizophrenia (EOS) (Frazier et al., 2012; Holmen et al., 2010; Sikich et al., 2008). In the present study, a total of 80 schizophrenia patients were sorted into two subgroups based on the onset age, with a total of 40 AOS and 40 EOS patients. The onset age was accessed from the medical records; onset ages as reported by patients or their family members were only used in the few cases where patients’ medical records were unavailable.

2.3. Peripheral blood sample collection

For peripheral samples, whole blood was collected using ethylendiaminetetraacetic acid (EDTA) anticoagulant tubes. Specimens were processed into plasma by centrifugation (800 × g, 10 min, 4 °C) within 2 h of collection to maintain the integrity of the sample. To preserve quality, we transferred the plasma into RNase/DNase-free microcentrifuge tubes and kept them at ~ 80 °C for long-term storage.
2.4. Fabrication of Escherichia coli proteome chips

We conducted the high-throughput protein expression, purification, and printing method according to our previous study (Chen et al., 2008). To print the *E. coli* proteome microarray, 4,300 purified proteins were spotted on each aldehyde slide in duplicates using the SmartArrayer 136™ (CapitalBio, Beijing) at 4°C for 8 h and then stored at 80°C before being probed with samples (Fig. 1B).

2.5. Experimental design and statistical rationale

The proteome chip was thawed using phosphate-buffered saline with 0.05% tween 20 (PBST) and blocked with 3 ml of 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at ambient temperature. After removing the blocking solution, 3 ml of a 4000-fold plasma sample diluted with 1% BSA in PBS was added and incubated at ambient temperature for 1 h. The chip was washed with PBST for 10 min 3 times, incubated with DyLight® 650-labeled goat anti-Human IgM antibody (A80-100D5; Bethyl Laboratories, Montgomery, TX) and DyLight® 550-labeled goat anti-Human IgG-Fc fragment antibody (A80-104D3; Bethyl Laboratories) at ambient temperature for 1 h. The final concentration was 0.33 µg/ml (1,500 fold) for IgM and 0.25 µg/ml (2,000 fold) for IgG. After antibody incubation, the chips were washed with PBST for 10 min 3 times and then dried by centrifugation at 164×g for 1 min at ambient temperature. Finally, the chips were read using a CapitalBio LuxScan™10 K microarray scanner (CapitalBio, Beijing). The excitation wavelength was 532 nm, and the emission wavelength was 570 nm (Fig. 1C).

Signal intensity for each spot was analysed using GenePix® Pro 6.0 software (Molecular Devices Corporation, Union City, CA). After processing and normalizing the duplicates in each chip by mean scaling, we used SAS 9.4 (SAS Institute, Cary, NC) to select the initial markers. Selection was based on significant differences (Student’s t-test) in 10 replicate tests of sets of random samples drawn from training (n = 30) and validation sets (n = 10). Based on the signal intensity, we selected the proteins that significantly differed between patients and controls and were also consistently assessed by eyeballing as being in the top 20 of the training sets in all replicates ( Supplementary Tables S1-S8 ). Eyeballing was based on the comparison of the intensity of the spot of interest with neighbouring spots on the original images; the eyeballing accuracy formula is listed in Supplementary Information. For any protein, eyeballing was only used as a selection criterion if accuracy of support vector machine learning was equal or higher than 70%.

Proteins with a standard deviation at least 3 times larger than the global mean across all random sampling replicate tests were selected. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) ver. 6.8 (https://david.ncifcrf.gov/) was used for the functional bioinformatics microarray analysis, including enrichment analysis in Gene Ontology (GO), using four major categories: biological process, cellular component, molecular function, and protein domain.

Twenty µg/ml of ABCB1 protein (TP710110; OriGene Technologies, Rockville, MD) was printed on an aldehyde-functional glass slide by a SmartArrayer 48™ Microarray Spotter (Capital Bio, Beijing) for immobilizing the ABCB1 autoantibody identification chip. Then the chip was treated with 3% BSA to reduce non-specific binding, followed by
probing with diluted plasma (1,500-fold). After washing with TBST, the chip was incubated with DyLight® 550-conjugated anti-Human IgG antibody for 1 h, and the fluorescence signal was measured with a CapitalBio LuxScan® 10 K microarray scanner (CapitalBio, Beijing). The acquired microarray images were analysed and indicated as 550 median-550 background using GenePix® Pro 6.0 software (Molecular Devices Corporation, San Jose, CA).

