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ARTICLE

Blood-borne extracellular vesicles of bacteria and intestinal cells in patients with psychotic disorders

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ABSTRACT

Background: Human cells and bacteria secrete extracellular vesicles (EV) which play a role in intercellular communication. EV from the host intestinal epithelium are involved in the regulation of bacterial gene expression and growth. Bacterial EV (bactEV) produced in the intestine can pass to various tissues where they deliver biomolecules to many kinds of cells, including neurons. Emerging data indicate that gut microbiota is altered in patients with psychotic disorders. We hypothesized that the amount and content of blood-borne EV from intestinal cells and bactEV in psychotic patients would differ from healthy controls.

Methods: We analyzed for human intestinal proteins by proteomics, for bactEV by metaproteomic analysis, and by measuring the level of lipopolysaccharide (LPS) in blood-borne EV from patients with psychotic disorders ($n=25$), tested twice, in the acute phase of psychosis and after improvement, with age- and sex-matched healthy controls ($n=25$).

Results: Patients with psychotic disorders had lower LPS levels in their EV compared to healthy controls ($p=.027$). Metaproteome analyses confirmed LPS finding and identified Firmicutes and Bacteroidetes as dominating phyla. Total amounts of human intestine proteins in EV isolated from blood was lower in patients compared to controls ($p=.02$).

Conclusions: Our results suggest that bactEV and host intestinal EV are decreased in patients with psychosis and that this topic is worthy of further investigation given potential pathophysiological implications. Possible mechanisms involve dysregulation of the gut microbiota by host EV, altered translocation of bactEV to systemic circulation where bactEV can interact with both the brain and the immune system.

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Extracellular vesicles; proteomics; psychotic disorders; lipopolysaccharides; brain-gut axis

Introduction

Emerging data indicate that the gut microbiota is altered in patients with psychotic disorders [1,2]. The human cells and bacteria in the intestine produce extracellular vesicles (EV) which can deliver functional biomolecules like proteins from one cell or bacteria to another [3]. Studies on human and mice organoids and mice have revealed mechanisms involved in the translocation of bacterial EV (bactEV) out of the intestinal lumen to the submucosa layer with immune and stem cells and further to the systemic circulation [4,5]. BactEV can influence the immune system in various ways [6,7]. A recent study showed that bactEV from the gut in mice are transported to various organs including the brain. In the brain, the bactEV deliver biomolecules to neurons [4]. Interestingly, the areas with the highest uptake of functional biomolecules from bactEV were predominantly the striatum and to a lesser extent the cortex and hippocampus [4]. This study used healthy mice, not involving a model of any disease, suggesting that this interspecies communication between the bacteria in the intestine and the brain might be a part of normal physiology. However, the striatum is a key brain structure in the pathophysiology of psychosis as increased synthesis of dopamine is a robust finding among patients with psychotic disorders including the prodromal stages of the disease [8–11]. Cell and animal models showed that EV can affect dopaminergic neuromodulation [12,13]. Thus, a possible mechanism in the pathophysiology of psychosis could be a disturbed transport of bactEV from the gut to the blood and thereby to the striatum where the delivered biomolecules affect dopamine synthesis.

Animal models have also shown beneficial effects such as reduced mortality after immunization with bactEV [14,15]. Psychotic disorders are associated with high morbidity and

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mortality and their biological origin remains partly unknown; there are thus several reasons why bactEV are promising research targets in psychotic disorders [16,17].

EV from host intestinal epithelial cells are delivered both at the apical (luminal) and basal side of the intestine and play a role in the regulation of microbiota, antigen presentation, food tolerance, inhibition of $CD4^+$ T-cell proliferation, and protection against infections [18–23]. However, the available mechanistic studies [18–23] are preclinical, and most clinical microbiota studies are association studies involving microbiota composition [24]. EV from gut bacterial and/or intestinal cells in human blood remain poorly characterized [25].

Here, we analyzed EV from peripheral blood, isolated in a previous publication, for human intestinal proteins and for bacterial content by metaproteomics and lipopolysaccharide (LPS) measurement, to test the hypothesis that the amount and content of EV from intestinal cells and bactEV differ between patients with psychotic disorders and healthy controls.

