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Autoantibodies against the C-terminus of Lipopolysaccharide binding protein are elevated in young adults with psychiatric disease

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ABSTRACT

Growing evidence implies interactions between infections, the immune system and vulnerability for psychiatric disease. This study applies an affinity proteomic-based method to investigate potential disease associated autoantibody signatures in serum from patients from the "Young Adults" section of the Department of General Psychiatry at Uppsala University Hospital (n = 395) and population-based controls (n = 102). We found serum levels of antibodies against Lipopolysaccharide Binding Protein (LBP), a protein that is important for mediating innate immune responses involving the toll-like receptor-4 (TLR-4), to be higher in patients compared to controls (Mann Whitney *U*-test $p = 5.248 \times 10^{-10}$). The patients were divided into three groups based on their relative levels of autoantibodies against LBP. The distribution of autism spectra disorders ($p = 2.0 \times 10^{-4}$) and hospital care for an infection as adults ($p = 0.036$) differed between the anti-LBP groups, with low incidence in the group of patients with the highest levels of anti-LBP who were diagnosed with primarily affective and anxiety disorders. In a sub-group analysis, the controls who screened positive for current or previous psychiatric diagnosis (n = 20) had higher anti-LBP compared to non-psychiatric controls with negative screening for psychiatric disorders (Mann Whitney *U*-test $p = 0.006$). Inflammatory markers were found to differ across anti-LBP groups and several pro-inflammatory markers, including IL-1β, were low in patients with high anti-LBP and serum LBP levels were lowest in patients with the highest levels of antibodies against LBP ($p = 3.5 \times 10^{-5}$). A cell-based model showed that polyclonal rabbit anti-LBP, obtained through purification *via* the same protein fragment used in the initial autoantibody analysis, could interfere with LBP signaling since addition of anti-LBP to the assay reduced both IL-1β and IL-6 release from activated monocytes in response to LBP and LPS ($p = 0.0001$ and $p = 0.02$). This novel finding of antibodies against LBP, where high levels were only found in young adults with psychiatric disease, merits further study. Our results suggest that these antibodies may have relevance for TLR4 based immune responses and vulnerability for both infection and psychiatric disorders.

1. Introduction

Epidemiological data confirms that autoimmune disease and severe infections are synergistic risk factors for psychiatric disease (Benros et al., 2011, 2012, 2013). A nationwide study identified a 64% increased risk of affective disorders post-treatment with anti-infective agents (Köhler et al., 2017). Another population-based study showed associations between childhood infections and the risk for psychotic illness. The strongest association was seen between hospital admission with bacterial infection and the subsequent development of a nonaffective psychosis during pre-adolescence (hazard ratio: 1.23) (Blomström et al., 2014).

Lipopolysaccharide Binding Protein (LBP) is an acute phase protein of 60 kDa produced mainly in the liver and secreted into the bloodstream. During acute infections, LBP levels are elevated as a response to immunological triggers such as bacterial derived lipopolysaccharides

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(LPS) (Tobias et al., 1986). LPS is the major component of the outer membrane compartments of gram-negative bacteria and is actively secreted through bacterial outer membrane vesicles (Beveridge, 1999). Several types of bacterial infections result in a sudden increase in LPS which induce immunological response with increased expression and release of pro-inflammatory cytokines and may result in tissue damage and sepsis (Beutler and Rietschel, 2003). Consequently, an early detection and processing of bacterial-derived LPS molecules is of high importance. LPS binds to the Toll-like receptor 4 (TLR4)-MD2 complex leading to the activation of signaling components such as NF-kB, and the production of cytokines (Raetz and Whitfield, 2002; Akira and Takeda, 2004; Park and Lee, 2013). LBP and CD14 are key molecules in recognizing and transferring LPS to the TLR4 receptor complex (Hailman et al., 1994). Recent findings, show that the N-terminal tip of LBP binds LPS and the C-terminal tip interacts with CD14 in order to transfer LPS to TLR4 (Ryu et al., 2017). As evidence for the importance of LBP function, a common mutation/SNP in the C-terminus leads to a reduced immune response and reduced expression of pro-inflammatory cytokines upon LPS stimulation *in vitro*. Furthermore, patients carrying a mutation close to this motif have an increased risk for septic shock (Eckert et al., 2013).

In this study, a panel of 224 protein fragments including fragments from LBP were used in an affinity proteomics-based method to investigate autoantibody signatures in young adult patients and volunteers. We then further characterized the antibody affinity and investigated potential links to specific clinical psychiatric and somatic phenotypes. Thereafter, we investigated the potential function of these antibodies *in vitro* using a cell assay designed to test the hypothesis that they could potentially modulate immunological responses. Our hypothesis is that LBP autoantibodies may be related to vulnerability for psychiatric disease, immune system activation, and susceptibility to infections.

2. Materials and methods

2.1. Ethics

The study was carried out in accordance with the Declaration of Helsinki and was approved by the Regional Ethical Review Board in Uppsala, Sweden. Informed and written consent was obtained from each participant.

2.2. Study population characteristics

Between August 2012 and September 2015, all new patients, 18–25 years of age, with primarily affective and anxiety disorders, receiving ambulatory care at the "Young Adults" section of Dept. of General Psychiatry at Uppsala University Hospital were asked to participate in Uppsala Psychiatric Patient Samples (UPP). The UPP infrastructure is described previously (Cunningham et al., 2017). The type, phase and degree of illness was variable as some patients were remitted from other medical units already in stabilized treatment while others were untreated. The large majority of patients reported current symptoms of depression and/or anxiety. In total, 1071 patients were approached and of them 441 (41.1%) accepted to participate. An investigation as to reasons for declining participation was recently published (Bixo et al., 2019). Whole blood was collected from non-fasting participants during office hours. Plasma and serum were isolated and stored frozen at − 80 °C at Uppsala Biobank. Other than sample and data availability, no other exclusion criteria were applied. Blood samples and sufficient data were available for this study from 395 participants. The control group was recruited from university employees and students and who had not received specialist level psychiatric care during their lifetime. Participants underwent a clinical health examination including blood pressure and body mass index (BMI) and answered questionnaires on socio-demographics, medical history, heredity and current medication.

Psychiatric diagnoses for patients and controls were assessed based on a clinical interview and a diagnostic interview (the M.I.N.I.-International Neuropsychiatric Interview (M.I.N.I. 6.0) (Sheehan et al., 1998) or the Swedish version of the Structural Clinical Interview for DSM IV axis I disorders (SCID-I) (First, 1996)) and based the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria (American Psychiatric Association, 2000). In a post-hoc analysis, data was reanalyzed using the DSM-V criteria. Depressive symptoms were assessed in all participants with the Montgomery-Åsberg Depression Self-Rating Scale (MADRS-S) in parallel with blood sample collection (Mattila-Evenden et al., 1996; Cunningham et al., 2011). For patients who gave consent (395 patients), additional data were taken from the available medical records in October to November 2019 when available, concerning i) current neuropsychiatric diagnoses (ADHD, ADD or autism spectra), and ii) history of hospitalization for infections.

