Extracellular vesicles in patients in the acute phase of psychosis and after clinical improvement- an explorative study

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Extracellular vesicles (EVs) are cell-derived structures that transport proteins, lipids and nucleic acids between cells, thereby affecting the phenotype of the recipient cell. As the content of EVs reflects the status of the originating cell, EVs can have potential as biomarkers. Identifying EVs, including their cells of origin and their cargo, may provide insights in the pathophysiology of psychosis. Here, we present an in-depth analysis and proteomics of EVs from peripheral blood in patients (n=25) during and after acute psychosis. Concentration and protein content of EVs in psychotic patients were twofold higher than in 25 age- and sex-matched healthy controls ($p < 0.001$ for both concentration and protein content), and the diameter of EVs was larger in patients ($p=0.02$). Properties of EVs did not differ significantly in blood sampled during and after the acute psychotic episode. Proteomic analyses on isolated EVs from individual patients revealed 1853 proteins, whereof 45 brain-related proteins were identified. Of these, levels of neurogranin (Q92686), neuron-specific calcium-binding protein hippocalcin (P84074), kalirin (O60229), beta-adducin (P35612) and ankyrin-2 (Q01484) were significantly different in psychotic patients compared to controls, but not different during and after psychosis. All five proteins are involved in regulation of plasticity of glutamatergic synapses. To summarize, our results show that peripheral EVs in psychotic patients are different from those in healthy controls and point at alterations in the glutamatergic system. We suggest that EVs allow investigation of blood-borne brain-originating biological material and that their role

as biomarkers in patients with psychotic disorders is worthy of further exploration.

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Extracellular vesicles in psychosis

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Abstract

Extracellular vesicles (EVs) are cell-derived structures that transport proteins, lipids and nucleic acids between cells, thereby affecting the phenotype of the recipient cell. As the content of EVs reflects the status of the originating cell, EVs can have potential as biomarkers. Identifying EVs, including their cells of origin and their cargo, may provide insights in the pathophysiology of psychosis. Here, we present an in-depth analysis and proteomics of EVs from peripheral blood in patients (n=25) during and after acute psychosis. Concentration and protein content of EVs in psychotic patients were twofold higher than in 25 age- and sex-matched healthy controls (p

40 <0.001 for both concentration and protein content), and the diameter of EVs was larger in
41 patients (p=0.02). Properties of EVs did not differ significantly in blood sampled during and after
42 the acute psychotic episode. Proteomic analyses on isolated EVs from individual patients
43 revealed 1853 proteins, whereof 45 brain-related proteins were identified. Of these, levels of
44 neurogranin (Q92686), neuron-specific calcium-binding protein hippocalcin (P84074), kalirin
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46 psychotic patients compared to controls, but not different during and after psychosis. All five
47 proteins are involved in regulation of plasticity of glutamatergic synapses. To summarize, our
48 results show that peripheral EVs in psychotic patients are different from those in healthy
49 controls and point at alterations in the glutamatergic system. We suggest that EVs allow
50 investigation of blood-borne brain-originating biological material and that their role as
51 biomarkers in patients with psychotic disorders is worthy of further exploration.

52 Introduction

53 Extracellular vesicles (EVs) are nanoscale (30-1000 nm) cell-derived double-lipid membrane
54 structures containing proteins, RNAs and lipids [1, 2]. They are secreted from cells by direct
55 budding of the cell membrane (microvesicles) or by exocytosis of multivesicular bodies
56 (exosomes)(Figure 1) . EVs have multiple functions, including removal of cellular waste and
57 signaling between cells [1, 2]. Cargo carried by the EVs is not random but controlled by the
58 originating cells [1, 2]. The proteins in EVs are common to the cells of origin, which allows to
59 identify the tissue of origin of EVs by analyzing cell-specific proteins [1, 2].

Evidence suggests that EVs from the brain are present in peripheral blood [3-5]. Hence, sampling of blood-borne EVs may be a non-invasive way to gain access to brain-derived biological material. Since evidence indicates that EVs are involved in brain plasticity and information storage [2, 6-9], research on EVs may reveal novel insights into brain disorders in which these processes are relevant, including acute psychosis. Also, EVs interact with the immune system [2] which is likely involved in the pathogenesis of schizophrenia [10]. Further, psychotic disorders are associated with abnormalities in several organ systems other than the brain [11], supporting the rational of investigating peripheral EVs in patients with psychosis . Thus, identifying EVs, their cells of origin and their cargo (i.e. proteins, RNAs and lipids) may uncover insights in the pathophysiology of psychosis and may serve as a source for biomarkers. In theory, EVs could also be used as therapeutic vehicles, as evidence indicates that their membrane proteins can guide them to specific recipient cells [1, 2]. To our knowledge, there are only four published studies on psychosis and EVs: one based on brain biopsies [12], a case report involving cerebrospinal fluid (CSF) analysis [13], and two studies showing altered insulin signaling in L1CAM positive EVs in patients with schizophrenia [14, 15].

In the present study, we investigated if concentration, size and protein content of EVs differed between psychotic patients and controls, and if the state of the disease affected these characteristics. We also explored whether brain-derived EVs could be detected in peripheral blood, and if the pattern of brain-related proteins was different in patients compared to controls. Finally, we used gene ontology (GO) analysis of the proteome to explore which protein categories were over-represented in significantly changed proteins.

Materials and Methods

Study participants

A total of 25 psychotic patients (six females, mean age 33.1 ±11.0 years), during a first episode of psychosis or during acute exacerbation of a known psychotic disorder, were recruited between December 2016 to December 2018 from the Østmarka acute inpatient psychiatric department, St. Olavs University Hospital, Trondheim, Norway. Exclusion criteria were affective psychoses, heart diseases, neurological diseases, pregnancy, rheumatic diseases, autoimmune diseases and cancer. In addition, patients with organic causes of psychosis were excluded. Diagnosis was assessed by ICD 10 Criteria for research and registered after discharge from hospital. Among the 25 patients, 12 (48%) had schizophrenia, 4 (16%) substance-induced psychotic disorder, 3 (12%) acute psychosis, 3 (12%) had unspecified psychosis and 3 (12%) had other psychotic disorders. Mean time since onset of the first psychotic episode was 63 months ±81 months (if no earlier episode, time since symptom debut of the present episode was registered).

A first blood sample was taken at inclusion during the acute phase of psychosis (T1). A second blood sample was drawn 6 weeks later or more (T2), when patients were clinically back to baseline or much/very much improved according to the Clinical Global Impression-Improvement Scale (CGI-I). The time between sample time points was 79±34 days (range: 42 – 162 days). The second blood sample was collected from 18 patients. Seven patients were lost to follow-up. Ongoing abuse of recreational drugs was screened for by questioning and a urine drug screen at first sampling time point and by questioning at second sampling point.

Healthy control persons (n=25) were recruited among the staff of the Department of Psychiatry, Østmarka, St. Olavs University Hospital, Trondheim. Controls were matched to psychotic patients according to sex and age (+/- 5 years). Mean age of healthy controls was 34.2 ±11.2 years. Exclusion criteria were the same as for the psychotic patient, including (self-reported) illegal substance use.

Scoring range

Clinical Global Impression-Severity Scale (CGI-S) scores were registered at both sampling points by the psychologist, board-certified psychiatrist or psychiatric resident in charge of the patient. The CGI-S ranges from 1 ("normal") to 7 ("among the most extremely ill") [16].

Blood sampling and EV isolation

Blood (15 ml) was collected by venipuncture in patients at the two sampling points, and in control persons, with EDTA as anti-coagulant. The samples were kept on ice and centrifuged (2000*g*, 30 min, 4 °C) within 2 hours to isolate cell free plasma. Plasma (6 ml) was transferred to Eppendorf tubes and centrifuged (10000*g*, 30 min, 4 °C). Supernatant was transferred to cryotubes, and both pellets and supernatants were frozen at -80 °C awaiting further analysis.