Metaproteomics is an emerging approach that can assess the complete suite of proteins (the metaproteome) in an environmental sample or ecosystem, such as host-symbiont interfaces – this, in contrast [26] with the proteome that defines the set of proteins in one single organism. The field is technically very demanding, that for both the experimental workflow [27] and the bioinformatics workflow [28], and we aimed to investigate if metaproteomics can be used to analyse non-bacterial enriched proteomics data, and thus to identify BactEV.

Methods

This is an add-on study based on EV isolated in a previous publication where we recruited 25 patients with psychotic disorders (Table 1). Patients were recruited during hospitalization for psychosis [29]. The first blood sample was taken during the acute phase of psychosis (T1). The patients were retested (T2) after minimum six weeks. Seven patients were lost to follow-up. All patients had a lower Clinical Global Impression-Severity Scale score at the second time point. Patients included were diagnosed with schizophrenia ($n=12$), substance-induced psychotic disorder ($n=4$), acute and transient psychotic disorders ($n=3$) and other psychotic disorders ($n=6$). Exclusion criteria were cardiac, rheumatic, autoimmune and neurological disorders, cancer, organic causes of psychosis, and pregnancy.

Blood sampling and EV isolation

Samples are prepared as described earlier [29]. Briefly, blood was drawn once from healthy controls and twice from patients [30]. The majority of samples were collected before lunch, data regarding fasting was not noted. Blood was collected in sterile EDTA tubes (Vacuette, Greiner Bio-One) and centrifuged fresh (2000 g, 30 min, 4 °C)(within 2 h) to isolate cell-free plasma. Plasma was centrifuged (10,000 g, 30 min, 4 °C). The supernatant was transferred to cryotubes, and were frozen at −80 °C awaiting further analysis. Pellet fractions were thawed in room temperature and resuspended in 100 µl phosphate-buffered saline (PBS). The samples were centrifuged again to remove any residual cells and debris, first at 2000 g (30 min, 4 °C). The supernatant was transferred and centrifuged at 10,000 g (30 min, 4 °C). The resulting supernatant was discarded, and the pellet was resuspended in ammonium bicarbonate buffer (100 µl, 100 mM) for further analysis. Samples for proteomics were frozen at −80 °C in Protein LoBind Eppendorf tubes before further sample processing. The mean concentration was 2.0×10^7 particles/ml plasma (SD 1.1×10^7, $n=68$) and with a mean size of 191 nm (SD 21 nm, $n=68$), as detailed previously [29].

The isolation method was calibrated to result in EV samples of the high recovery, low specificity category of MISEV2018 guidelines [31]. The method is submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV200067) [32].

Proteomic analysis of isolated EV

To address the presence of human-originating intestinal proteins in the isolated EV, the proteomic raw data from an earlier study was analysed [29] (available in PRIDE, dataset identifier PXD016293) [33], focusing on proteins enriched in intestines as based on the Human Protein Atlas (https://www.proteinatlas.org) [34].

Metaproteomic reanalysis to identify bactEV

The raw files obtained [29] were reanalysed applying a metaproteomic approach [35]. To encompass all possible bacterial species that could be potential candidates an unbiased analysis of a large set of different microorganisms is required. We searched against a concatenated target-decoy database of the human proteome (20,000 proteins) combined with the integrated gene catalogue (IGC, approximal 10 million gut microbe genes) [36]. This catalogue includes data from 1267 sequenced samples and is expected to represent a close-to-complete data sets of genes for most gut microbes [37]. We also need to include the human proteome as the majority of proteins in the analyzed samples are expected to be human and a repository of common contaminants [38]. The reanalysis result files, including raw files, search files and database

Table 1. Description of study participants.

	Psychotic patients (T1)	After improvement (T2)	Healthy controls
Number of participants	25	18	25
Age in years, mean (SD)	33.1 (11.0) years		34.2 (11.2)
Sex	19 males, 6 females	14 males, 4 females	19 males, 6 females
Clinical global impression-severity scale score	6.5 (0.65)	3.8 (1.23)	
Time since debut of first psychotic episode	63 (81) months		
Time between sampling points (T1 and T2)		79 (34) days. Range 42–162 d	

Values given as mean (SD) if not specified otherwise.

used are available at the PRIDE partner repository (dataset identifier PXD029074) [33].