2.3. Suspension bead array for autoantibody profiling

Autoantibody profiling in serum was carried out using the previously described suspension bead array technology (Schwenk et al., 2008). Based on the planar array results, previous in-house screenings and targets from literature, a total of 224 protein fragments representing 127 proteins were selected for this study (Uhlén et al., 2005). The protein fragments with the length of about 80–140 amino acids were coupled to color-coded magnetic beads (MagPlex, Luminex corp.) as described elsewhere (Ayoglu et al., 2016). Two protein fragments for LBP were used in this study. One was 111 amino acids (AA) long obtained from the N-terminal LBP (position 113–224) and another 136 AA long sequence (position 296–432) found at the C-terminal part of the protein. The coupled protein fragments were then mixed together to create a bead array in suspension. In total 395 patient serum samples and 102 control samples were distributed on two 384 well plates including replicates and blank samples and incubated with protein containing sample buffer in a dilution of 1:250 (1 h, shaking, RT). Diluted samples were then incubated with the suspension bead array for 2 h at room temperatures. Following incubation, all unbound antibodies were washed away using 3 × 100 μl PBS-T (0.05% Tween) using an automated plate washer (EL406 Washer Dispenser – BioTek). Subsequently, the beads were incubated with 50 μl secondary R-PE conjugated anti-human IgG (R-phycoerythrin conjugated goat anti-human IgG, Invitrogen). After washing, bound autoantibodies were reported as median fluorescent intensities (MFI) using a FlexMap 3D instrument (Luminex corp.). Inter-assay variation of both plates had a CV of 13% and 7%, respectively.

2.4. Analysis of inflammatory markers

LBP was measured in serum from participants using a commercially available assay according to the manufactures protocol (MULTI-ARRAY Assay System, K151IYC-1, Meso Scale Discovery).

Electro chemiluminescent signals were detected corresponding to cytokine targets using a SECTOR Imager instrument (Meso Scale).

The plasma levels of inflammatory markers were analyzed with two methods: Meso Scale MULTI-SPOT (Meso Scale Discovery, MSD) described above and proximity extension assay (PEA; Proseek multiplex Inflammation panel I, Olink Bioscience) according to the manufacturer's protocol. The MesoScale analyzing kits we used were: Vascular Injury V-plex (K15198D), Cytokine V-plex (K151A0H), Pro-inflammatory V-plex (K15049D and Chemokine V-plex (K15047D).

For PEA analysis, 1 μl of plasma was analyzed according to manufacturer instructions on 96-well plates containing 90 samples and 6 controls (three inter-plate controls and three negative controls). The samples were evenly distributed over the plates and analyzed at the same time point, randomly distributed across eight plates. The PEA analyses were performed by a certified service manager (SciLife Lab, Uppsala). The methods and part of the data set are described earlier (Söderquist et al., 2019). For the cytokine analysis using PEA technologies the data was initially normalized for plate differences using a

median normalization, applied separately for each protein. Data normalization was performed using GenEx software in the Olink Wizard (www.olink.com). The quantification cycle (Cq) values generated in the real-time PCR PEA analysis were used to calculate the normalized protein expression (NPX) in three steps. The NPX data was presented on a Log2 scale. Across all 92 assays, the mean intra-assay and inter-assay variations observed were 7% and 18%, respectively.

2.5. Assay to evaluate the effect of rabbit anti-LBP on human monocyte pro-inflammatory cytokine release in vitro

The protein fragment and antibody towards C-terminal LBP were obtained *via* the Human Protein Atlas Project. Here the protein fragment (136 AA, 296–432) was designed, used for immunization, and purification of the corresponding antibody, obtaining a polyclonal rabbit-anti-human LBP antibody (HPA001508). A cell-based model was used to test if these antibodies could interfere with LBP signaling. Antibodies against collagen 1A were also used as a control (anti-COL1A, HPA011795). Human monocytes were isolated from healthy blood donors at Uppsala university hospital. After PBMC gradient separation (Ficoll-Paque PLUS density gradient media, GE health Care, Sweden), CD14+/CD16- cells were purified according to the manufacturers protocol (MACS Miltenyi Biotec, product number 130-117-337). The purified monocytes were cultured for one day at 37 °C in a humidified cell incubator, then counted for dead cells and frozen in populations of 14 million cells per vial with at least 98% live cells. For each cell experiment, one vial of cells was thawed at room temperature in RPMI 1640 medium supplemented with glutamine and 2% heat inactivated fetal bovine serum (Sigma Aldrich). After spin down and a PBS wash, cells were re-suspended in serum free media and transferred to a 96-well culture dish (Nunclon Delta) with 100,000 cells/well. Human recombinant Lipopolysaccharide binding protein (rhLBP RnD systems, 870-LP) and s-LPS (E.Coli O55:B5, L5418, Sigma Aldrich) were added to the cells in each well to induce cytokine responses. The antibodies were added in two concentrations to evaluate their impact on cytokine production. After a four-hour incubation at 37 °C in a humidified cell incubator, the cells were harvested, centrifuged and the supernatant was kept at − 20 °C prior to cytokine analysis. Cell supernatants were thawed and cytokine levels analyzed using the V-PLEX Proinflammatory Panel 1 Human Kit (Meso Scale Discovery, K15049D-1) according to the manufacturer's protocol. we used a custom made Proinflammatory Panel 1 Human Kit including the inflammatory markers linked to TLR4 signaling; interferon gamma (IFN-γ), interleukin − 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) was used. Electro chemiluminescent signals were measured corresponding to included cytokine targets using a SECTOR Imager instrument (Meso Scale). All treatments were performed in triplicates and the assay was repeated twice.

2.6. Statistical analysis

Statistical analyses were performed using the software SPSS (version 23.0) and R version 3.4.2. Visualizations were done using R and R studio (Team, 2013/5/1). Patient and control characteristics are compared in Table 1 where significance values are derived using Mann-Whitney *U* test for continuous variables and likelihood ratio test for categorical data. For the suspension bead array, the median of each individual sample across all antigens was calculated together with the median absolute deviation (MAD) for each data point in the profile and converted into scores as ordinal variable (0–9). defined as: 0: below detection; 1: below the median absolute deviation (MAD); 2: < 5 MAD; 3: > 5 MAD; 4: > 10 MAD; 5: > 15 MAD; 6: > 20 MAD; 7: > 40 MAD; 8:> 60 MAD; 9:> 80 MAD. The anti-LBP level as the ordinal variable was compared between patients and controls using Mann-Whitney *U*-test.

As the control group was smaller, we first conducted a *t*-test with bootstrapping of 1000 samplings (simple) using SPSS. Thereafter, we also performed a permutation test with 1 million iterations. In each iteration, measurements were randomly assigned to the case or control group, keeping the original group sizes (395 cases, 102 controls) and computing the mean difference in anti-LBP between the groups, thus creating a null distribution of mean differences to compare our true observation with. The permutation test was implemented in R 3.6.3. Further, as some controls screened positive for psychiatric disorders in the interview, a pre-specified sub group analysis was performed to compare anti-LBP reactivity between controls with and without history of psychiatric disorders.

All participants in this study were then grouped according to their reactivity against LBP into three groups defined arbitrarily as: Low anti-LBP: score 0–2, (MAD below factor 5) n = 359, Intermediate anti-LBP: score 3 (MAD between factor 5 and 10), n = 88 group, and High anti-LBP: score 4–9 (MAD above factor 10), n = 50.

All available clinical data was explored looking for more specific clinical phenotypes with association to elevated anti-LBP levels. These analyses included the continuous variables BMI, age, MADRS-S score; and the categorical variables: gender, medication, current diagnosis.

Table 1
Characteristics and inflammatory markers in controls and psychiatric patients.