Pellet fractions were thawed and resuspended in 100 µl phosphate-buffered saline (PBS), and pellet samples originating from the same blood sample were pooled. The samples were centrifuged again to remove any residual cells and debris, first at 2000*g* (30 min, 4 °C). The supernatant was transferred to a pre-weighed Eppendorf tube and centrifuged at 10000*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.

123 *Characterization of isolated EV samples*

124 The protein concentration in isolated EV samples was determined by Qubit Quant-IT Protein
125 Assay Kit (Thermo Fisher Scientific, cat. no. Q33211) on a Qubit Fluorometer 2.0. EVs were
126 analyzed for size and concentration using Nanoparticle Tracking Analysis (NTA, Nanosight LM10,
127 Malvern Panalytical Ltd, Malvern, UK). EVs were diluted 100-fold in sterile PBS and three one-
128 minute movies were recorded on the NTA (detection threshold 4, auto blur size, max jump
129 distance).

130 *Statistical analyses*

131 A two-sample *t*-test was used to compare the mean values of main characteristics of EVs (size,
132 concentration and protein content) between the patients in the acute phase of psychosis and
133 healthy controls, whereas a paired sample *t*-test was used to examine if main characteristics of
134 EV changed from the acute psychotic phase (T1) to improvement of the psychotic episode (T2).
135 To assess if a longer history of psychosis affected main characteristics of EVs, we compared
136 patients with <1 year since onset of first psychosis with patients with 1 year or more since onset
137 of first psychosis using two sample *t*-test. A two-sample *t*-test was also used to assess if drug
138 abuse at the acute phase changed the characteristics of EVs.

139 *Proteomics of isolated EVs*

140 The protein composition of EVs was determined by LC-MS/MS analysis [17]. Sample containing
141 30 µg of protein as determined by Qubit was diluted to 25 µL in ammonium bicarbonate buffer
142 (100 mM), digested by trypsin and desalted as described earlier [18]. The sample was loaded and
143 desalted on a pre-column (Acclaim PepMap 100, 2cm x 75µm ID nanoViper column, packed with 3µm

C18 beads) at a flow rate of 5µl/min for 5 min with 0.1% TFA. Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 250 nl/min, 120 min run) on a 25 cm analytical column (PepMap RSLC, 25cm x 75 µm ID EASY-spray column, packed with 2µm C18 beads with pore size 100Å). Solvent A and B were 0.1% FA (vol/vol) in water and 100% ACN respectively. The gradient composition was 5%B during trapping (5min) followed by 5-7%B over 0.5min, 7–22%B for the next 59.5min, 22-35%B over 22 min, and 35–90%B over 5min. Elution of very hydrophobic peptides and conditioning of the column were performed during 10 minutes isocratic elution with 80%B and 15 minutes isocratic elution with 5%B. The eluting peptides from the LC-column were ionized in the electrospray and analyzed by the Q-Exactive HF. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Q-Exactive HF Tune 2.9 and XCalibur 4.1 Survey full scan MS spectra (from m/z 375-1500) were acquired in the Orbitrap with resolution R = 120 000 at m/z 200, automatic gain control (AGC) target of 3e6 and a maximum injection time (IT) of 100ms. The 12 most intense eluting peptides above an intensity threshold of 50 000 counts, and charge states 2 to 5, were sequentially isolated to a target value (AGC) of 1e5 and a maximum IT of 110ms in the C-trap, and isolation width maintained at 1.6 m/z (offset of 0.3 m/z), before fragmentation in the HCD (Higher-Energy Collision Dissociation) cell. The minimum AGC target for fragmentation were set at 5.5e3. Fragmentation was performed with a normalized collision energy (NCE) of 28 %, and fragments were detected in the Orbitrap at a resolution of 60 000 at m/z 200, with first mass fixed at m/z 120. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 20s with “exclude isotopes” on. Lock-mass internal calibration (m/z 445.12003) was enabled. The spray and ion-source parameters were as follows. Ion spray voltage = 1800V, no sheath and auxiliary gas flow, and capillary temperature = 275 °C.

In total, 68 samples were submitted to proteomic analysis, and each sample was analysed a single time with mass spectrometry. The samples were divided in three groups: patient samples at first time point (n=25); patient samples at second time point (n=18); and control samples from age-matched healthy persons (n=25).

Database search parameter and acceptance criteria for identification

The raw data was converted to Mascot Generic Format (mgf) peak lists with MS convert with peak picking of MS2 to convert to centroid data[19]. Peak lists obtained from MS/MS spectra were identified using X!Tandem (X!Tandem Vengeance, v2015.12.15.2). The search was conducted using SearchGUI (v3.3.15). Protein identification was conducted against a concatenated target/decoy database of Homo sapiens (reference proteome downloaded from UniProtKB [PMID 14681372] in March 2018) with porcine trypsin (P00761) added as likely contaminant (40660 entries in concatenated database, based on 20330 entries from uniprot.org). The decoy sequences were created by reversing the target sequences in SearchGUI[20]. The identification settings were as follows: specific trypsin digest with a maximum of 2 missed cleavages; 10 ppm as MS1 and 0.02 Da as MS2 tolerances; fixed modifications: Carbamidomethylation of C (+57.021464 Da); variable modifications: Oxidation of M (+15.994915 Da); fixed modifications during refinement procedure: Carbamidomethylation of C (+57.021464 Da); variable modifications during refinement procedure: Acetylation of protein N-term (+42.010565 Da), Pyrolidone from E (--18.010565 Da), Pyrolidone from Q (--17.026549 Da), Pyrolidone from carbamidomethylated C (--17.026549 Da).

187 Peptides and proteins were inferred from the spectrum identification results using
188 PeptideShaker (v1.16.38) [21]. Peptide Spectrum Matches (PSMs), peptides and proteins were
189 validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy hit distribution. Post-
190 translational modification localizations were scored using the D-score[22]. All samples were
191 processed in parallel in PeptideShaker to provide data for all identified proteins across all
192 samples, with individual quantitative measures for each sample. The average precursor
193 intensity, an average of MS1 signal for all spectra allocated to a given protein in a given sample,
194 was used for label-free quantitative evaluation.

195 *Proteomic data analysis and submission of data to a repository*

196 For quantification, average precursor intensities were normalized by dividing the intensity on
197 the sum of intensities within individual samples. Statistical analysis was performed using
198 Perseus (version 1.6.5.0) [23]. Identification of significant differences in protein detection
199 between sample groups were analysed in Perseus, using Student's *t*-test with correction for
200 multiple hypothesis testing by using permutation-based FDR < 0.01 and artificial within group
201 variance s0=0.1. Missing values were imputed from a normal distribution with a 1.8 standard
202 deviation shift from the average and a width of 0.3.

203 Gene ontology of identified proteins was analysed by PANTHER classification system (version
204 15.0, released on 2020-04-07) [24]. The EV proteome was screened for brain-enriched proteins
205 and membrane-proteins as determined in the human protein atlas [25, 26]. To control for co-
206 isolation of lipoprotein and chylomicrons, we searched proteomic results for apolipoproteins
207 [27]. The mass spectrometry data along with the identification results have been deposited to

the ProteomeXchange Consortium [28] via the PRIDE partner repository [29] available at http://proteomecentral.proteomexchange.org/cgi/GetDataset with the dataset identifier PXD016293.

Ethics

The study was approved by the Regional Ethics committee, South East Norway (2016/949) and performed according to the ethical principles of the Helsinki declaration. All participants gave their written, informed consent after a board-certified psychiatrist or psychologist had checked that they were able to do so.

Results

Clinical Global Impression-Severity Scale

The CGI-S score was used to evaluate the severeness of psychosis, and the change in state for patients in the two sample time points. CGI-S score showed a decline from a median of 7 (defined as “among the most extremely ill patients”), range 5 to 7, during the acute psychotic period (T1) to a median of 4 (“moderately ill”), range 2-6, at T2. All patients had a lower CGI-S score at the second time point.