3. Results

3.1. Descriptive data of participants

The demographic profiles of the participants are presented in Table 1. There were no significant differences in age, gender proportion, and body mass index (BMI) between EOS and AOS groups compared to HC. However, there were significant differences in mean age (34.1 ± 9.0 and 42.3 ± 9.7, p < 0.0001), onset age (16.9 ± 2.5 and 27.7 ± 7.1, p < 0.0001), and duration of illness (17.2 ± 8.5 and 14.7 ± 9.9, p < 0.0050) between the AOS and EOS groups.

3.2. Research framework

The framework of this study is shown in Fig. 1. In this research, we printed all E. coli K12 proteins to make E. coli proteome chips (Fig. 1B). The chips were used to identify proteins that differentiate HC from EOS, AOS and SZ. From the differentiation proteins, we formed combination panels to identify the best protein combination panel for classification. Enrichment analysis was also conducted for the proteins that significantly classify the comparison groups. According to the enrichment result, we discovered and validated the ABCB1 IgG level difference between HC and AOS.

3.3. Proteins that differentiate between schizophrenia patients and healthy controls

Fig. 2A shows the representative E. coli proteomes microarray images of EOS, EOS, and HC. We aligned the spots by GenePix® Array List file, and then the signal intensity was produced by GenePix® Pro 6.0, and defined as foreground mean minus background mean. After extracting the signal intensity data, the further analysis flow chart is shown in Fig. 1C. The comparison between groups for IgG and IgM, include Sz vs HC, EOS vs HC, AOS vs HC and EOS vs AOS. We resampled the training set and the validation set for 10 times. Based on the protein signal intensity, we ranked the proteins with t-test p < 0.05 in training set. If the proteins were always in the top 20 among all the 10 times resampled training sets, they were selected as differentiation proteins (for details, see the Supplementary Table S1-S8). For those selected differentiation proteins, area under the curve (AUC), accuracies, specificities, and sensitivities were calculated in both training set and validation set by using SVM (Table 2). In this part, we set the criteria: AUC of both the training set and the validation set need to be above 0.7. Overview of all groups’ results, EOS vs HC (IgG) did not have any differentiation proteins pass our criteria. Although EOS vs HC (IgM) had two differentiation proteins, and AOS vs EOS (IgG) also had one candidate proteins; however, the AUC in validation set are both < 0.7. Thus, we did not further work on those three groups and focused on the analysis of other groups with eyeballing all differentiate protein images to evaluate their accuracies.

Table 3 shows the analysis result that the differentiation proteins yjjU, livG, pldA, livG, and ftsE in AOS vs HC (IgG) group were > 70%, indicating only those five proteins are really promising, and they are for differentiating IgG in AOS vs HC. Fig. 2B shows the representative images of the five proteins in the IgG of AOS and HC. We further used SVM to find the best combination panel among these five proteins. Table 4 summarizes the AUC and accuracy of all combination among the five proteins in both training data set and validation data set. Four combinations show that all their AUC, accuracies, specificity and sensitivity are larger than 0.7 in both training and validation sets, including panel I (grol, pldA, livG and ftsE), panel II (grol, yjjU and livG, ftsE), panel III (grol, yjjU and livG) and panel IV (yjjU, livG and ftsE). Among the four combinations, only the combination of yjjU, livG and ftsE acts as the best combination panel for differentiating IgG in AOS vs HC with 0.8 AUC, 80% accuracy, 90% specificity and 70% sensitivity in the validation set. To evaluate if these three proteins are specific to differentiate schizophrenia from HC, we also cross checked our previous study in bipolar I disorder (Chen et al., 2015). In comparison with bipolar patients, these three proteins are specific to AOS, which present the different higher-expressed hits of antibodies.