Metaproteomic data analysis and taxonomic profiling

Statistical analyses of metaproteomic results were performed with Perseus (version 1.6.14) [39]. The number of validated peptide-to-spectrum-matches (PSMs) was normalized by dividing the validated PSMs for each protein with the sum of validated PSMs for statistical analysis. Differences in protein detection between sample groups were analysed using Student's *t*-test, corrected for multiple hypothesis testing (permutation-based FDR < 0.05, artificial within group variance s0 = 0.1). Missing values were imputed from a normal distribution with a 1.8 standard deviation shift from the average and a width of 0.3. Peptides matched to microbial proteins that were significantly different between groups were extracted from peptide identification results. We analyzed taxa with the Unipept desktop application [35], to validate microbial proteins to specific species. In addition, the peptides that constituted bacterial proteins that differed between groups and the dominating bacteria phyla in the samples were aligned to known sequence databases using NCBI Blast to verify bacteria origin [40]. To identify potentially sources of bacterial contamination in our samples, results were also search for bacteria from human skin [41] and typical contamination bacteria in laboratories and low biomass samples [42,43].

LPS analysis

Since EV from Gram-negative bacteria contain inner membrane and cytoplasmic components in addition to the outer LPS-containing membrane, LPS can be used as a marker for Gram-negative bacteria [25]. The content of LPS in the isolated EV was determined by the PyroGene recombinant factor C endotoxin detection end-point assay (Lonza, Belgium). Two to four dilutions were prepared for each sample, and the test was performed in a 96-well format with three technical replicates of each dilution. To assure data reliability with respect to test interference, 0.5 EU/mL control standard endotoxin from the kit was spiked into each dilution in three technical replicates. Based on the kit protocol, spike recovery between 50% and 200% was accepted. The released fluorescence substrate was measured fluorometrically at 440 nm (excitation 380 nm) using a fluorescence microplate reader (TECAN, Infinite M200 PRO). The detection range of the assay was 0.005–5 EU/mL.

Statistical analyses

Initial data for LPS/vesicle contained extreme outliers (modified *z* scores ranging from 7 to 43) considered to be caused by contamination or error in the analyses and therefor were removed before further analyses ($n = 2$ in the healthy control group, $n = 1$ in patients at T1 and $n = 1$ in patients at T2) [44].

The distribution of data was assessed by the Kolmogorov–Smirnof test and statistical tests were chosen based on this. We used an independent *t*-test to compare LPS measurements between healthy controls and patients at T1 (data for the healthy control group were slightly skewed and data for the patient group at T1 were normally distributed) [45]. To compare LPS measurements in patients before and after improvement we used related samples Wilcoxon signed rank test as data at T2 was highly skewed and the analyses only involved 16 pairs.

For proteomic results we performed an independent *t*-test (unpaired) between patients at T1 and healthy controls with correction for multiple hypothesis testing by using permutation-based FDR <0.01 and artificial within group variance $s0 = 0.1$, to evaluate if the total level of intestinal human proteins differed between groups. To evaluate if the intestine proteins were affected by the status of psychosis, we used a paired *t*-test.

We compared the spectral counts of the microbial proteins, as identified by the metaproteomic searches for patients at T1 and healthy controls by the Mann Whitney *U* test [36]. To evaluate if disease status affected the microbial constituents at T1 and T2, we used Wilcoxon related sample signed rank test.

Statistical analysis was performed using Perseus (version 1.6.14.0) [39] and IBM SPSS Statistics for Windows (version 26.0.).

Ethics

The study was approved by the Regional Ethics committee, South East Norway (2016/949). All participants gave written informed consent.

Results

LPS

As LPS is found in the outer membrane of bactEV from Gram-negative bacteria, we measured LPS in our EV as an indicator of the bactEV content in the blood (Figure 1) [25].

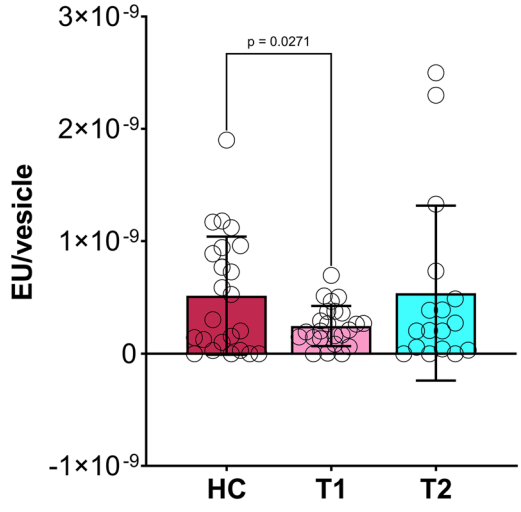

Figure 1. Scatter plot with mean (bar) and standard deviation (error bars) of LPS in EV in patients at T1 and T2 and healthy controls (HC).