Size, concentration and protein content

The isolated EV fractions were analyzed to evaluate total amount and EV characteristics in each group. Mean size and concentration of EVs and protein concentration in EV fractions are shown in Figure 2. The protein concentration per EV was equal for all groups, and averaged at $1.2 \cdot 10^{-6}$ $\pm$ $6.7 \cdot 10^{-7}$ µg protein/vesicles. NTA analysis showed that most vesicles in the samples was

between 75 and 200 nm in size, but larger vesicles were also present. Exosomes are defined as vesicles 30-150 nm in size [2], and the isolated samples are likely a mixture of exosomes and microvesicles. We found that the size, concentration and protein content of EVs from psychotic patients differed significantly from healthy controls (Table 1A). There was no apparent difference between T1 and T2 in the psychotic patients (Table 1B). There were no significant differences either in EV characteristics between patients with a short (< 1 year) versus longer (≥ 1 year) history of psychosis (Table 1C).

Substance use

About 40% of patients with schizophrenia spectrum disorders also have a substance use disorder [30]. Substance use disorders are highly correlated to smoking [31] and linked with poor outcomes in symptom severity and service use in patients with psychosis [32]. We studied if a recent intake of illegal substances affected EV parameters. Nine and 3 patients had used illegal drugs within 1 week before T1 and T2, respectively. Within the psychosis group there was no significant change in size of EVs in the group without illegal substance use the week before sampling (191nm) compared to patients with illegal substance use 1 week before sampling (203nm) (n=25, mean change 12 nm, p=0.156, equal variance not assumed) at T1. There were no differences comparing mean concentrations in patients without ($2,46x10^7$ particles/ml) and with illegal substance use 1 week before sampling ($2,19x10^7$ particles/ml) at T1 (mean change $2,75\ x10^6$ particles/ml n=25, p=0.573). There was no significant change in mean protein content in EV fractions in patients without (28,54 µg/ml) and with illegal substance use 1 week before sampling (27,65 µg/ml) (n=25, mean change 0,89 µg/ml, p=0.897).

EV proteomes

The protein cargo of EVs are central to understand their origin, function and classification. Shotgun proteomics of all EV samples resulted in 1853 identified proteins with more than 1 identified peptide across all samples by 26,537 unique peptides, using a false positive rate (FDR) of 1% (Table S1). Of these, 1658 (89%) proteins were identified in all three sample groups [33]. 118 proteins were identified at one or two time points from the psychotic patient samples, while not detected in the control group (Figure 3A). To verify the EV origin, the proteomes were compared to the 100 most frequent proteins found in exosomes from Exocarta [34]. We identified 93 of these in our sample material, without difference between patient samples and controls. This includes known EV markers such as Alix (Q8WUM4), Tsg101 (Q99816), CD9 (P21926), CD81 (P60033) and CD63 (P08962), demonstrating that the isolated EV fractions in this study contained EVs, likely both microvesicles and exosomes. The enrichment of the EV proteomes was also confirmed by comparing the 1853 proteins identified with the human proteome by GO enrichment analysis. The GO-terms *extracellular exosomes* and *vesicle-mediated transport* were among the mostly enriched GO-terms (Table S2A), verifying that the EV isolation process yielded an EV-enriched fraction.

Differentially expressed proteins were identified by comparing proteins in groups pairwise by normalized average precursor intensity of identified proteins (Figure 3B). No proteins were identified as differentially expressed between T1 and T2, although COP9 signalosome complex subunit 6 (Q7L5N1) had significant p-value ($p < 0.001$) and close to significant fold change (0.66). In T1 and/or T2, 119 and 40 proteins were differentially expressed compared with healthy controls (HC), respectively (complete lists in Table S3). Combined, 131 proteins were differently expressed in T1 and/or T2 compared with HC, 102 proteins had increased

abundance in T1 and/or T2 compared with HC, while 29 proteins had a lower abundance. The relative variance and distribution for each protein between samples within one group was considered to evaluate different heterogeneity in the three sample groups, and were similar for HC, T1 and T2 groups. Thus, the group heterogeneity was similar for HC, T1 and T2.

The proteins that were differentially expressed in psychotic patients compared with healthy controls were submitted to GO overrepresentation analysis, to identify enriched GO terms for the changed proteins (significantly enriched GO terms compared to the human proteome, FDR threshold at 0.05) (Table S2B and C).

Proteins with higher abundance in psychotic patients had overrepresented GO-terms related to localization and transport inside and out of the cell, as well as leukocyte and neutrophil activation. GO terms enriched for proteins that had a higher abundance in HC samples were represented by lipoprotein processes, the immunoglobulin complex and complement pathway. Of note, the GO terms *main axon* and *postsynapse* were enriched in the proteins with higher abundance in healthy controls and represented 9 proteins (Table S4).

The mass spectrometry data along with the identification results have been deposited to the ProteomeXchange Consortium [28] via the PRIDE partner repository [29] available at http://proteomecentral.proteomexchange.org/cgi/GetDataset with the dataset identifier PXD016293 and Project DOI 10.6019/PXD016293.

Lipoproteins

Lipoproteins are an important constituent of EVs and occur also in plasma as lipid particles that can co-isolate with EVs. Sixteen apolipoproteins were identified by proteomics. Significant

differences with higher levels in healthy controls were found for Apolipoprotein L1, B-100, A-I and A-IV (Table S5). The apolipoproteins contributed with 1.0 to 6.0 % of the total signal intensity for each sample analysed by proteomics (average 2.3 %), indicating that the overall contribution of lipoproteins in the samples are low. The percentage contribution of apolipoprotein spectra was higher in healthy controls (2.6±1.0) compared with T1 (2.0±0.9) ($p = 0.04$, unpaired *t*-test), but not T2. The GO term for chylomicron and lipid-particle formation was enriched in proteins more abundant in healthy controls, also corroborating that the concentration of lipid particles in HC compared with psychotic patients is proportionally higher.

Brain proteins

We investigated whether brain-related proteins could be identified in the isolated EVs, and if these were different in healthy persons and patients with psychosis. The Human Protein Atlas contains information regarding the expression profiles of human genes both on the mRNA and protein level. Protein evidence is calculated for each gene based on three different sources: UniProt protein existence; a Human Protein Atlas antibody- or RNA based score; and evidence based on PeptideAtlas [25, 26]. We identified 45 proteins in our EV proteome (Table S6) that have an elevated expression in the brain compared to other tissue types according to The Human protein Atlas[25, 26]. The sum of spectral intensities for all brain-enriched proteins showed no difference between patients and controls. However, 5 proteins had significant different abundancies in healthy controls compared to patients (Figure 4, Table 2).

Membrane-bound protein candidates for immunolabeling of brain-derived EVs

313 To enable selective isolation of EVs from the brain, surface proteins can be targeted either in
314 immunoaffinity chromatography or fluorescence activated cell sorting (FACS). In addition, the
315 proteins need to be tissue specific. In the Human Protein Atlas a mRNA and protein expression
316 summary for different organs is calculated for each gene, hereby giving a measure of organ
317 specificity for proteins, which is here used to identify brain-specific proteins.

318 NCAM1 and L1CAM are two proteins that may be used for immunolabeling of brain-derived
319 EVs[35-39]. NCAM1 and L1CAM was not detected in this study. However, we identified other
320 membrane-related proteins with high specificity to the brain [26]; Plexin B3 (Q9ULL4) is
321 expressed in cerebral cortex, with high expression in neurons and intermediate expression in
322 glial cells. C type lectin domain 2 family L (P0C7M8) is expressed in cerebral cortex with
323 intermediate expression in neuropil and glia. Myelin basic protein (P02686) has high expression
324 in glia cells in the caudate. We also detected purinergic receptor P2Y12, a protein with high
325 expression in microglia but also in other immune cells [26].

326 Discussion

327 We present the first characterization of blood-based EVs isolated from psychotic patients with
328 extensive peripheral blood EV proteomes for psychotic patients and healthy persons [40]. Size,
329 concentration and protein concentration in EVs were all increased in psychotic patients
330 compared to controls and remained unaltered with clinical improvement. Use of illegal
331 substances or duration of the psychotic disorder had no influence on EV characteristics,
332 indicating that the findings correlate to other factors than an unhealthy life style which is
333 common in patients with psychosis [41]. This could suggest that our findings are related to the

334 psychiatric condition itself rather than representing confounders. Investigating if EV alterations
335 are inherent to psychotic disorders or are due to lifestyle factors, medications or high stress
336 levels will be an important area for future research.