	Controls	Patients	*p*-value
Number of individuals	102	395	
Age; Mean (SD)	25.6 (9.1)	21.4 (2.2)	< 0.001
Gender Male: Female	30:72	87:308	ns
BMI; Mean (SD)	22.6 (3.0)	23.5 (4.9)	ns
Smoker	1	100	< 0.001
Any psychiatric medication	6	253	< 0.001
Current major depressive disorder	0	174	< 0.001
Life-time depressive episode (unipolar)	14	277	< 0.001
Bipolar disorder type I, II or not specified	0	59	< 0.001
Alcohol/substance abuse or dependency	5	40	< 0.001
Life-time psychotic disorder	0	3	ns
Any current anxiety disorder (DSM IV)	7	245	< 0.001
Anorexia	0	10	ns
Bulimia nervosa	0	27	> 0.01
ADHD/ADD	0	55	< 0.001
Autism spectrum disorder	0	33	< 0.001
MADRS-S score; Mean (SD)	6.7 (5.6)	22.8 (9.3)	< 0.001
Sheehan Disability Score; Mean (SD)	3.1 (5.2)	17.2 (6.7)	< 0.001
Systemic autoimmune disease	2	11	ns
Celiac disease (self-reported)	2	4	ns
CRP (mg/L); Median (Range)	0.56 (0.02–68)	0.87 (0.03–155)	ns
Serum LBP (ng/ml); Median (Range)	2147 (11–26168)	1840 (62–21703)	ns
Hospitalization for infection as adult	na	24	na
Number of self-reported weeks sick due to infection within a year; Mean (SD)	na	1.9 (1.4)	na

Body mass index (BMI), Diagnostic and Statistical Manual of Mental Disorders version 4 (DSM-IV), ADHD/ADD: Attention deficit disorder with or without hyperactivity. Montgomery Åsberg Depressive Rating Scale –Self reported (MADRS-S:) SD (standard deviation), ns, not significant, na: not available, P-values are based on results from likelihood ratio test or Mann Whitney test. Systemic autoimmune disease includes inflammatory bowel disease, rheumatoid arthritis and psoriasis. C-reactive protein (CRP), Lipopolysaccharide binding protein (LBP).

Diagnostic codes were taken from the medical records in Uppsala University Hospital from patients until Nov 2019. As sufficient data was only available for adulthood within the state financed care, our analysis was focused on infections that required hospitalization.

Measured inflammatory markers with > 80% of the samples (both cases and controls) with values above lowest level of detection (LLOD) were managed as continuous variables and tested for differences between anti-LBP groups using a non-parametric test (Kruskal-Wallis). Inflammatory markers with 20–80% of samples with detectable levels were converted into the categorical variable (Yes = above LLOD, No = Below LLOD) and tested for different frequencies between anti-LBP groups using Likelihood ratio test. Inflammatory markers with less than 20% of samples with detectable levels were excluded. A summary of all the continuous, categorized and excluded inflammatory markers are presented in Supplementary Table 2. Analyses were performed for cases and controls combined and then again as a *post hoc* test excluding controls.

The distribution of the data was further explored using different thresholds for grouping and heatmaps. The heatmap was created to visualize the relationship between anti-LBP groups and inflammatory markers (z-scores). High levels or scores are coded orange while low levels or scores are coded in purple.

For the human monocyte cell-assay, the mean and the SD of the triplicates from one representative experiment was calculated. We did Student's *t*-test with significance levels set to $p < 0.05$.

3. Results

An affinity based proteomic approach was used to profile the autoantibody repertoire of 395 patients and 102 volunteers, see Table 1 for population description. The standardized psychiatric diagnostic interview revealed 20 controls who screened positive for previous or current psychiatric conditions (consisting of mild depression and anxiety disorders) and six of these controls were medicated with psychiatric medication without current symptoms.

3.1. Suspension bead array analysis

After data processing and analysis, potential disease associated autoantibodies were identified (data not shown). Among the top candidates, we identified autoantibodies towards the C-terminal fragment of LBP with a higher mean rank of the ordinal values in patients compared

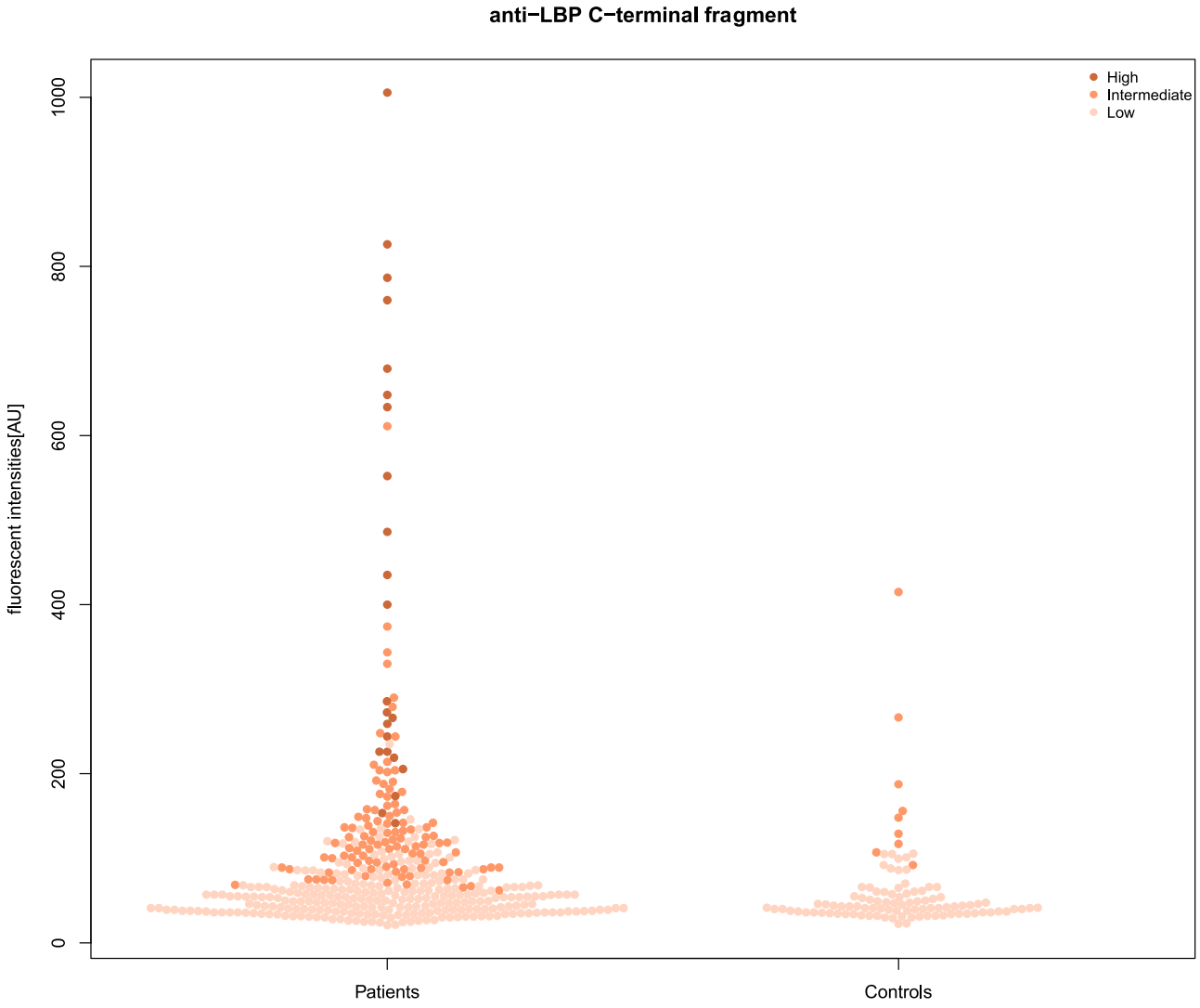


Fig. 1. Autoantibody reactivity towards the c-terminal fragment of lipopolysaccharide binding protein (LBP). Shown are the relative levels of anti-LBP antibodies in patients and controls. Color codes indicate the three groups based on LBP autoantibody reactivity adjusted for individual background signal using median absolute deviation (MAD); Low (MAD < 5), Intermediate (MAD < 10), High (MAD > 10) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

to controls (Mann Whitney *U*-test, $p = 5.248 \times 10^{-10}$, Fig. 1). Anti-LBP levels were also higher in controls (n = 20) who screened positive for current or previous psychiatric diagnosis compared to controls with negative screening for psychiatric disorders (Mann Whitney *U*-test $p = 0.006$, data not shown. The mean difference between cases and controls in reactivity (anti-LBP score) was 0.786, as calculated with 1000 bootstraps in *t*-test, $p < 0.001$). Furthermore, permutation test revealed that the difference between cases and controls was not due to chance, $p = 1.0 \times 10^{-6}$. No reactivity was found for the N-terminal LBP fragment.