Microbial proteins

The metaproteomic analysis resulted in 2744 proteins identified with more than one peptide across all analyzed samples, whereof 178 protein identities were mapped to bacterial proteins in the IGC. Patients at T1 had a lower number of PSMs mapped to bacterial proteins (mean $2.6\times10^{-3}\pm1.3\times10^{-3}$, median 2.4×10^{-3}) compared to healthy controls (mean $3.2\times10^{-3}\pm1.3\times10^{-3}$, median 3.3×10^{-3}) ($N=50$, $p=.049$, Independent samples Mann Whitney). Unipept identified in total 24,741 peptides, whereof 17,510 (71%) is from eukaryotic organisms, 1116 peptides (5%) were mapped to bacteria, 5 (0.002%) were mapped to Archaea and 6109 peptides (25%) were mapped as undefined. Firmicutes, therein *Clostridiales*, and Bacteroidetes were the most frequent identified bacterial phyla across all samples (Figure 2), the content of these did not differ between groups. Six microbial proteins were initially identified as significantly different between groups, based on normalized spectra. Of these, four were only presented by a small number of spectra (<5 across all samples), while two proteins, with entries in IGC: *159268001-stool2_revised_scaffold1240_2_gene6335* and *MH0124_GL0029177,* were identified by 395 and 236 spectra, respectively. We therefore selected these two proteins for extended manual curation, to qualify the identifications (Table S1). Identified peptides for these two proteins were evaluated by NCBI Protein BLAST to verify microbial origin and identify the specific organisms these proteins originated from, as a quality control. However, although links could be found from the identified peptides to bacterial species, the highly similar or mass equivalent peptide sequences could be mapped to serum albumin and glyceraldehyde 3-phosphate dehydrogenase, proteins that are both highly conserved between species and are expected to have a high abundance in human samples, as peripheral blood is. Thus, we cannot conclude that these two proteins are solely originating from bactEV. We also did individual quality control of the 858 peptide sequences determined to belong to the phyla Bacteroidetes and Firmicutes by the Unipept desktop, which were the most frequent bacteria phyla identified in our samples. Three of the 858 peptides were revealed to most likely be derived from human albumin, one of the peptides had a 100% peptide coverage with bacterial protein and 90% coverage with human 'natural killer cell receptor2B4' and and