3.4. Enrichment of proteins that differentiate IgGs between AOS and HC

Since only group of AOS vs HC in IgG was classified well, we further analysed the enrichment of proteins that differentiate IgGs between AOS and HC to know the significant characteristics of those proteins. From 10 times random sample t-test, 49 proteins showed p < 0.05 and the protein difference larger than the global difference mean 3SD in all 10 time random-samples (for details, see the Supplementary Table S9). To further examine those IgG proteins that were significantly effective in distinguishing AOS from HC, we carried out gene ontology (GO) and protein domain enrichment analysis and present the results in Supplementary Material and Fig. 3.

In the biological process, those 49 proteins are enriched in the lipid catabolic process (p-value < 0.05). ATP-binding cassette (ABC) transporter complex are enriched in cellular component. In the molecular function, ATPase activity, ATP binding and transcription factor activity, sequence-specific DNA binding are enriched. Five protein domains related to ABC transporter, ATPase and kinase were also enriched. It is worth noting that ABC transporter has been reported in schizophrenia by immunohistochemical study (Bernstein et al., 2016). ABCB1, an important molecular component of BBB, was reported to have significantly reduced expression in the schizophrenia by immunohistochemical study (Bernstein et al., 2016). However, another study reported that ABCB1 activity on BBB was increased in the patients with chronic schizophrenia (de Klerk et al., 2010). According to Interpro database, the domains of ABCB1 are also categorized into ABC transporter-like, AAA+ ATPase, and F-loop NTPase.

In our study here, we found the target proteins of the antibodies in EOS patients are enriched in ABC transporter related domains, and these antibodies are not in normal control. This indicates that EOS, but not normal people, has the antibodies against ABC transporter related domains. We hypothesize that the immunohistochemically reduced ABCB1 expression observed in the report (Bernstein et al., 2016) was actually reflected the blocking of autoantibodies against ABCB1 in the schizophrenia patients. It is interesting that in the same report, increased brain levels of proinflammatory cytokines in schizophrenia patients was observed (Bernstein et al., 2016), which is possible due to the autoantibody attack to ABCB1 on BBB. It is also worth noting that in our best

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**Table 1**

Demographic characteristics and clinical profiles of participants.

| Characteristics | Patients with schizophrenia (AOS (N = 40) vs EOS (N = 40)) | Healthy controls (N = 40) |
|-----------------|----------------------------------------------------------|--------------------------|
| Male            | 25/62.5%                                                 | 27/67.5%                 |
| Age (years)     | 42.3 ± 9.7                                               | 34.1 ± 9.0               |
| Onset age (years)| 27.7 ± 7.3                                               | 16.9 ± 2.5               |
| BMI (kg/m²)     | 26.2 ± 5.9                                               | 25.3 ± 4.4               |

* AOS vs EOS, p < 0.05. AOS: adult-onset of schizophrenia; EOS: early-onset of schizophrenia; BMI: body mass index.
combination panel for differentiate between AOS and HC, two out of three proteins (ftsE and livG) are categorized into the domains of AAA+ ATPase, ABC transporter-like, ABC transporter conversed site and P-loop_NTPase. These indicate the levels of antibodies against ABCB1 in the AOS and HC plasma samples could be very different.

### 3.5. AOS patients have higher human ABCB1 autoantibody level than healthy controls

To validate our hypothesis that autoantibody against ABCB1 is associated with AOS, Fig. 4 displays the strategy to detect the anti-human ABCB1 autoantibody in plasma. First, the human ABCB1 proteins were printed on a glass aldehyde slide. Then, the antibodies in plasma probed on the glass slide. The anti-ABCB1 antibody was further identified via probing the Dylight® 550 conjugated anti-Human IgG. The anti-ABCB1 antibody was examined whether the presence in both in the plasma of AOS patients and healthy controls. The dots-plat shows the quantitative of fluorescence signal intensity between the AOS and healthy group. The level of anti-ABCB1 IgG antibody in AOS group were significantly higher than the health group. This result validates our

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**Table 2**

Differentiation of the top 20 ranked proteins as a panel in the training and validation sets for each pair of comparison groups by support vector machine (SVM) surveillance. 