337 *Brain-enriched proteins derived from EVs*

338 We identified several brain-specific and brain-enriched proteins in isolated EVs from psychotic
339 patients and healthy controls. Brain-specific proteins have previously been found in EVs from
340 patients with malignant glioma, Alzheimer's disease (AD), frontotemporal dementia and
341 healthy controls [3, 4], indicating that EVs originating from the brain can enter the bloodstream.
342 The most likely route is via the brain glymphatic system that can transport large molecules and
343 cells [42]. Transport via the blood-brain barrier (BBB) may also contribute this, as preclinical
344 evidence suggests an inflammatory dose-dependent transcytosis of EVs through the BBB [43,
345 44]. Comparing the average content of all brain-enriched proteins, we noticed no difference
346 between patients and controls. Although a crude estimate, this suggest that the difference
347 between patients and controls in terms of total production, clearance by the glymphatics and
348 passage of brain EVs through the BBB is minor.

349 *Neurogranin, neuron-specific calcium-binding protein hippocalcin, kalirin, beta-adducin and*
350 *ankyrin-2*

351 We found five brain proteins with different abundances in patients and controls; all these
352 proteins are involved in the regulation of glutamatergic synapses. GO analysis pointed in the
353 same direction as analysis of individual brain proteins; the GO terms *main axon* and
354 *postsynapse* had higher abundance in healthy controls. The formation of neuronal circuits

355 during brain development and their subsequent modification during lifetime require plasticity
356 at excitatory synapses, manifested by changes in synaptic strength [45]. Long-term potentiation
357 (an increase in synaptic strength) and long-term depression (a decrease in synaptic strength)
358 are synapse-specific forms of plasticity [45]. Two important postsynaptic processes are involved
359 in the plasticity of glutamatergic synapses: modifications in the amount of α-amino-3-hydroxy-
360 5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and morphological alterations of
361 dendritic spines mainly mediated by actin filament [46]. Dendritic spines are the postsynaptic
362 structural correlate of excitatory synapses [47].

363 AMPAR is a glutamate receptor that mediates the majority of fast synaptic excitation in the
364 central nervous system. To modulate the synaptic transmission strength, AMPAR are
365 transported to or from synapses [45]. An excitatory synapse containing *N*-methyl-D-aspartate
366 receptors (NMDAR), but no AMPAR, is termed a silent synapse because of its low activity [47].
367 One of the main regulators of AMPAR endocytosis is stimulation of NMDAR [45].

368 Hippocalcin, a Ca^{2+}-sensing protein, is found in the retina and the brain [25, 26, 45] and was
369 increased in EVs from our psychotic patients (Figure 4B). Hippocalcin is required for long-term
370 depression in the synapse, and a suggested mechanism is that hippocalcin recruits AMPAR to
371 endocytic sites in response to NMDAR mediated Ca^{2+} signals [45].

372 Kalirin levels were also increased in EVs from patients (Figure 4C). Kalirin expression is enriched
373 in the forebrain. Its most abundant isoform, kalirin-7, is localized to dendritic spines on cortical
374 pyramidal neurons, where it plays a key role in morphological and functional plasticity at
375 excitatory synapses and facilitates actin remodeling such that overexpression increases the

number of dendritic spines [48]. Kalirin-7 interacts with the protein product of, *DISC1*, modulating the response to NMDAR activation [49, 50]. When the DISC1 protein are disrupted it predisposes the carrier to a number of mental health disorders including schizophrenia [50-52]. In our study, the levels of neurogranin, beta-adducin, and ankyrin-2 were lower in EVs from psychotic patients (Figure 4A, D and E). The expression of beta-adducin is mainly restricted to the brain and hematopoietic tissues [25, 26] and regulates dendritic spine stability through actin-based synapse formation and spectrin-based synapse stabilization [53]. Ankyrin-2 is a member of the ankyrin family of proteins that link the integral membrane proteins to the underlying spectrin-actin cytoskeleton and is a key presynaptic target of casein kinase 2 to maintain synapse stability [54]. Neurogranin, a neuron-specific and postsynaptic protein, increases synaptic strength in an activity- and NMDAR-dependent manner [55]. Decreased neurogranin levels lead to accelerated spine elimination and impaired recruitment of AMPAR to silent synapses [47].

Our results indicate weakening of the glutamatergic synapse in psychotic patients; the high levels of kalirin being an exception. The role of the detected synapse proteins as well as underlying mechanisms of synaptic plasticity in general, remains little understood[56]. However, the literature does indicate an important function of EVs in synapse regulation [8, 9, 57, 58] as is also apparent in our study.

Glutamatergic neurotransmission and psychotic disorders

One of the main hypotheses regarding the pathophysiology of psychotic disorders is abnormal glutamatergic neurotransmission and NMDAR hypofunction [59]. This is supported by the fact

that psychosis typically starts during adolescence, a period involving modification of synapses [60]. Further, NMDAR antagonists can produce psychotic symptoms [59, 61]. Growing genetic data supports the association between schizophrenia and glutamatergic synapse hypofunction [62-64]. Two large genome wide studies have identified the GO term “abnormal long-term potentiation” on their top list of gene sets enriched in schizophrenia [65, 66]. Two reviews also pointed at variants in genes belonging to the postsynaptic density at the glutamatergic synapse [67, 68]. Animal models have provided possible mechanisms linking NMDAR hypofunction to the perceptual disturbances and abnormal associative learning in schizophrenia [69, 70]. To summarize, evidence points at dysfunction of the glutamatergic synapse as a possible mechanism in the pathophysiology of psychosis. Our results support data suggesting glutamatergic dysfunction in psychosis and indicate a role of EVs in disease-related synaptic regulation.

Protein candidates for immunolabeling of brain-derived EVs

Our proteomic analysis revealed presence of brain-derived EVs in the blood, in a mixture of EVs from other tissues. The isolation of brain-derived EVs from the blood EV population can enable more detailed analysis on EVs originating directly from the brain. To enable such isolation, affinity methods based on antibodies recognizing surface proteins can be applied. Some authors have proposed that membrane proteins L1CAM, NCAM1, or glutamine aspartate transporter may be suitable protein markers for immunolabeling of brain-derived EVs [5, 35-39]. However, these proteins have a low specificity for the brain and are not suited for the isolation of pure fractions of brain-derived EVs[25, 26]. Our study identified membrane proteins with high brain specificity [26]. These proteins are promising candidates to isolate brain-derived

EVs, representing different brain-related cells and compartments. Although purinergic receptor P2Y12 also is highly expressed in peripheral immune cells, this protein is interesting as a target protein for immunolabeling of EVs due to its high expression in microglia. EVs from microglia will be relevant to investigate in the future, as evidence suggests that microglia might contribute to neuroinflammation in psychosis but the usefulness of PET and the translocator protein tracer to assess microglia activation in patients with psychosis has been challenged [10].

GO analysis

GO overrepresentation analysis of significantly changed proteins revealed that immunoglobulin complex, complement pathway and lipoprotein particle-related proteins were overrepresented GO terms for proteins with significantly lower abundance in patients with psychosis. These proteins are large and abundant in human plasma[40]. The apparent increase in these GO pathways could be caused by a proportionally larger co-precipitation of free proteins and a lower overall EV concentration in healthy controls. Cholesterol and lipid-soluble proteins are also present within EVs and the detected apolipoproteins may originate from the EVs themselves [71]. Also, there is increasing evidence that EVs carry complement factors as cargo and on their surface, thereby contributing to both pro- and anti-inflammatory immune states [72]. Of note, genetic variations in some complement genes and changed levels of complement components are associated with psychosis [73].