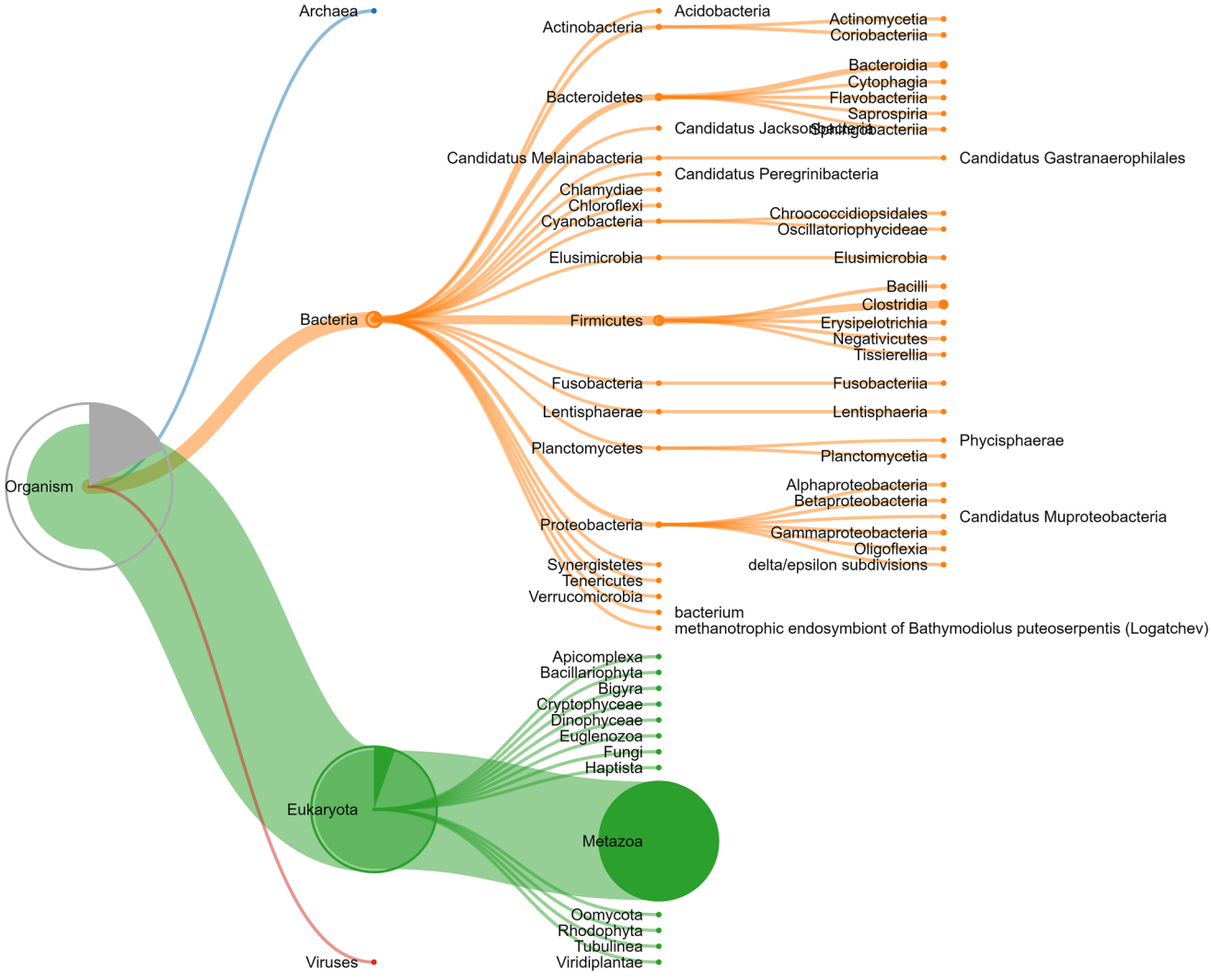

Figure 2. Treeview of identified peptides and species match in Unipept, where the thickness of the line indicates the amount of unique peptide evidence at each taxonomic level.

should therefore considered to be doubtful. The rest of the peptides were confirmed as true bacterial identifications.

None of the most frequent human skin bacteria (*Proprionibacterium species*, *Corynebacterium* species, and *Staphylococcus species*) [41] were detected in our samples by Unipept. Of the 59 genera detected by Salter et al. as typical contaminants in low-mass biosamples belonging to the phylum Proteobacteria; [42] *Burkholderia* was identified with 17 peptides, *Oxalobacter* with 16 peptides, *Acinetocanter* with three peptides, *Enterobacter* with 123 peptides, and *Escherichia* with 11 peptides. Of the phylum Actinobacteria none of the typical bacterial contaminants from the Salters study were detected in our study [42]. Within the Firmictues phylum identified as typical contaminating bacteria by Salter et al. only *Paenibacillus* and *Streptococcus* were identified with 3 and 26 peptides indicating that the main part of the species of Firmicutes in our samples are not originating from contamination. Of the bacteria shown to be involved in contamination within the phylum Bacteroidetes [42] only *Flavobacterium* were identified by two peptides also indicating that the main population of the identified Bacteroidetes is not due to contamination. A newer study investigating contamination in modern and ancient laboratory facilities showed that contaminating bacteria in modern laboratories, as ours, typically belongs to the phyla Fimicutes and Proteobacteria and that the bacteria with increased abundance were Erythrobacteraceae (phylum Proteobacteria) and *Staphylococcus* taxa (phylum Firmicutes) [43]. Erythrobacteraceae and *Staphylococcus* taxa were not identified in our study.

Intestine-enriched proteins

The number of PSMs identified for intestinal proteins was significantly higher in healthy controls compared with T1 [34]. Three individual proteins were significantly different between healthy controls and T1: Apolipoprotein A4 (APOA4) and P0C671 Chromosome 6 open reading frame 222 (C6orf222) were lower and Sorcin was elevated in psychotic patients (Figure 3).