|                | SZ vs HC |                        | EOS vs HC |                        | AOS vs HC |                        | EOS vs AOS |                        |
|----------------|----------|------------------------|----------|------------------------|----------|------------------------|----------|------------------------|
|                | IgG      | Training set          | IgM      | Training set          | IgG      | Training set          | IgM      | Training set          |
|                | ftsE, inaA, kefG, livG, napF, nikiE, pldA, prkB, rfbC, rffC, ycnN, yjjU | AUC: 0.98, Accu: 0.93, Sens: 0.99, Spes: 1.00, Validation set: 0.76, 0.77, 0.80, 0.70 | malY, maxG, uidR, ybaJ, ybdH, ybiU, yjjC | AUC: 0.96, Accu: 0.89, Sens: 0.85, Spes: 0.97 | Validation set: 0.70, 0.73, 0.70, 0.80 |
|                | IgG      | Training set          | IgM      | Training set          | IgG      | Training set          | IgM      | Training set          |
|                | None     | AUC: –, Accu: –, Sens: –, Spes: –, Validation set: –, –, –, – | uidR, fliN | AUC: 0.92, Accu: 0.85, Sens: 0.73, Spes: 0.97 | Validation set: 0.62, 0.50, 0.40, 0.60 |
|                | IgG      | Training set          | IgM      | Training set          | IgG      | Training set          | IgM      | Training set          |
|                | groL, ycnN, yjjU, pldA, napF, livG, rfbC, nikiE, ftsE, kefG, yjiR, yjbl, yscT, rffC | AUC: 0.97, Accu: 0.93, Sens: 0.97, Spes: 0.90 | malY, maxG, thiC, ybaJ, ybdH, yfeM, yjjC | AUC: 0.93, Accu: 0.85, Sens: 0.93, Spes: 0.77 | Validation set: 0.77, 0.65, 0.70, 0.60 |
|                | IgG      | Training set          | IgM      | Training set          | IgG      | Training set          | IgM      | Training set          |
|                | yjbl     | AUC: 0.63, Accu: 0.65, Sens: 0.87, Spes: 0.43 | uidR, fliN | AUC: 0.74, Accu: 0.62, Sens: 0.63, Spes: 0.60 | Validation set: 0.74, 0.70, 0.80, 0.60 |

*The top 20 ranking proteins with a p-value < 0.05 were considered as a prospective panel. SVM was then used to perform the comparison between groups in training and validation. SVM: support vector machine; SZ: patients with schizophrenia; AOS: adult-onset of schizophrenia; EOS: early-onset of schizophrenia; HC: healthy control; AUC: area under curve; Sen: sensitivity; Spe: specificity; Accu: accuracy; IgG: immunoglobulin G; IgM: immunoglobulin M.*
Table 3
Eyeballing evaluation of accuracy of differentiation proteins.

| Protein list | Average | Protein list | Average |
|--------------|---------|--------------|---------|
| IgG tfsE      | 59.44   | IgM malY     | 45.14   |
| inaA         | 61.11   | marG        | 33.89   |
| keG          | 62.78   | uidR        | 42.78   |
| livG         | 61.67   | ybaJ        | 42.78   |
| napf         | 62.22   | ybdH        | 40.00   |
| nikE         | 59.44   | ybuC        | 39.44   |
| plA          | 63.33   | yjcC        | 52.78   |
| prkt         | 60.00   |             |         |
| rfibC        | 53.89   |             |         |
| rfC          | 55.56   |             |         |
| ycfN         | 62.78   |             |         |
| yjiU         | 62.33   |             |         |

EOS vs HC

| IgG | yjb1 | 55.83 | IgM | uidR | 50.00 |
|-----|------|-------|-----|------|-------|
| –   | –    | 53.33 | flN |      |       |