GO terms overrepresented in proteins significantly higher in patient samples compared with healthy controls were terms related to localization and transportation within and out of the cell, as well as proteins related to activation of neutrophils and other leukocytes. This

difference may indicate more active secretion and loading of EVs in psychotic patients. According to two recent meta-analyses, the neutrophil-to-lymphocyte ratio is increased in patients with non-affective psychosis and schizophrenia [74, 75], and several studies have shown other types of inflammation and immune alterations in psychotic patients [10, 11, 75] in line with our GO analyses. Possible mechanisms could be promotion of inflammation and immune activation by EVs trough their regulatory role or inflammation stimulating EV secretion from immune cells and tissues [76].

Methodological considerations

Our study cohort consisted of acutely admitted patients with a primary diagnosis within the psychosis spectrum. This cohort reflects the real-life setting from an acute and emergency psychiatric treatment facility [77]. Although a psychiatric cohort as ours is thus subject to heterogeneity, there are valid arguments against categorizing psychotic disorders into too many different diagnostic entities [78-80]. Even the validity of the distinction between a primary psychosis with comorbid drug abuse and drug-induced psychosis has been questioned [81-84]. From a pragmatic point of view, we therefore conclude that our cohort was suitable for the aim of our study, i.e. investigating EVs in patients in the acute phase of psychosis and after improvement.

Regarding the isolation of EVs, our method is simple, and the EV population identified here was likely a mixture of microvesicles and exosomes. As the yield of EVs from a normal-sized peripheral blood sample is modest, our available sample volumes were not sufficient for dividing the samples into more defined, smaller, fractions, e.g. by a density gradient separation method. However, by comparison with Exocarta and gene ontology, we demonstrated that the obtained samples are highly enriched in EVs. The majority of protein aggregates from abundant

plasma proteins are expected to be discarded with the supernatant. Preliminary experiments were also done on selected samples, to isolate smaller vesicles from the supernatant by ultracentrifugation at 110,000*g*, to evaluate if this could provide us with more pure exosome fractions. However, proteomic analysis of such samples revealed predominately abundant plasma proteins, and none of the expected exosome protein markers that were identified in the 10,000*g* fraction (data not shown). We conclude that the isolation method used in this study yields samples enriched with important exosomal protein markers and with sufficient EV amounts for in-depth characterization of individual samples.

Strengths and limitations

Our study has limitations that should be acknowledged. First, this was an explorative study with a small and heterogenous patient cohort. Second, we were unable to control for weight, smoking and metabolic factors that are expected to be unequally distributed in psychotic patients and controls. Third, our study may have been subject to selection bias with the most paranoid and anxious patients declining consent; and finally, 28% of patients with psychosis was lost to follow-up. On the positive side, this is the first study that characterizes peripheral EVs in psychosis, i.e. a novel and promising opportunity to identify liquid biomarkers for a major psychiatric disorder.

Conclusions

Blood-borne EVs differ substantially between patients with psychotic disorders and healthy controls. Also, amounts of several proteins involved in the regulation of plasticity of glutamatergic synapses were altered in the psychotic patients supporting evidence of

483 glutamatergic dysfunction in psychosis and indicate a role of EVs in disease-related synaptic
484 regulation. Thus, collecting peripheral EVs allows access to brain-originating biological material.
485 We suggest that this approach may provide novel insights about the underlying processes of
486 psychotic disorders.

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494 References

495 1. van Niel, G., G. D'Angelo, and G. Raposo, *Shedding light on the cell biology of extracellular*
496 *vesicles.* Nat Rev Mol Cell Biol, 2018. **19**(4): p. 213-228.
497 2. Yanez-Mo, M., et al., *Biological properties of extracellular vesicles and their physiological*
498 *functions.* J Extracell Vesicles, 2015. **4**: p. 27066.
499 3. Galbo, P.M., Jr., et al., *Circulating CD9+/GFAP+/survivin+ exosomes in malignant glioma patients*
500 *following survivin vaccination.* Oncotarget, 2017. **8**(70): p. 114722-114735.
501 4. Goetzl, E.J., et al., *Decreased synaptic proteins in neuronal exosomes of frontotemporal*
502 *dementia and Alzheimer's disease.* FASEB J, 2016. **30**(12): p. 4141-4148.
503 5. Kapogiannis, D., et al., *Association of Extracellular Vesicle Biomarkers With Alzheimer Disease in*
504 *the Baltimore Longitudinal Study of Aging.* JAMA Neurol, 2019.
505 6. Pastuzyn, E.D., et al., *The Neuronal Gene Arc Encodes a Repurposed Retrotransposon Gag*
506 *Protein that Mediates Intercellular RNA Transfer.* Cell, 2018. **172**(1-2): p. 275-288 e18.
507 7. Goldie, B.J., et al., *Activity-associated miRNA are packaged in Map1b-enriched exosomes*
508 *released from depolarized neurons.* Nucleic Acids Res, 2014. **42**(14): p. 9195-208.
509 8. Fowler, C.D., *NeuroEVs: Characterizing Extracellular Vesicles Generated in the Neural Domain.* J
510 Neurosci, 2019. **39**(47): p. 9262-9268.
511 9. Chivet, M., et al., *Exosomes secreted by cortical neurons upon glutamatergic synapse activation*
512 *specifically interact with neurons.* J Extracell Vesicles, 2014. **3**: p. 24722.

10. Kroken, R.A., et al., *Constructing the Immune Signature of Schizophrenia for Clinical Use and Research; An Integrative Review Translating Descriptives Into Diagnostics.* Front Psychiatry, 2018. **9**: p. 753.
11. Pillinger, T., et al., *Is psychosis a multisystem disorder? A meta-review of central nervous system, immune, cardiometabolic, and endocrine alterations in first-episode psychosis and perspective on potential models.* Mol Psychiatry, 2018.
12. Banigan, M.G., et al., *Differential expression of exosomal microRNAs in prefrontal cortices of schizophrenia and bipolar disorder patients.* PLoS One, 2013. **8**(1): p. e48814.
13. Mobarrez, F., et al., *Microparticles and microscopic structures in three fractions of fresh cerebrospinal fluid in schizophrenia: case report of twins.* Schizophr Res, 2013. **143**(1): p. 192-7.
14. Kapogiannis, D., et al., *Insulin-signaling abnormalities in drug-naive first-episode schizophrenia: Transduction protein analyses in extracellular vesicles of putative neuronal origin.* Eur Psychiatry, 2019. **62**: p. 124-129.
15. Wijtenburg, S.A., et al., *Brain insulin resistance and altered brain glucose are related to memory impairments in schizophrenia.* Schizophr Res, 2019. **208**: p. 324-330.
16. Guy, W., *Clinical Global Impression*, E. Department of Health, and Welfare, Editor. 1976: ECDEU Assessment Manual for Psychopharmacology.
17. Choi, D.S., et al., *Proteomics of extracellular vesicles: Exosomes and ectosomes.* Mass Spectrom Rev, 2015. **34**(4): p. 474-90.
18. Haslene-Hox, H., et al., *A new method for isolation of interstitial fluid from human solid tumors applied to proteomic analysis of ovarian carcinoma tissue.* PLoS One, 2011. **6**(4): p. e19217.
19. Chambers, M.C., et al., *A cross-platform toolkit for mass spectrometry and proteomics.* Nat Biotechnol, 2012. **30**(10): p. 918-20.
20. Barsnes, H. and M. Vaudel, *SearchGUI: A Highly Adaptable Common Interface for Proteomics Search and de Novo Engines.* J Proteome Res, 2018. **17**(7): p. 2552-2555.
21. Vaudel, M., et al., *PeptideShaker enables reanalysis of MS-derived proteomics data sets.* Nat Biotechnol, 2015. **33**(1): p. 22-4.
22. Vaudel, M., et al., *D-score: a search engine independent MD-score.* Proteomics, 2013. **13**(6): p. 1036-41.
23. Tyanova, S., et al., *The Perseus computational platform for comprehensive analysis of (prote)omics data.* Nat Methods, 2016. **13**(9): p. 731-40.
24. Mi, H., A. Muruganujan, and P.D. Thomas, *PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees.* Nucleic Acids Res, 2013. **41**(Database issue): p. D377-86.
25. Uhlen, M., et al., *Proteomics. Tissue-based map of the human proteome.* Science, 2015. **347**(6220): p. 1260419.
26. The Human protein Atlas. *The brain-specific proteome*. n.d. [cited 2018 November, 22, 2018]; Available from: http://www.proteinatlas.org/humanproteome/tissue/brain.
27. Karimi, N., et al., *Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins.* Cell Mol Life Sci, 2018. **75**(15): p. 2873-2886.
28. Vizcaino, J.A., et al., *ProteomeXchange provides globally coordinated proteomics data submission and dissemination.* Nat Biotechnol, 2014. **32**(3): p. 223-6.
29. Vizcaino, J.A., et al., *2016 update of the PRIDE database and its related tools.* Nucleic Acids Res, 2016. **44**(D1): p. D447-56.
30. Hunt, G.E., et al., *Prevalence of comorbid substance use in schizophrenia spectrum disorders in community and clinical settings, 1990-2017: Systematic review and meta-analysis.* Drug Alcohol Depend, 2018. **191**: p. 234-258.