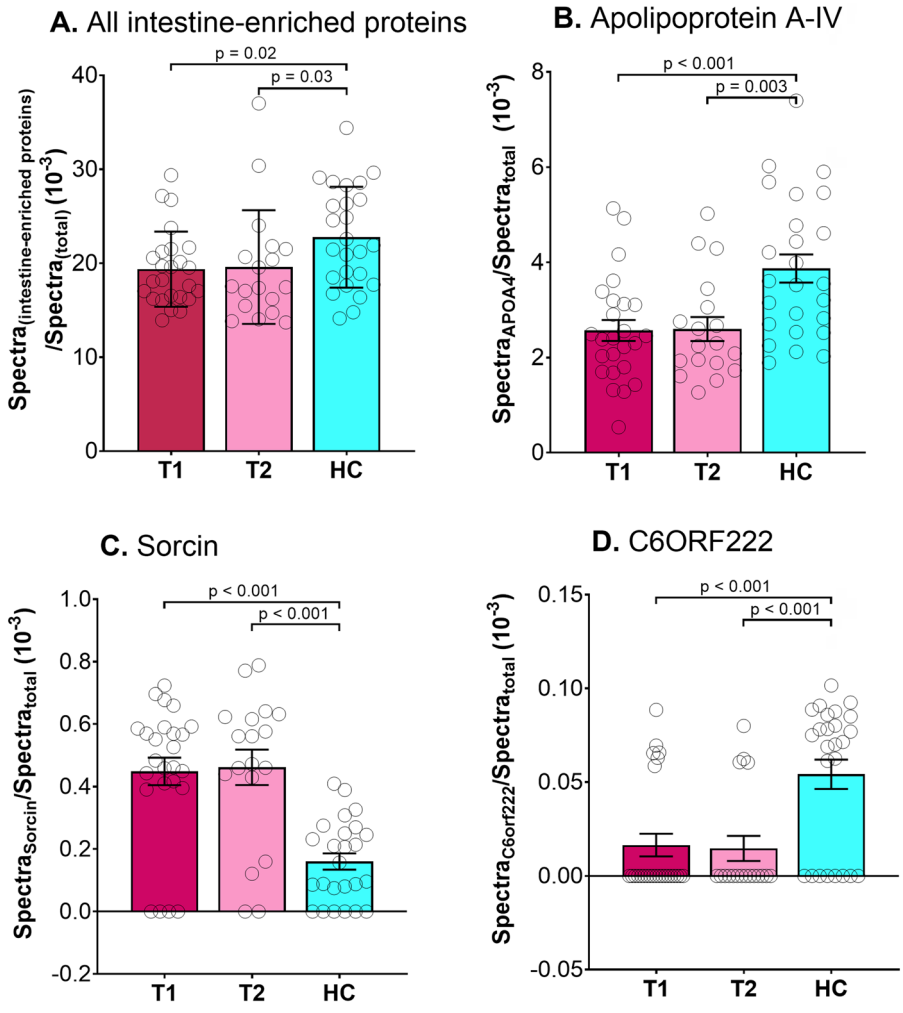

Figure 3. Scatter plot for normalized average precursor intensity with mean (bar) and standard deviation (error bars) for human intestinal proteins in the isolated EV. (A) The sum of normalized average precursor intensities across all intestine-elevated proteins. (B–D) normalized average precursor intensity for the three individual intestine proteins identified as significantly different between psychotic patients during psychosis (T1) and/or in improved state (T2) (correction for multiple hypothesis testing by using permutation-based FDR <0.01 and artificial within group variance s0 = 0.1) Individual values (per patient) shown as circles.

Discussion

Bacteria-derived EV

Mean levels of LPS in EV were lower in patients with psychotic disorders compared to healthy controls, indicating lower levels of bactEV.

In mice, there is evidence of a 'bactEV axis' from the intestinal lumen to the striatum [4]. If this is true also in humans, a disturbance of the amount or type of bactEV, that is transported to the striatum, could be involved in the increased dopamine synthesis in psychotic disorders [8,12,13].