3.2. Serum LBP levels are lower in individuals with high anti-LBP

Total serum LBP (s-LBP) was measured in patients and controls (495 samples were analyzed, two samples had too low volume for the s-LBP analysis). Serum LBP levels differ between anti-LBP groups ($p = 3.5 \times 10^{-5}$, Fig. 2). Post-hoc tests found that patients with highest levels of antibodies against LBP had lower s-LBP than both Intermediate and Low anti-LBP groups ($p = 3.5 \times 10^{-5}$ and $p = 0.006$, respectively), while the Intermediate anti-LBP group has higher S-LBP when compared to the Low anti-LBP group ($p = 0.030$). These findings were unchanged when controls were excluded from the analysis (data not shown). S-LBP levels were also associated with elevated BMI ($p = 7.4 \times 10^{-5}$) but did not differ between patients and controls, and were not significantly different between diagnostic groups (data not shown).

3.3. Exploratory analysis of specific clinical characteristics within the patient group and anti-LBP

Patients with high anti-LBP were diagnosed with primarily affective and anxiety disorders and reported low function and high levels of depressive symptoms but the distribution of these factors did not differ significantly between the different anti-LBP groups. The frequency of patients with autism spectrum diagnoses differed between groups where highest frequency ($p = 0.002$), 18.4% (14 of 76) was found in the Intermediate anti-LBP group compared to Low and High groups where frequencies were 6.8% (18 of 263) and 2% (1 of 50) respectively (see

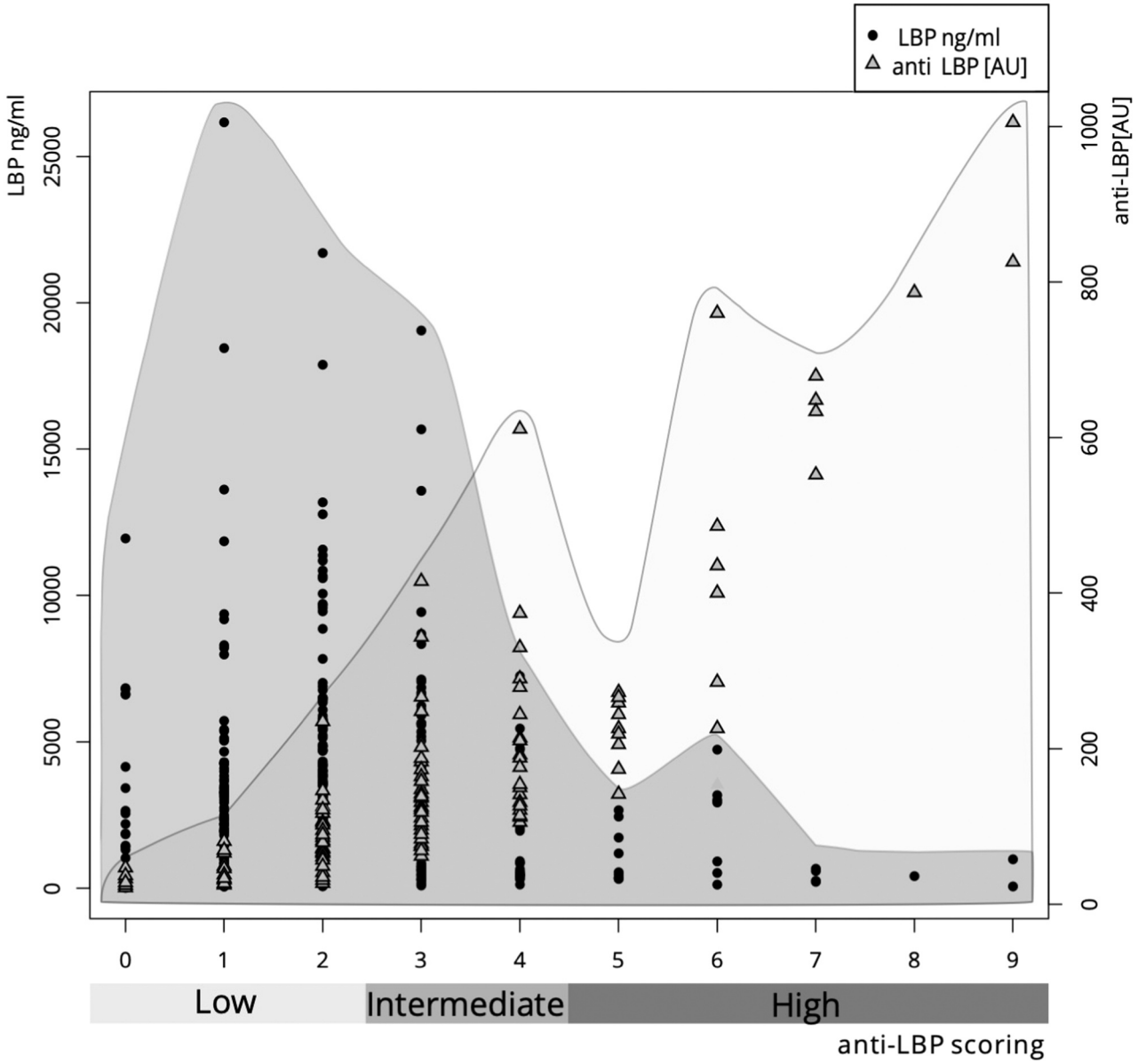


Fig. 2. Relationship between serum levels of total Lipopolysaccharide Binding Protein (s-LBP) and anti-LBP levels. Shown are the levels of total s-LBP (ng/ml) in relation to the levels of fluorescent intensities as arbitrary units [AU] within the 10 different anti-LBP scores (0–9). Total s-LBP (ng/ml) is shown in black circles, anti-LBP levels are shown in gray triangles.

Supplementary Table 1). Supplementary Table 1 shows the distribution of the clinical data and potential confounders from patients over anti-LBP groups where the high group is further divided into two as shown in the heat map. No significant differences in BMI, gender, age, smoking and psychiatric medication (Y/N) were found between the three anti-LBP groups. Finally, hospital records were available which included information on hospitalization due to infection in adult years for 389 patients. Of these, 24 patients needed hospitalization for infection in adult years and their distribution differed between anti-LBP groups ($p = 0.036$) where the frequency was higher in the Intermediate group 13.2% (10 of 76) compared to both Low 4.6% (12 of 263) and High groups 4% (2 of 50) respectively. In the low LBP group 42% (5 of 12) were hospitalized for viral infections. In the intermediate or high anti-LBP the infections were all bacterial, four in the gastrointestinal tract, three in the kidneys and 75% (9 of 12) of these infections occurred months to years after the sample was drawn for this study. The timing of