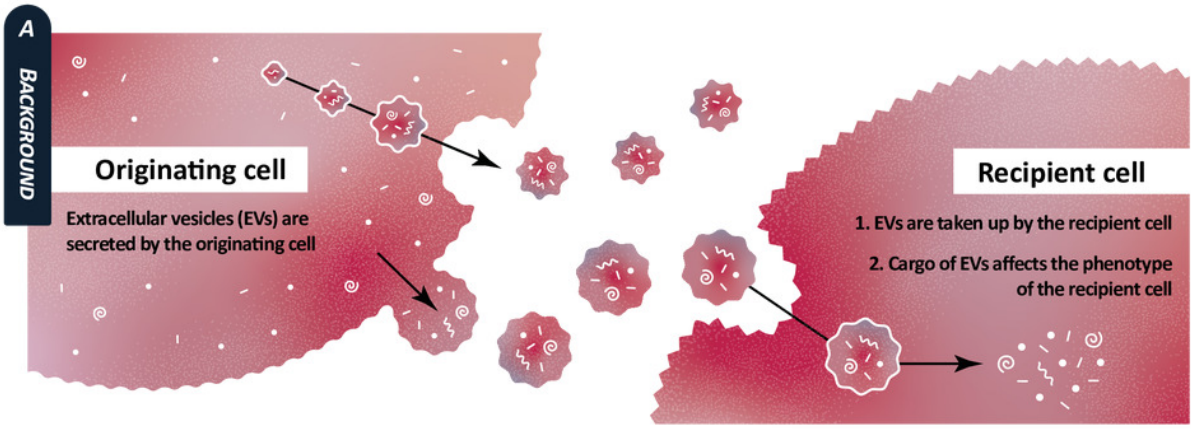
31. Smith, P.H., C.M. Mazure, and S.A. McKee, *Smoking and mental illness in the U.S. population.* Tob Control, 2014. **23**(e2): p. e147-53.
32. Abdel-Baki, A., et al., *Symptomatic and functional outcomes of substance use disorder persistence 2 years after admission to a first-episode psychosis program.* Psychiatry Res, 2017. **247**: p. 113-119.
33. Hulsen, T., J. de Vlieg, and W. Alkema, *BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams.* BMC Genomics, 2008. **9**: p. 488.
34. *Exocarta*. 2018 October]; Available from: http://exocarta.org/exosome_markers_new.
35. Mustapic, M., et al., *Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes.* Front Neurosci, 2017. **11**: p. 278.
36. Fiandaca, M.S., et al., *Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study.* Alzheimers Dement, 2015. **11**(6): p. 600-7 e1.
37. Goetzl, E.J., et al., *Low neural exosomal levels of cellular survival factors in Alzheimer's disease.* Ann Clin Transl Neurol, 2015. **2**(7): p. 769-73.
38. Goetzl, E.J., et al., *Cargo proteins of plasma astrocyte-derived exosomes in Alzheimer's disease.* FASEB J, 2016. **30**(11): p. 3853-3859.
39. Kapogiannis, D., et al., *Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease.* FASEB J, 2015. **29**(2): p. 589-96.
40. Braga-Lagache, S., et al., *Robust Label-free, Quantitative Profiling of Circulating Plasma Microparticle (MP) Associated Proteins.* Mol Cell Proteomics, 2016. **15**(12): p. 3640-3652.
41. Jakobsen, A.S., et al., *Dietary patterns and physical activity in people with schizophrenia and increased waist circumference.* Schizophr Res, 2018. **199**: p. 109-115.
42. Louveau, A., et al., *Structural and functional features of central nervous system lymphatic vessels.* Nature, 2015. **523**(7560): p. 337-41.
43. Matsumoto, J., et al., *Transmission of alpha-synuclein-containing erythrocyte-derived extracellular vesicles across the blood-brain barrier via adsorptive mediated transcytosis: another mechanism for initiation and progression of Parkinson's disease?* Acta Neuropathol Commun, 2017. **5**(1): p. 71.
44. Andras, I.E., et al., *Extracellular vesicles of the blood-brain barrier: Role in the HIV-1 associated amyloid beta pathology.* Mol Cell Neurosci, 2017. **79**: p. 12-22.
45. Hanley, J.G., *The Regulation of AMPA Receptor Endocytosis by Dynamic Protein-Protein Interactions.* Front Cell Neurosci, 2018. **12**: p. 362.
46. Bosch, M. and Y. Hayashi, *Structural plasticity of dendritic spines.* Curr Opin Neurobiol, 2012. **22**(3): p. 383-8.
47. Han, K.S., S.F. Cooke, and W. Xu, *Experience-Dependent Equilibration of AMPAR-Mediated Synaptic Transmission during the Critical Period.* Cell Rep, 2017. **18**(4): p. 892-904.
48. Penzes, P. and C. Remmers, *Kalirin signaling: implications for synaptic pathology.* Mol Neurobiol, 2012. **45**(1): p. 109-18.
49. Hayashi-Takagi, A., et al., *Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1.* Nat Neurosci, 2010. **13**(3): p. 327-32.
50. Tropea, D., et al., *Mechanisms underlying the role of DISC1 in synaptic plasticity.* J Physiol, 2018. **596**(14): p. 2747-2771.
51. Thomson, P.A., et al., *Balanced translocation linked to psychiatric disorder, glutamate, and cortical structure/function.* NPJ Schizophr, 2016. **2**: p. 16024.