AOS vs HC

| IgG | grol  | 70.00 | IgM | malY | 61.67 |
|-----|-------|-------|-----|------|-------|
| ycnB| 68.33 | marG  | 51.67 |
| yjiU| 70.00 | thcC  | 59.17 |
| plA | 71.67 | ybaJ  | 59.17 |
| napf| 69.17 | ybdH  | 57.50 |
| livG| 70.83 | yfcM  | 65.00 |
| rfbC| 67.50 |       |      |
| kefC| 69.17 |       |      |
| nikE| 65.83 |       |      |
| ftSE | 70.00 |       |      |
| yjiR| 65.00 |       |      |
| yjb1| 64.17 |       |      |
| ycfN| 69.17 |       |      |
| rfcC| 61.67 |       |      |

EOS vs AOS

| IgG | None | IgM | uidR | 56.67 |
|-----|------|-----|------|-------|
|     |      | flN | 57.50 |

a The proteins that passed the eyeballing evaluation were selected for further analysis as candidate biomarkers of schizophrenia. SZ: patients with schizophrenia; EOS: early-onset of schizophrenia; AOS: adult-onset of schizophrenia; HC: healthy control; IgG: immunoglobulin G; IgM: immunoglobulin M.

4. Discussion

In the present study, we employed a total of 4,300 purified E. coli proteins as an E. coli proteome microarray to reveal the antibody profiles of schizophrenia and distinguish adult-onset schizophrenia patients from mentally healthy controls. We identified a set of protein combination that is able to distinguish AOS from HC and found that higher antibody expression against ABCB1 occurred specifically in adult-onset schizophrenia patients in comparison with healthy controls. Our results suggest that the E. coli proteome microarray is a promising platform for profiling the antibody signature of schizophrenia. Here, proteome microarrays serve as antigen microarrays, which have proven to be effective for determining antibody-protein interactions based on linear and conformational epitopes. This allows rapid, cost-efficient and user-friendly screening of novel biomarkers for clinical use (Ramachandran et al., 2008). The results of the present study also showed that the E. coli proteome microarray is able to identify serological antibody biomarkers for AOS and reveal their potential binding domain.

It is commonly accepted that microbiota regulate social behaviour via stress response neurons in the brain through the gut–brain axis (Wu et al., 2021), and weak BBB function in schizophrenia patients might enlarge the influence of microbiota-immune-brain communication on stress-related psychiatric behaviours. More importantly, schizophrenia patients have been reported to have the leaky gut problem (Barber et al., 2019; Maes et al., 2019b), which can cause translocate E. coli protein from the intestine to the blood in turn may activate immune response and produce E. coli antibodies. Thus, we discovered the AOS and EOS are varied, more prone to present antibodies against ABC transporter related domains compared to healthy individuals, which in turn may drive schizophrenia symptoms.

Moreover, the concentration of cytokines is increased in schizophrenia (Fillman et al., 2016; Miller et al., 2011), causing B cells activated by T-cell-produced cytokines to change their weak binding with immunoglobulin-μ to the highly specified IgG (Glass et al., 2017) as brain-reactive antibodies (Borda et al., 2004; Dahm et al., 2014; Deluarno et al., 2016; Jones et al., 2014; Levin et al., 2010; Margari et al., 2013; Tanaka et al., 2003). In previous studies, a higher percentage of IgG antibodies targeting N-methyl-aspartate receptors (NMDAR) was considered of clinical and pathogenic significance (Hara et al., 2018) in the plasma in patients experiencing first-episode psychosis (Ezeoke et al., 2013) or cognitive impairments (Dulman et al., 2008; Hammer et al., 2014) and were diagnosed with schizophrenia (Hara et al., 2018), compared to healthy individuals. On the other hand, lower IgG synthesis within the central nervous system (CNS) and cerebrospinal fluid (CSF) of patients with schizophrenia was found to be related to psychosis (Melkersson and Bensing, 2018). These consistent and intriguing results might indicate that different levels of IgG are clustered within in different types of schizophrenia, as the IgG panels developed by the present study also suggest.