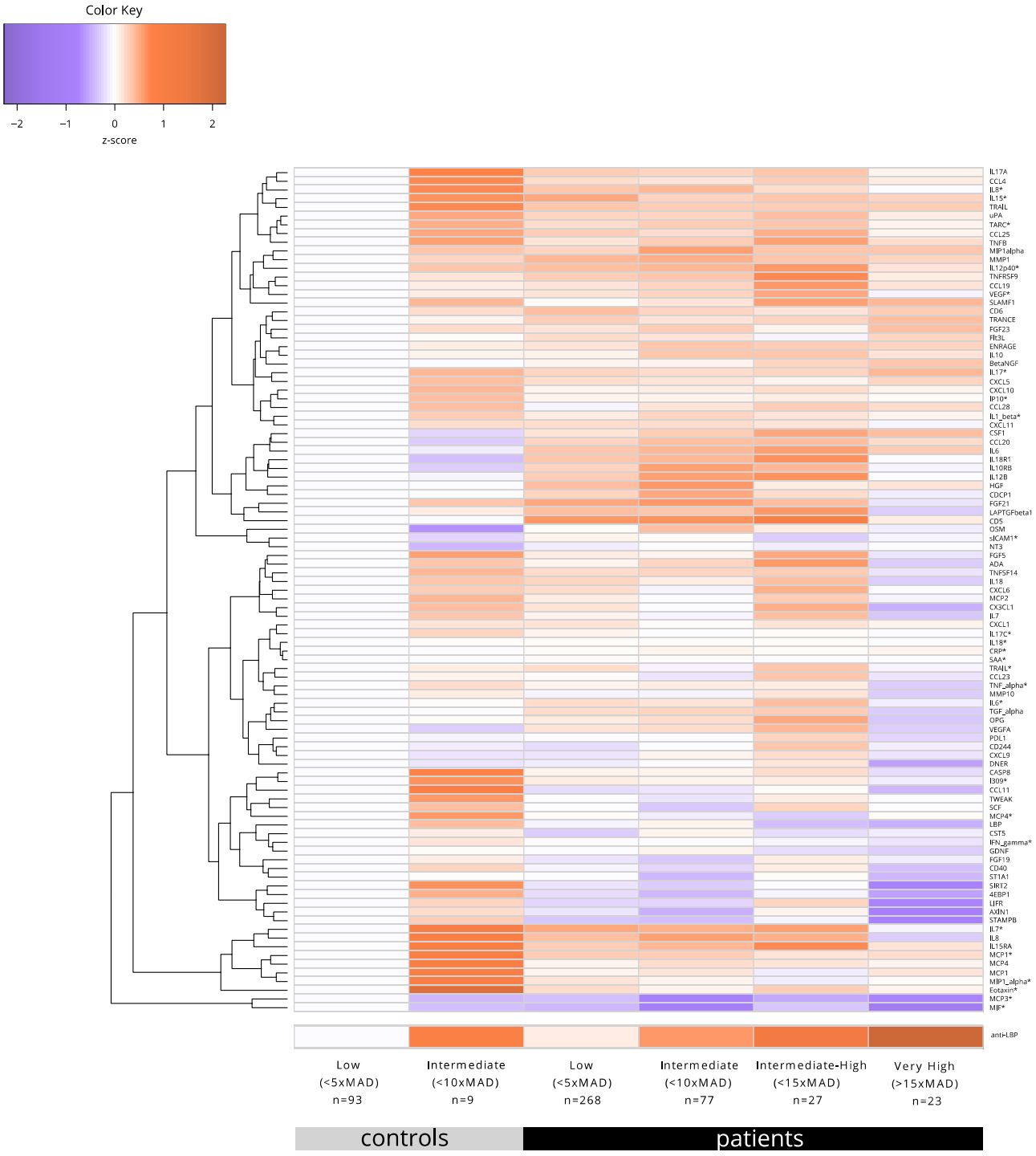


Fig. 3. Exploratory analysis of inflammatory markers. Shown is a heatmap visualizing z-scores of all analyzed inflammatory markers using both Olink and MesoScale (*) panels. Groupings are made based on patients and controls as well as the respective median absolute deviation (MAD) cutoff for anti-LBP reactivity defined as; Low (< 5xMAD), Intermediate (< 10xMAD), Intermediate-High (< 15xMAD), and Very High (> 15xMAD). All markers are color coded according to their relationships to levels in controls with low anti-LBP. Higher levels or scores are colored in orange, while lower levels or scores are colored in purple. Clustering was performed using the R package g plots (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

blood sampling in relation to the time of infection that required hospitalization is summarized in Supplementary Table 2.

3.4. Plasma levels of inflammatory markers differ across anti-LBP groups

Levels of IL-1β ($p = 0.032$), IL-8 ($p = 0.027$), TNF-β ($p = 0.026$), β-NGF ($p = 0.019$), SLAMF1 ($p = 0.018$), MIP-1α ($p = 0.009$), and IL-10 ($p = 0.006$) differed between three anti-LBP groups. It was observed that the majority of inflammatory markers are found at lowest levels in the group with highest anti-LBP levels. A nonlinear pattern emerged where highest levels of the majority of inflammatory markers were found in the Intermediate group.

A heat map was created to visualize the distribution of data for patients and controls and relationship between anti-LBP group, LBP and other inflammatory markers of interest (Fig. 3). Lower levels of inflammatory markers is most pronounced in the 23 individuals with a very high anti-LBP, MAD above factor 15, and these individuals appeared to show a distinctly different immunomarker profile when compared to lower levels of antibodies. To visualize this, the High anti-LBP group is further split into "Intermediate-High" and "Very High" in the heatmap.

3.5. Cytokine levels are inhibited by rabbit anti-LBP in monocyte cell culture after LPS and LBP treatment

Secreted levels from human cultured monocytes (CD14+/CD16- PBMCs) were measured using an inflammatory marker panel from MesoScale. After addition of LPS (5 ng/ml) the levels of IL-6, IFN-γ, and IL-1β increased dramatically. The levels further increased by 1.5–2-fold after addition of LBP (10 ng/ml) (Fig. 4a). Introduction of a polyclonal rabbit antibody against LBP (HPA HPA001508, 100 μg/ml) clearly lowered the levels of secreted inflammatory markers in a dose related manner. Secreted levels of IL-8 and TNF-α also increased after addition of LPS (5 ng/ml), as expected. The levels of IL-8 and TNF-α did not, however, increase much further after LBP nor were they significantly influenced by the addition of anti-LBP. The addition of a non-specific antibody, anti-collagen antibody (100 μg/ml), did not inhibit the additive effect of LBP addition on inflammatory marker secretion (Fig. 4b). Bars are mean values of triplicate wells +/− standard deviation of one representative experiment.

4. Discussion

This study for the first time identified autoantibodies towards LBP in young adults with psychiatric disorders using an affinity proteomics-based approach to explore autoantibody signatures. These antibodies are potentially disease associated as they were detected with a higher prevalence in patients compared to controls. Antibody levels were additionally higher among the small subgroup of controls who screened positive for previous or current milder forms of psychiatric disease, and the highest levels of antibodies against LBP were measured in young adults suffering from psychiatric symptoms that require specialist level of care and the diagnoses were primarily affective and anxiety disorders, which strengthened our interest in further characterization. Patients with higher levels of autoantibodies towards LBP had lower total LBP protein in their serum which is in line with the hypothesis that these antibodies may interfere with LBP function. Exploratory analysis identified other inflammatory markers, IL-8 and Il-1β that were lower in patients with high anti-LBP. The *in vitro* assay confirmed that antibodies against LBP can interfere with cell signaling and reduce the release of several inflammatory markers including IL-6 and IL-1β.