52. Sachs, N.A., et al., *A frameshift mutation in Disrupted in Schizophrenia 1 in an American family with schizophrenia and schizoaffective disorder.* Mol Psychiatry, 2005. **10**(8): p. 758-64.
53. Engmann, O., et al., *DARPP-32 interaction with adducin may mediate rapid environmental effects on striatal neurons.* Nat Commun, 2015. **6**: p. 10099.
54. Bulat, V., M. Rast, and J. Pielage, *Presynaptic CK2 promotes synapse organization and stability by targeting Ankyrin2.* J Cell Biol, 2014. **204**(1): p. 77-94.
55. Zhong, L., et al., *Neurogranin enhances synaptic strength through its interaction with calmodulin.* EMBO J, 2009. **28**(19): p. 3027-39.
56. Dieterich, D.C. and M.R. Kreutz, *Proteomics of the Synapse--A Quantitative Approach to Neuronal Plasticity.* Mol Cell Proteomics, 2016. **15**(2): p. 368-81.
57. Fruhbeis, C., et al., *Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication.* PLoS Biol, 2013. **11**(7): p. e1001604.
58. Ashley, J., et al., *Retrovirus-like Gag Protein Arc1 Binds RNA and Traffics across Synaptic Boutons.* Cell, 2018. **172**(1-2): p. 262-274 e11.
59. Balu, D.T. and J.T. Coyle, *The NMDA receptor 'glycine modulatory site' in schizophrenia: D-serine, glycine, and beyond.* Curr Opin Pharmacol, 2015. **20**: p. 109-15.
60. Keshavan, M.S., et al., *Changes in the adolescent brain and the pathophysiology of psychotic disorders.* Lancet Psychiatry, 2014. **1**(7): p. 549-58.
61. Thiebes, S., et al., *Glutamatergic deficit and schizophrenia-like negative symptoms: new evidence from ketamine-induced mismatch negativity alterations in healthy male humans.* J Psychiatry Neurosci, 2017. **42**(4): p. 273-283.
62. Fromer, M., et al., *De novo mutations in schizophrenia implicate synaptic networks.* Nature, 2014. **506**(7487): p. 179-84.
63. Kirov, G., et al., *De novo CNV analysis implicates specific abnormalities of postsynaptic signalling complexes in the pathogenesis of schizophrenia.* Mol Psychiatry, 2012. **17**(2): p. 142-53.
64. Schizophrenia Working Group of the Psychiatric Genomics, C., *Biological insights from 108 schizophrenia-associated genetic loci.* Nature, 2014. **511**(7510): p. 421-7.
65. Pocklington, A.J., et al., *Novel Findings from CNVs Implicate Inhibitory and Excitatory Signaling Complexes in Schizophrenia.* Neuron, 2015. **86**(5): p. 1203-14.
66. Pardinas, A.F., et al., *Common schizophrenia alleles are enriched in mutation-intolerant genes and in regions under strong background selection.* Nat Genet, 2018. **50**(3): p. 381-389.
67. Hall, J., et al., *Genetic risk for schizophrenia: convergence on synaptic pathways involved in plasticity.* Biol Psychiatry, 2015. **77**(1): p. 52-8.
68. Soler, J., et al., *Genetic variability in scaffolding proteins and risk for schizophrenia and autism-spectrum disorders: a systematic review.* J Psychiatry Neurosci, 2018. **43**(4): p. 223-244.
69. Ranson, A., et al., *Top-Down Suppression of Sensory Cortex in an NMDAR Hypofunction Model of Psychosis.* Schizophr Bull, 2019. **45**(6): p. 1349-1357.
70. Clifton, N.E., K.L. Thomas, and J. Hall, *The effect of ketamine on the consolidation and extinction of contextual fear memory.* J Psychopharmacol, 2018. **32**(2): p. 156-162.
71. Raposo, G. and W. Stoorvogel, *Extracellular vesicles: exosomes, microvesicles, and friends.* J Cell Biol, 2013. **200**(4): p. 373-83.
72. Karasu, E., et al., *Extracellular Vesicles: Packages Sent With Complement.* Front Immunol, 2018. **9**: p. 721.
73. Woo, J.J., et al., *The complement system in schizophrenia: where are we now and what's next?* Mol Psychiatry, 2019.
74. Mazza, M.G., et al., *Neutrophil-lymphocyte ratio, monocyte-lymphocyte ratio and platelet-lymphocyte ratio in non-affective psychosis: A meta-analysis and systematic review.* World J Biol Psychiatry, 2019: p. 1-13.

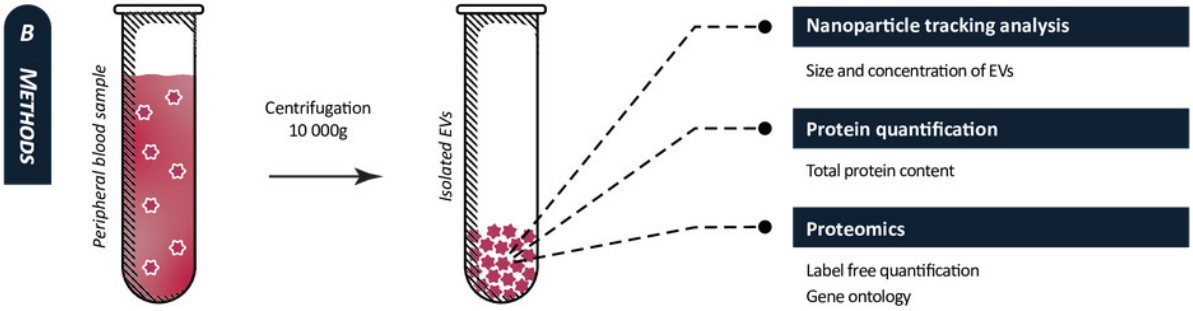
75. Karageorgiou, V., G.P. Milas, and I. Michopoulos, *Neutrophil-to-lymphocyte ratio in schizophrenia: A systematic review and meta-analysis.* Schizophr Res, 2019. **206**: p. 4-12.
76. Slomka, A., et al., *Large Extracellular Vesicles: Have We Found the Holy Grail of Inflammation?* Front Immunol, 2018. **9**: p. 2723.
77. Zealberg, J.J. and K.T. Brady, *Substance abuse and emergency psychiatry.* Psychiatr Clin North Am, 1999. **22**(4): p. 803-17.
78. Guloksuz, S. and J. van Os, *The slow death of the concept of schizophrenia and the painful birth of the psychosis spectrum.* Psychol Med, 2018. **48**(2): p. 229-244.
79. Pries, L.K., et al., *Evidence That Environmental and Familial Risks for Psychosis Additively Impact a Multidimensional Subthreshold Psychosis Syndrome.* Schizophr Bull, 2018. **44**(4): p. 710-719.
80. Castagnini, A.C., P. Munk-Jorgensen, and A. Bertelsen, *Short-term course and outcome of acute and transient psychotic disorders: Differences from other types of psychosis with acute onset.* Int J Soc Psychiatry, 2016. **62**(1): p. 51-6.
81. Mauri, M.C., et al., *Primary psychosis with comorbid drug abuse and drug-induced psychosis: Diagnostic and clinical evolution at follow up.* Asian J Psychiatr, 2017. **29**: p. 117-122.
82. Caton, C.L., et al., *Stability of early-phase primary psychotic disorders with concurrent substance use and substance-induced psychosis.* Br J Psychiatry, 2007. **190**: p. 105-11.
83. Wilson, L., et al., *Clinical characteristics of primary psychotic disorders with concurrent substance abuse and substance-induced psychotic disorders: A systematic review.* Schizophr Res, 2018. **197**: p. 78-86.
84. Wearne, T.A. and J.L. Cornish, *A Comparison of Methamphetamine-Induced Psychosis and Schizophrenia: A Review of Positive, Negative, and Cognitive Symptomatology.* Front Psychiatry, 2018. **9**: p. 491.

Figure 1

Overview of the background, methods, study design and main findings



EVs are secreted by most cells. Surface proteins are distinct and correlated to originating cells. EVs are taken up by recipient cells near by or distant from originating cells. EVs carry proteins, lipids and nucleotides that can change the phenotype of recipient cells.



D MAIN FINDINGS

- Psychotic patients had approximately twice as high concentrations of EVs as healthy controls. EVs were also larger in psychotic patients.
- Brain-specific proteins were identified in EVs, indicating that EVs might serve as a liquid brain biopsy.
- Five brain related proteins - all involved in regulation of glutamatergic synapses - distinguished psychotic patients from healthy controls.
- Gene ontology (GO) analyses confirm EV origin and reveal differences between patients and controls in several GO categories.

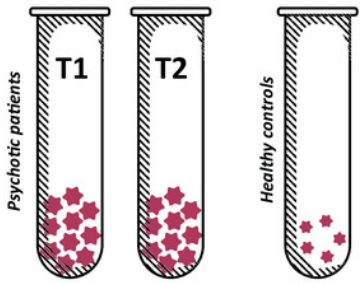


Figure 2

Concentration, size and protein content of EVs

Vesicle concentration (A), vesicle concentration versus size from nano-tracking analysis (B), vesicle diameter (C) and protein concentration (D) of isolated EV fractions for psychotic patients during psychosis (T1) and in improved state (T2) and healthy controls (HC). P-values are given for significant differences. Bar shows mean value with standard deviation error bars.