Our analysis results indicate the most effective combination panel of IgGs (yjiU, livG, and tfsE), and show possible molecular mimicry to ABCB1. A study of immunolocalized ABCB1 protein expression in twelve brain regions in schizophrenia patients found that ABCB1 is located in multiple brain vessels and at habenular neurons known for purinergic neurotransmission (Bernstein et al., 2016). More importantly, the ABCB1 gene polymorphisms had a consistent impact on the autonomic nervous system, antipsychotic drug dosage, and therapeutic responses of schizophrenia patients of different ethnicities (Azam et al., 2021; Bernstein et al., 2016; Haltori et al., 2018; Haltori et al., 2020; Vijayan et al., 2012; Yan et al., 2020). Another study reported that ABCB1 activity on BBB was increased in the chronic schizophrenia patients (de Klerk et al., 2010). The several studies of pharmacogenetics also identified the expression of ABCB1 might have effect on the risperidone treatment response and no association with improvement of the condition in positive and general symptoms in SZ (Cendrosi et al., 2020; Jovanovic et al., 2016; Suzuki et al., 2014). However, the ABCB1 expression and gene variants on plasma drug levels, treatment response and adverse effects between the AOS and EOS remain unclear.

Although we performed antibody profiling of schizophrenia patients using advanced screening tools, some methodological limitations may apply to the present study. First, while there were no differences in age, gender, and BMI between EOS and AOS patients compared to HC, we still could not exclude the possibility that smoking, dietary habits, and physical activity levels might exert confounding effects on the results. Second, the included schizophrenia patients were all in a chronic but stable period during the study period. The effects of various psycho-pharmacological treatments might affect the production of immunoglobulin.

We used E. coli proteome microarrays to probe human plasma samples to identify proteins that allow distinguishing between different onset age schizophrenia patients from healthy controls. This could present a unique perspective for revealing antibody profiles in schizophrenia. We found that different onset ages were linked to different sets of differentiation protein panels. In particular, AOS was classified by a panel of yjiU, livG and tfsE for differentiating IgG in AOS patients from HC. In contrast, no suitable differentiation proteins were found for EOS patients. These intriguing findings suggest different immune responses and immunoglobulin levels in AOS patients, indicating different
### Table 4

Area under curve (AUC), sensitivity, specificity and accuracy in the training and validation set of all combinations among the five proteins of IgG that passed eyeballing validation.