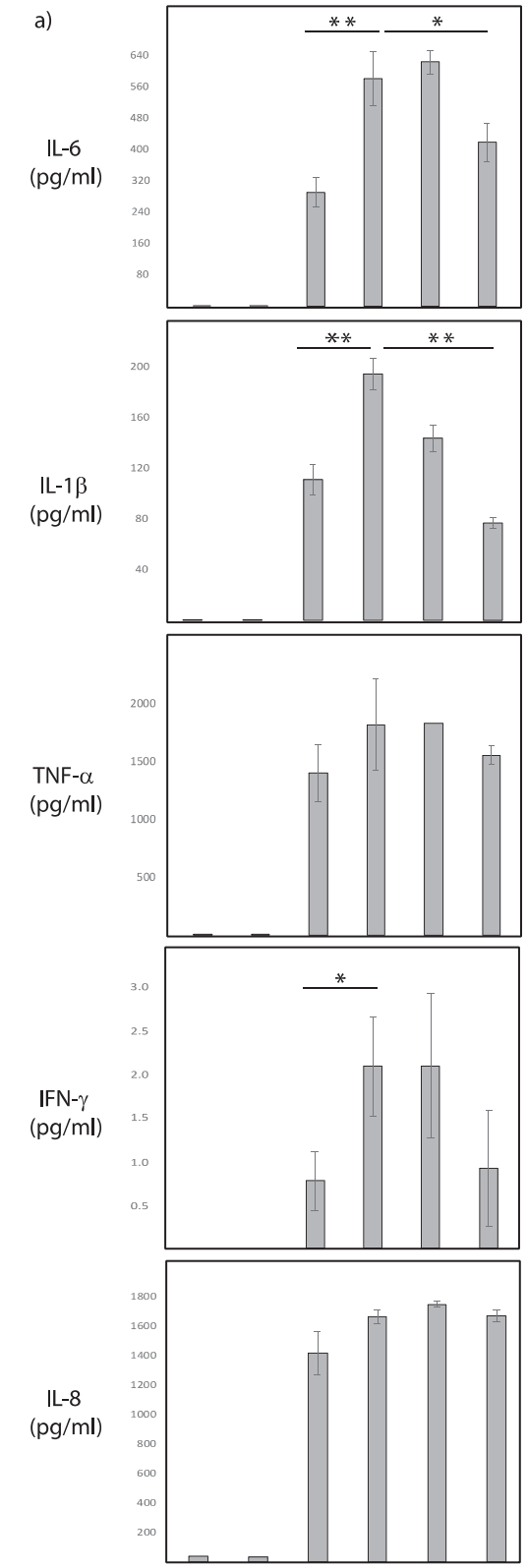
To our knowledge, autoantibodies against LBP have not previously been described in patients with psychiatric disorders. LBP is a 60 kDa protein, produced mainly in the liver and subsequently secreted into the bloodstream (Tobias et al., 1986). It plays a major role in the LPS activated immune response by recognition of LPS and transfer of LPS towards the TLR4 receptor (Park and Lee, 2013). Previous research has implied a role for several TLRs in psychiatric diseases (Hung et al., 2014; Enstrom et al., 2010; McKernan et al., 2011; Oliveira et al., 2015). A recent population-based study by Köhler et al. (2017) identified an increased risk and a hazard rate ratio of 2.59 of developing affective disorders after an infection requiring hospitalization. A more recent study has made a similar observation with an odds ratio (OR) of 1.72, linking a lifetime diagnosis of psychiatric disorders and infections (Nudel et al., 2019).

In the present study, autoantibodies against LBP were associated with lower serum LBP levels which agrees with a potential functional role for the antibody. Serum LBP (s-LBP) levels themselves were, however, not significantly different between patients and controls or associated with diagnostic groups. A previous study showed that while s-LBP levels were correlated with CD14 levels they did not significantly differ between cases and controls (Severance et al., 2013). Levels in the schizophrenia group were, however, significantly increased compared to levels in the bipolar disorder group. Furthermore, s-LBP levels were higher in females and correlated with CD14 levels, BMI and antibodies to gluten. Our data could confirm an association between s-LBP and BMI.

LPS-activated monocytes release inflammatory markers such as IL-1β (Schumann et al., 1994). Our primary analysis of selected inflammatory markers reveled an intriguing pattern with highest levels of inflammatory markers including IL-1β as well as IL-8, in patients with intermediate anti-LBP titers and lowest levels were found in patients with the highest antibody titers suggesting a potentially inhibitive function of anti-LBP antibodies. These observations are in line with a previous study reporting an antibody neutralization of LBP and prevention of LPS binding to monocytes after LBP antibody introduction, protecting from lethal endotoxemia in a mouse model (Heumann et al., 1992).

With the finding of lower LBP and proinflammatory markers in patients with high anti-LBP we wondered if the antibodies may interfere with LBP function. We were able to model this proposed mechanism using a CD14+monocyte assay. Stress conditioning with LPS and LBP resulted in an increase in inflammatory cytokine secretion and subsequent introduction of anti-LBP reduced cytokine secretion. In earlier work, using ten and fifty times higher concentrations of antibody compared to the current paper, it has been demonstrated that monoclonal anti-LBP antibodies could nearly completely inhibit TNF-α release from monocytes (Gutsmann et al., 2001). The concentrations of antibodies in our *in vitro* assay are still well within physiological levels and several thousand times lower than total IgG levels in serum (Agarwal and Cunningham-Rundles, 2007). The antibodies used in our cell assay are produced using the same protein fragment used in the screening. Within the C-terminal cleft of LBP lies a sequence which has been recently reported to be essential for LPS transfer to CD14 and TLR4. Further, a mutation in this cleft significantly reduces the expression of IL-6 and TNF-α post LPS induction (Ryu et al., 2017). One of these mentioned mutations effecting the amino acid at position R322 lies within our identified potential minimal binding epitope (data not shown) suggesting a functional similarity between antibody binding and blockage of this basic patch at the tip of the LBP C-terminal domain. Genetic variants of LBP gene have been demonstrated to modify its function in relation to LPS induced inflammatory reactions. In 2013, Eckert et al. (2013) found that a specific SNP (rs2232613, allelic frequency 0.08) increased the risk of sepsis and sepsis related death after pneumonia.

Our study population was a mixed group in terms of diagnosis and we chose to analyze the range of autoantibody and inflammatory marker variation within the whole population to maximize the power of the dataset which led to the finding of high levels of anti-LBP only in patients. The biological differences between the anti-LBP groups were however not clearly mirrored by distinctive clinical phenotypes. The patients with highest levels of anti-LBP have a common denominator in affective and anxiety disorders with significant impact on emotional regulation and function. A finding we found interesting, but difficult to