EV from specific gut bacteria can elicit immunosuppressive responses [6], promote wound healing [6], protect against colitis [46], provide anti-tumor effects [7], and increase BDNF expression in neurons and offer antidepressant effects in mice [47]. Mice immunized with bactEV from different bacteria also live longer than control mice [14]. Thus, identification of bactEV and their constitution and origin in the blood may allow novel insights into which and how bacteria might be involved in the pathophysiology of psychosis and might contribute to increased mortality. BactEV-based vaccines against infections have been approved for use in humans [48]. A possible mechanism behind the increased mortality due to infections in patients with psychotic disorders could be a disturbance in the natural bactEV amount and/or content reducing the natural immunity against infections as a lack of natural effective vaccines [49].

We did not measure free LPS, however, it should be noted that LPS besides being a well-known trigger of inflammation, LPS has the capacity to reprogram the immune system with prolonged exposure, including suppression of inflammatory cytokines, in particular TNF-α, IL6 and IL-12p40 [49]. This is in contrast to patients with schizophrenia who commonly have increased IL-1β, IL6, and TNF-α levels [50]. Patients with schizophrenia have a markedly increased mortality owing to, among other things, infections, and cardiovascular diseases [16,49], but interestingly, mice treated with LPS from specific bacteria to reach tolerance are protected against systemic infections [51], and also show improved glucose and lipid metabolism [52], suggesting a possibility for novel treatment approaches in patients with psychotic disorders.

The size of EV in our study and in a previous study of bact EV in gastrointestinal patients by Tulkens et al. was similar, and the latter investigators also found LPS levels in healthy controls similar to our study although they used a sophisticated protocol for bactEV enrichment [29,53].

The results from the metaproteome analysis confirm the LPS result by finding less bacteria peptides in the EV from patients compared to healthy controls. Although none of the individual microbial proteins identified as differently expressed, could be verified as true bacterial findings, Unipept analysis revealed a large number of peptides likely originating from bacterial species.

Our results identify Firmicutes and Bacteroidetes as the dominating phyla in our samples and the manual curation verifies bacteria origin for 854 of 858 peptides. Firmicutes and Bacteroidetes are the dominating phyla in the gut, representing 90% of gut microbiota [54]. We expect our analyses to be exposed to some contamination, however, the profile of our bacterial finding does not match the typical profile of contamination in laboratories [43,55]. Together, this supports that our material contains bactEV originating from the intestine.

Thus, we conclude that metaproteomics in a non-enriched sample is possible, but improved sample preparations are recommended to yield better coverage and a statistical basis for identifying bacterial proteins in the isolated EV.

Intestinal-derived EV

The total level of human intestinal proteins was lower in EV from patients compared to healthy controls. A search through our previous findings in the overrepresented Gene Ontology Term analyses support the validity of the observed difference in total intestinal proteins between the groups. In these analyses, the categories 'regulation of digestive system process' (GO:0044058), 'regulation of intestinal absorption' (GO:1904478), 'regulation of intestinal cholesterol absorption' (GO:0030300), 'regulation of intestinal lipid absorption' (GO:1904729) were found to be downregulated in patients with psychotic disorders compared to healthy controls [29]. Notably, no organ-specific processes, except for those related to the intestine, were identified in these analyses, with the exception of processes primarily associated with the brain, such as 'main axon' and 'postsynapse' [29]. In addition, the overall brain-related proteins did not exhibit similar changes (see also Figure 3(F) in [29]), indicating that the EV change in intestinal proteins are organ-specific. Overall, these findings suggest that there is a difference in the production or transport of EV from intestinal cells in psychotic patients, as evidenced by the lower levels of intestinal proteins in their EV compared to healthy controls.

EV originating from epithelial intestinal cells play a role in antigen presentation and are important in the defense against pathogens [23]. Evidence from humans and mice indicates that EV from intestinal epithelial cells are the main contributor to miRNA in feces [19,56]. These intestinal miRNAs in EV from the host are internalized by bacteria in the intestine, thereby regulating bacterial gene expression and growth. Deficiency of host epithelial-originated miRNAs result in a more diverse microbiota and changed the intestinal barrier integrity in mice [19]. These effects can be countered by fecal transplantation containing intestinal cell-originating miRNAs [19]. Thus, a disturbance in the hosts regulating capacity by EV could explain the altered microbiota in patients with psychotic disorders [1,2]. Interestingly, there is a genetic correlation between schizophrenia and inflammatory bowel disease [57]. Evidence suggests that EV have a role in the development of inflammatory bowel disease including the inflammatory and microbiota regulatory aspects of the pathophysiology [58] The host intestinal EV proteome is changed in inflammatory bowel disease [59] as also possibly indicated in our study of patients with psychotic disorders.