| Group | Protein list | Training set | Validation set |
|-------|--------------|--------------|----------------|
|       |              | AUC | Accu | Sen | Spe | AUC | Accu | Sen | Spe |
| AOS   | groL, pldA, yjjU, livG, ftsE | 0.88 | 0.83 | 0.97 | 0.70 | 0.68 | 0.77 | 0.70 | 0.70 |
|       | yjjU, livG, pldA, ftsE | 0.89 | 0.80 | 1.00 | 0.60 | 0.82 | 0.80 | 0.90 | 0.70 |
| HC    | groL, yjjU, livG, ftsE | 0.96 | 0.87 | 0.97 | 0.77 | 0.80 | 0.75 | 0.80 | 0.70 |
|       | groL, pldA, yjjU, ftsE | 0.95 | 0.88 | 0.93 | 0.83 | 0.70 | 0.70 | 0.60 | 0.80 |
|       | groL, pldA, yjjU, livG | 0.89 | 0.88 | 0.97 | 0.80 | 0.76 | 0.70 | 0.60 | 0.80 |
|       | groL, yjjU, pldA | 0.88 | 0.77 | 0.83 | 0.70 | 0.71 | 0.70 | 0.60 | 0.80 |
|       | groL, yjjU, livG | 0.72 | 0.87 | 0.97 | 0.77 | 0.71 | 0.75 | 0.80 | 0.70 |
|       | groL, yjjU, ftsE | 0.91 | 0.83 | 0.93 | 0.73 | 0.72 | 0.65 | 0.60 | 0.70 |
|       | groL, pldA, livG | 0.60 | 0.85 | 0.67 | 0.83 | 0.73 | 0.75 | 0.80 | 0.70 |
|       | groL, pldA, ftsE | 0.88 | 0.85 | 0.97 | 0.73 | 0.72 | 0.70 | 0.60 | 0.80 |
|       | groL, livG, ftsE | 0.83 | 0.82 | 0.93 | 0.70 | 0.71 | 0.70 | 0.60 | 0.80 |
|       | yjjU, pldA, livG | 0.90 | 0.83 | 0.93 | 0.70 | 0.65 | 0.70 | 0.80 | 0.60 |
|       | yjjU, pldA, ftsE | 0.88 | 0.82 | 0.97 | 0.67 | 0.66 | 0.60 | 0.70 | 0.60 |
|       | pldA, livG, ftsE | 0.88 | 0.82 | 0.97 | 0.67 | 0.66 | 0.60 | 0.70 | 0.60 |
|       | groL, pldA | 0.83 | 0.80 | 0.93 | 0.67 | 0.66 | 0.65 | 0.80 | 0.50 |
|       | groL, yjjU | 0.77 | 0.75 | 0.97 | 0.53 | 0.74 | 0.75 | 0.80 | 0.70 |
|       | groL, livG | 0.60 | 0.70 | 0.93 | 0.43 | 0.71 | 0.65 | 0.80 | 0.50 |
|       | groL, ftsE | 0.70 | 0.68 | 0.93 | 0.43 | 0.71 | 0.65 | 0.80 | 0.50 |
|       | pldA, yjjU | 0.65 | 0.67 | 1.00 | 0.33 | 0.83 | 0.65 | 1.00 | 0.30 |
|       | pldA, livG | 0.88 | 0.82 | 0.97 | 0.67 | 0.66 | 0.70 | 0.80 | 0.60 |
|       | plAA, ftsE | 0.83 | 0.83 | 0.97 | 0.50 | 0.86 | 0.80 | 0.90 | 0.70 |
|       | pldA, ftsE | 0.85 | 0.77 | 0.93 | 0.60 | 0.76 | 0.65 | 0.70 | 0.60 |
|       | yjjU, livG | 0.78 | 0.72 | 1.00 | 0.43 | 0.80 | 0.75 | 0.80 | 0.70 |
|       | yjjU, ftsE | 0.70 | 0.67 | 1.00 | 0.33 | 0.83 | 0.65 | 1.00 | 0.30 |
|       | livG, ftsE | 0.78 | 0.77 | 0.93 | 0.60 | 0.82 | 0.80 | 0.90 | 0.70 |
|       | ftsE | 0.65 | 0.67 | 1.00 | 0.33 | 0.83 | 0.65 | 1.00 | 0.30 |
|       | groL | 0.63 | 0.65 | 0.87 | 0.43 | 0.73 | 0.65 | 0.80 | 0.50 |
|       | livG | 0.73 | 0.72 | 1.00 | 0.43 | 0.82 | 0.80 | 0.90 | 0.70 |
|       | pldA | 0.76 | 0.70 | 0.90 | 0.50 | 0.78 | 0.75 | 0.80 | 0.70 |
|       | yjjU | 0.65 | 0.67 | 1.00 | 0.33 | 0.79 | 0.60 | 0.90 | 0.30 |

AOS: adult-onset of schizophrenia; HC: healthy control; IgG: immunoglobulin G; AUC: area under curve; Accu: accuracy; Sen: sensitivity; Spe: specificity. The protein in bold type means the combination panel and all their AUC, accuracy, sensitivity and specificity are larger than 0.7 in both training and validation sets.

**Fig. 3.** Enrichment analysis of differentiated proteins between AOS and HC groups. Using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) as a bioinformatic tool for functional annotation. The figure shows the Gene Ontology (GO) partitioning into the categories of biological process, cellular component, molecular function and InterPro protein domain, among 49 proteins that were significantly enriched ($p < 0.05$, i.e. $-\log_{10} p = 1.30$).
mechanisms for AOS and EOS patients due to environmental effects. Based on our study results, the specific antibodies expressions in peripheral blood would help to facilitate the diagnosis of AOS patients by developing a rapid test kit for the detection of specific IgG using antigen yGy, livG and fsf.

5. Data availability

The datasets used and analyzed in the current study are not publicly available due to conditions on participant consent and other ethical restrictions. The raw data that supports the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Due to ethical restrictions and conditions on participant consent, the datasets in the current study are not publicly available.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbi.2022.07.162.

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