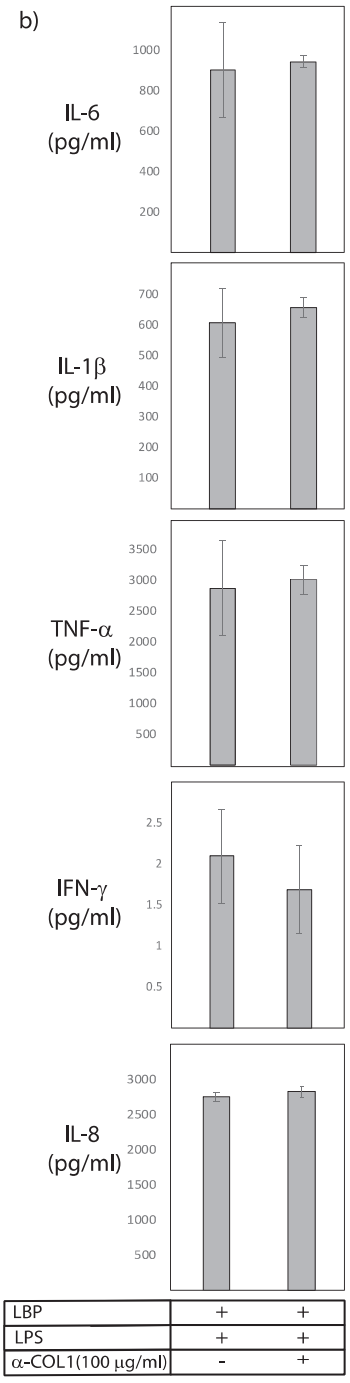


Fig. 4. Monocyte cultures of human CD14+/CD16- cells with addition of Lipopolysaccharide (LPS), Lipopolysaccharide Binding Protein (LBP) and antibodies against LBP and Collagen 1 alpha. The polyclonal rabbit anti-LBP antibody was specific for the c-terminal LBP fragment. Experiments in (a) were done in triplicate wells, performed twice. Bars are mean values and standard deviation from one representative experiment. The control experiment in (b), testing a non-related antibody against Collagen 1 alpha (COL1), with a concentration of 100 ug/ml, which is the same as the highest concentration of anti-LBP used in (a). Results from Student's T-tests are shown; $*p < 0.05$ and $**p < 0.01$.

interpret, was that patients with autism spectrum disorder (ASD) were overrepresented in the intermediately elevated anti-LBP group but nearly absent in the high group. Alterations in innate immunity pathways are shown earlier in gene expression data from patients with autism spectrum disorder with links to infection susceptibility (Nazeen et al., 2016). To assess infection susceptibility in our cohort, hospital records were scanned for information about serious infections and antibiotic use in adult years and found that hospitalization for infections was also associated to intermediately elevated anti-LBP levels in patients but did not overlap with the ASD group. Interestingly, hospitalization in the majority of the patients occurred after blood was drawn for this study, which speaks against anti-LBP as a consequence of the infection. Patients hospitalized for infections were also nearly absent in the high anti-LBP group ($n = 2$). Our findings could indicate that elevated anti-LBP confers vulnerability to psychiatric disease by reducing innate response to infection. An alternative explanation that is also congruent with our findings is that repeated infections due to susceptibility for infection and/or specific infectious agents induces the production of antibodies against LBP that may even have a protective roll by reducing inflammatory responses.

Our study has several limitations. Firstly, autoantibody screening was performed with a single type of analysis (single binder assay) using a protein fragment and not the full-length protein. Secondly, the control population was smaller than the patient population, therefor we included more advanced methods in the statistical analysis to see that the results hold for permutation tests. Further, a population of control individuals were screened positive for psychiatric diseases. These individuals reported milder forms of disorders that are more common in the population and did not have the same functional handicap as is seen in the patients requiring specialist psychiatric care. We chose to retain these individuals within the control group in accordance with our analysis plan but could demonstrate the differences in biomarkers in the post-hoc analyses. The population in this study differs from many other studies due to the young age of the participants. There was no difference between patients with or without current psychiatric medication, however, further analysis of the effects from individual types of medications was not conducted due to low power. The recruitment base is young adults seeking ambulatory care within general psychiatry, therefore, patients with severe autism spectra disorders or psychosis are not represented as they are generally referred to other units. Thirdly, during our cell-based assay we observed differences in the effect of LPS on the cytokine release from the cells. It has been postulated that LPS can induce CD14 aggregation in plasma membrane lipid rafts indicating an inflammatory response independent from TLR4 (Rosadini and Kagan, 2017). Further extended titration of the concentrations of LPS, LBP and antibodies may have revealed a greater dynamic for IL-8 and TNF-α release that was found to be highly elevated already with the addition of LPS alone. Genetic analysis was beyond the scope of this study and future studies are needed to address if there may be an association between genetic variants of LBP, anti-LBP, autism and frequency of infections in psychiatric populations.

This study identifies high levels of autoantibodies against LBP as potential disease associated autoantibody signature in both patients with early onset of psychiatric symptoms but not controls. High levels of anti-LBP in patients with primarily affective and anxiety disorders were found to have very low levels of s-LBP and other pro-inflammatory markers. Intermediate levels of antibodies were found in a small subgroup of controls who screened positive milder forms of current or previous psychiatric disease. Our functional *in vitro* assay showed that commercial rabbit antibodies against the same LBP fragment could modulate LBP action. A clear understanding of the role of these antibodies in the development of psychiatric disease is elusive. Our data, however, provides clues as the incidence of both autism spectrum disorders and hospitalization for infection show associations with anti-LBP levels. These combined results suggest that LBP autoantibodies may have functional relevance and may be related to vulnerability for psychiatric disease and/or susceptibility to serious infections.

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Conflicts of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2021.105162.

References

Agarwal, S., Cunningham-Rundles, C., 2007. Assessment and clinical interpretation of reduced IgG values. Ann. Allergy Asthma Immunol. 99, 281–283.
Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499–511.
Diagnostic and Statistical Manual of Mental Disorders: DSM-IV-TR, fourth ed., 2000 American Psychiatric Association, Washington, DC.
Ayoglu, B., Mitsios, N., Kockum, I., Khademi, M., Zandian, A., Sjöberg, R., Forsström, B., Bredenberg, J., Lima Bomfim, I., Holmgren, E., Grönlund, H., Guerreiro-Cacais, A.O., Abdelmagid, N., Uhlén, M., Waterboer, T., Alfredsson, L., Mulder, J., Schwenk, J.M., Olsson, T., Nilsson, P., 2016. Anoctamin 2 identified as an autoimmune target in multiple sclerosis. Proc. Natl. Acad. Sci. USA 113, 2188–2193.
Benros, M.E., Mortensen, P.B., Eaton, W.W., 2012. Autoimmune diseases and infections as risk factors for schizophrenia. Ann. N.Y. Acad. Sci. 1262, 56–66.
Benros, M.E., Nielsen, P.R., Nordentoft, M., Eaton, W.W., Dalton, S.O., Mortensen, P.B., 2011. Autoimmune diseases and severe infections as risk factors for schizophrenia: a 30-year population-based register study. Am. J. Psychiatry 168, 1303–1310.
Benros, M.E., Waltoft, B.L., Nordentoft, M., Østergaard, S.D., Eaton, W.W., Krogh, J., Mortensen, P.B., 2013. Autoimmune diseases and severe infections as risk factors for mood disorders: a nationwide study. JAMA Psychiatry 70, 812–820.
Beutler, B., Rietschel, E.T., 2003. Innate immune sensing and its roots: the story of endotoxin. Nat. Rev. Immunol. 3, 169–176.
Beveridge, T.J., 1999. Structures of gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. 181, 4725–4733.
Bixo, L., Cunningham, J.L., Ekselius, L., Öster, C., Ramklint, M., 2019. 'Sick and tired': patients reported reasons for not participating in clinical psychiatric research. Health Expect.
Blomström, Å., Karlsson, H., Svensson, A., Frisell, T., Lee, B.K., Dal, H., Magnusson, C., Dalman, C., 2014. Hospital admission with infection during childhood and risk for psychotic illness–a population-based cohort study. Schizophr. Bull. 40, 1518–1525.
Cunningham, J.L., Wernroth, L., von Knorring, L., Berglund, L., Ekselius, L., 2011. Agreement between physicians' and patients' ratings on the Montgomery-Asberg Depression Rating Scale. J. Affect. Disord. 135, 148–153.
Cunningham, J.L., Zanzi, M., Willebrand, M., Ekselius, L., Ramklint, M., 2017. No regrets: young adult patients in psychiatry report positive reactions to biobank participation. BMC Psychiatry 17, 21.
Eckert, Jana K., Kim, Young J., Kim, Jung I., Gürtler, K., Oh, D.-Y., Sur, S., Lundvall, L., Hamann, L., van der Ploeg, A., Pickkers, P., Giamarellos-Bourboulis, E., Kubarenko, Andriy V., Weber, Alexander N., Kabesch, M., Kumpf, O., An, H.-J., Lee, J.-O., Schumann, Ralf R., 2013. The crystal structure of Lipopolysaccharide binding protein reveals the location of a frequent mutation that impairs innate immunity. Immunity 39, 647–660.
Enstrom, A.M., Onore, C.E., Van de Water, J.A., Ashwood, P., 2010. Differential monocyte responses to TLR ligands in children with autism spectrum disorders. Brain Behav. Immun. 24, 64–71.
First, M.S.R., Gibbon, M., Williams, J., 1996. Structured Clinical Interview for DSM-IV Axis I Disorders, Clinician Version (SCID-CV). American Psyhiatric Press, Washington, D.C.
Gutsmann, T., Müller, M., Carroll, S.F., MacKenzie, R.C., Wiese, A., Seydel, U., 2001. Dual role of Lipopolysaccharide (LPS)-binding protein in neutralization of LPS and