After fecal transplantation, mice receiving feces from patients with schizophrenia developed psychomotor hyperactivity, impaired learning and memory, reduced levels of glutamate and higher glutamine and GABA in the hippocampus [60,61]. Thus, transplantation of healthy donor feces for

psychotic disorders could be an avenue for future research, although the lasting effect of fecal transplantation might be limited if a disturbance in the microbiota regulation by intestinal EV and their miRNA is an underlying problem

In our study, following correction for multiple hypothesis testing, patients had higher levels of the intestine proteins Sorcin and C6orf222 and lower levels of ApoA4. Sorcin, a calcium-binding protein, has been suggested as an early marker of neurodegeneration [62]. A study demonstrated lower levels of this protein in the brain tissue from 15 patients with schizophrenia [63]. Moreover, in line with the increased levels in our data, another recent study showed a higher level of Sorcin in the intestinal biopsies of patients with irritable bowel syndrome compared to the controls [64]. C6orf222 (also named BNIP5) has a high specificity to intestinal cells. To our knowledge, studies regarding the function of C6orf222 or its relation to EV have not been conducted. ApoA4 is a lipid-binding protein primarily synthesized in the small intestines and is secreted into the intestinal lymphatics during fat absorption. Studies have showed inconsistently altered levels of APoA4 (not EV bound) in patients with schizophrenia [65–67]. Approximately 25% of ApoA4 in the blood are bound to lipids, mainly HDL [68]. ApoA4 has been identified in EV, but the proportion of EV-bound ApoA4 is unknown [69,70]. LPS induces the expression of ApoA4 in mice [71]. If this is true also in humans, low level of LPS could lead to lower levels of ApoA4, as in our study. ApoA4 deficiency is associated with atherosclerosis and diabetes [72,73], and these conditions are more frequent in patients with psychotic disorders [16,74–76]. Our findings suggest that C6orf222, ApoA4 and Sorcin are protein candidates for further studies regarding their function and role in EV and the potential impact on the pathophysiology of psychosis.

Limitations

The number of participants in our study was low, and our EV isolation approach favored high yield over the specific selection of EV populations [29]. Small EV are frequently reported isolated by >100,000 g. Proteomic analysis of fractions isolated both by 10,000 g and 110,000 g was performed as preliminary work for this study. Here, known EV markers were identified solely in the 10,000 g fraction. We suggest that this may be caused by aggregation of EV during freezing, and the 10,000 g EV-enriched fraction was chosen for further studies, as presented. We did not perform specialized metaproteomic wet-lab procedures [77] and our bacterial analyses could be prone to contamination as pyrogen-free glasses was not used throughout all steps of the laboratory process. Moreover, we did not collect data on fasting, which should be considered in future studies. On the positive side, this is the first study exploring intestinal and bacterial EV in patients with psychotic disorders, opening a promising research field.

Conclusions

Our data suggest that blood-borne EV originating from the intestine, both from bacteria and human intestinal cells, differ between patients with psychotic disorders and healthy controls. If confirmed in future studies with protocols enriching for bactEV, it is possible that bactEV could be involved in several aspects of the pathophysiology of psychotic disorders which could have implications for the development of new treatments.

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Geolocation information

Latitude: 63° 25′ 49.854″ N. Longitude: 10° 23′ 42.1908″ E. Elevation: 10.653

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Daniel Kondziella, Neurologist and clinician-scientist based at Rigshospitalet, Copenhagen University Hospital, with an interest in acute and critical care neurology including coma and disorders of consciousness; >150 papers in PubMed.

Data availability statement

The raw data from the mass spectrometry are published at the PRIDE partner repository: http://proteomecentral.proteomexchange.org/cgi/GetDataset Dataset identifier PXD016293. The procedure for EV isolation is published at EV-TRACK knowledgebase https://evtrack.org ID: EV200067.

The metaproteomic data has been submitted to ProteomeXchange *via* the PRIDE database (http://proteomecentral.proteomexchange.org/cgi/GetDataset). The data set is named 'Metaproteomic analysis of human plasma extracellular vesicles', with project accession: PXD029074.

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