enhancement of LPS-induced activation of mononuclear cells. Infect. Immun. 69, 6942–6950.
Hailman, E., Lichenstein, H.S., Wurfel, M.M., Miller, D.S., Johnson, D.A., Kelley, M., Busse, L.A., Zukowski, M.M., Wright, S.D., 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. 179, 269–277.
Heumann, D., Gallay, P., Barras, C., Zaech, P., Ulevitch, R.J., Tobias, P.S., Glauser, M.P., Baumgartner, J.D., 1992. Control of lipopolysaccharide (LPS) binding and LPS-induced tumor necrosis factor secretion in human peripheral blood monocytes. J. Immunol. 148, 3505.
Hung, Y.-Y., Kang, H.-Y., Huang, K.-W., Huang, T.-L., 2014. Association between toll-like receptors expression and major depressive disorder. Psychiatry Res. 220, 283–286.
Köhler, O., Petersen, L., Mors, O., Mortensen, P.B., Yolken, R.H., Gasse, C., Benros, M.E., 2017. Infections and exposure to anti-infective agents and the risk of severe mental disorders: a nationwide study. Acta Psychiatr. Scand. 135, 97–105.
Mattila-Evenden, M., Svanborg, P., Gustavsson, P., Asberg, M., 1996. Determinants of self-rating and expert rating concordance in psychiatric out-patients, using the affective subscales of the CPRS. Acta Psychiatr. Scand. 94, 386–396.
McKernan, D.P., Dennison, U., Gaszner, G., Cryan, J.F., Dinan, T.G., 2011. Enhanced peripheral toll-like receptor responses in psychosis: further evidence of a pro-inflammatory phenotype. Transl. Psychiatry 1, e36.
Nazeen, S., Palmer, N.P., Berger, B., Kohane, I.S., 2016. Integrative analysis of genetic data sets reveals a shared innate immune component in autism spectrum disorder and its co-morbidities. Genome Biol. 17, 228.
Nudel, R., Wang, Y., Appadurai, V., Schork, A.J., Buil, A., Agerbo, E., Bybjerg-Grauholm, J., Børglum, A.D., Daly, M.J., Mors, O., Hougaard, D.M., Mortensen, P.B., Werge, T., Nordentoft, M., Thompson, W.K., Benros, M.E., 2019. A large-scale genomic investigation of susceptibility to infection and its association with mental disorders in the Danish population. Transl. Psychiatry 9, 283.
Oliveira, J., Etain, B., Lajnef, M., Hamdani, N., Bennabi, M., Bengoufa, D., Sundaresh, A., Chaabane, A.B., Bellivier, F., Henry, C., Kahn, J.-P., Charron, D., Krishnamoorthy, R., Leboyer, M., Tamouza, R., 2015. Combined effect of TLR2 gene polymorphism and early life stress on the age at onset of bipolar disorders. PLoS One 10 e0119702-e0119702.
Park, B.S., Lee, J.-O., 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp. Mol. Med 45, e66.
Raetz, C.R.H., Whitfield, C., 2002. Lipopolysaccharide Endotoxins. Annu. Rev. Biochem. 71, 635–700.
Rosadini, C.V., Kagan, J.C., 2017. Early innate immune responses to bacterial LPS. Curr. Opin. Immunol. 44, 14–19.
Ryu, J.-K., Kim, S.J., Rah, S.-H., Kang, J.I., Jung, H.E., Lee, D., Lee, H.K., Lee, J.-O., Park, B.S., Yoon, T.-Y., Kim, H.M., 2017. Reconstruction of LPS transfer cascade reveals structural determinants within LBP, CD14, and TLR4-MD2 for efficient LPS recognition and transfer. Immunity 46, 38–50.
Schumann, R.R., Rietschel, E.T., Loppnow, H., 1994. The role of CD14 and lipopolysaccharide-binding protein (LBP) in the activation of different cell types by endotoxin. Med. Microbiol. Immunol. 183, 279–297.
Schwenk, J.M., Gry, M., Rimini, R., Uhlen, M., Nilsson, P., 2008. Antibody suspension bead arrays within serum proteomics. J. Proteome Res. 7, 3168–3179.
Severance, E.G., Gressitt, K.L., Stallings, C.R., Origoni, A.E., Khushalani, S., Leweke, F. M., Dickerson, F.B., Yolken, R.H., 2013. Discordant patterns of bacterial translocation markers and implications for innate immune imbalances in schizophrenia. Schizophr. Res. 148, 130–137.
Sheehan, D.V., Lecrubier, Y., Sheehan, K.H., Amorim, P., Janavs, J., Weiller, E., Hergueta, T., Baker, R., Dunbar, G.C., 1998. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. J. Clin. Psychiatry 59 (Suppl 20), 34–57, 22-33;quiz.
Söderquist, F., Sundberg, I., Ramklint, M., Widerström, R., Hellström, P.M., Cunningham, J.L., 2019. The relationship between daytime salivary melatonin and gastrointestinal symptoms in young adults seeking psychiatric care. Psychosom. Med. 81, 51–56.
Team, R.C., 2013/5/1. R: A language and environment for statistical computing. 3, 201.
Tobias, P.S., Soldau, K., Ulevitch, R.J., 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. 164, 777–793.
Uhlén, M., Björling, E., Agaton, C., Szigyarto, C.A.-K., Amini, B., Andersen, E., Andersson, A.-C., Angelidou, P., Asplund, A., Asplund, C., Berglund, L., Bergström, K., Brumer, H., Cerjan, D., Ekström, M., Elobeid, A., Eriksson, C., Fagerberg, L., Falk, R., Fall, J., Forsberg, M., Björklund, M.G., Gumbel, K., Halimi, A., Hallin, I., Hamsten, C., Hansson, M., Hedhammar, M., Hercules, G., Kampf, C., Larsson, K., Lindskog, M., Lodewyckx, W., Lund, J., Lundeberg, J., Magnusson, K., Malm, E., Nilsson, P., Ödling, J., Oksvold, P., Olsson, I., Öster, E., Ottosson, J., Paavilainen, L., Persson, A., Rimini, R., Rockberg, J., Runeson, M., Sivertsson, Å., Sköllermo, A., Steen, J., Stenvall, M., Sterky, F., Strömberg, S., Sundberg, M., Tegel, H., Tourle, S., Wahlund, E., Waldén, A., Wan, J., Wernérus, H., Westberg, J., Wester, K., Wrethagen, U., Xu, L.L., Hober, S., Pontén, F., 2005. A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol. Cell. Proteom. 4, 1920–1932.