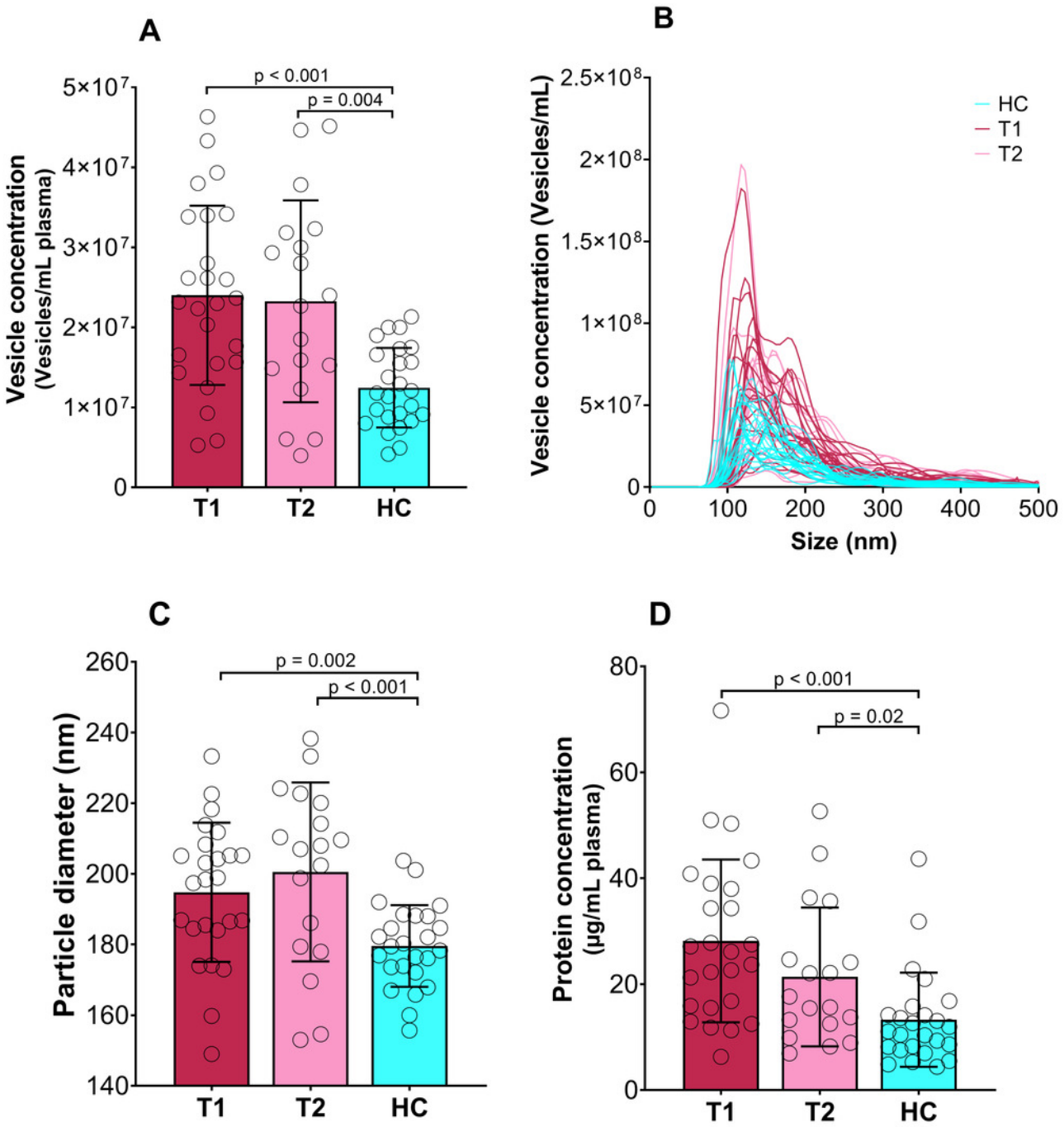

A
p < 0.001
p = 0.004
Vesicle concentration (Vesicles/mL plasma)
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
T1
T2
HC
B
Vesicle concentration (Vesicles/mL)
2.5×10^8
2×10^8
1.5×10^8
1×10^8
5×10^7
0
HC
T1
T2
0
100
200
300
400
500
Size (nm)
C
p = 0.002
p < 0.001
Particle diameter (nm)
260
240
220
200
180
160
140
T1
T2
HC
D
p < 0.001
p = 0.02
Protein concentration (µg/mL plasma)
80
60
40
20
0
T1
T2
HC

Figure 3

Overall proteomic findings

A) Venn diagram showing total number of identified proteins in psychotic patients during psychosis (T1) and in improved state (T2) and healthy controls (HC) and the overlap between sample groups. B) Volcano plot showing the p-value versus the fold change of all proteins for all three groups compared pairwise with each other (Student's t-test with multiple hypothesis correction, lines showing significance threshold (Significant at p < 0.05, Artificial within groups variance s0=0.1)).

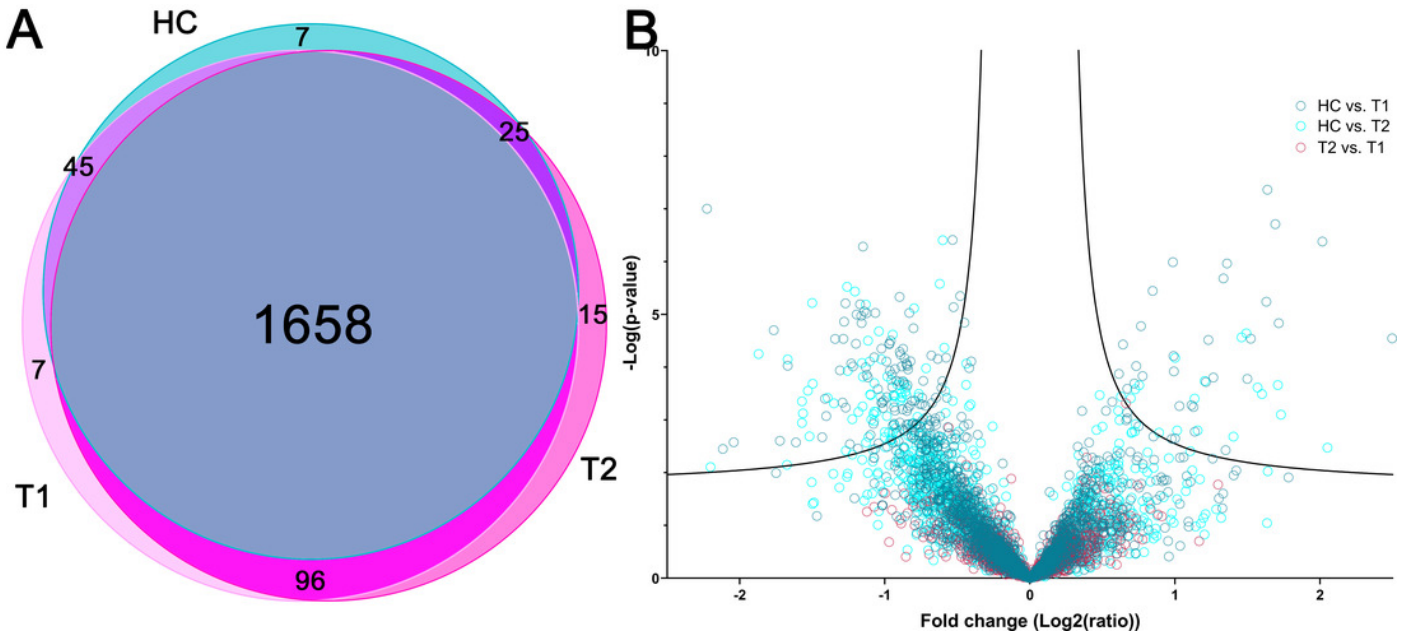


Figure 4

Total amount of brain proteins and significantly changed brain proteins

Scatter plot for normalized (divided by total sum within each sample) average precursor intensity with mean (bar) and standard deviation (error bars) for the five brain-related proteins identified as significantly different between psychotic patients during psychosis (T1) and/or in improved state (T2) and healthy controls (HC). A) Neurogranin (Q92686), B) Neuron-specific calcium-binding protein hippocalcin (HPCA, P84074), C) Kalirin (O60229), D) Beta-adducin (P35612), E) Ankyrin-2 (Q01484) and F) all 55 brain-enriched proteins

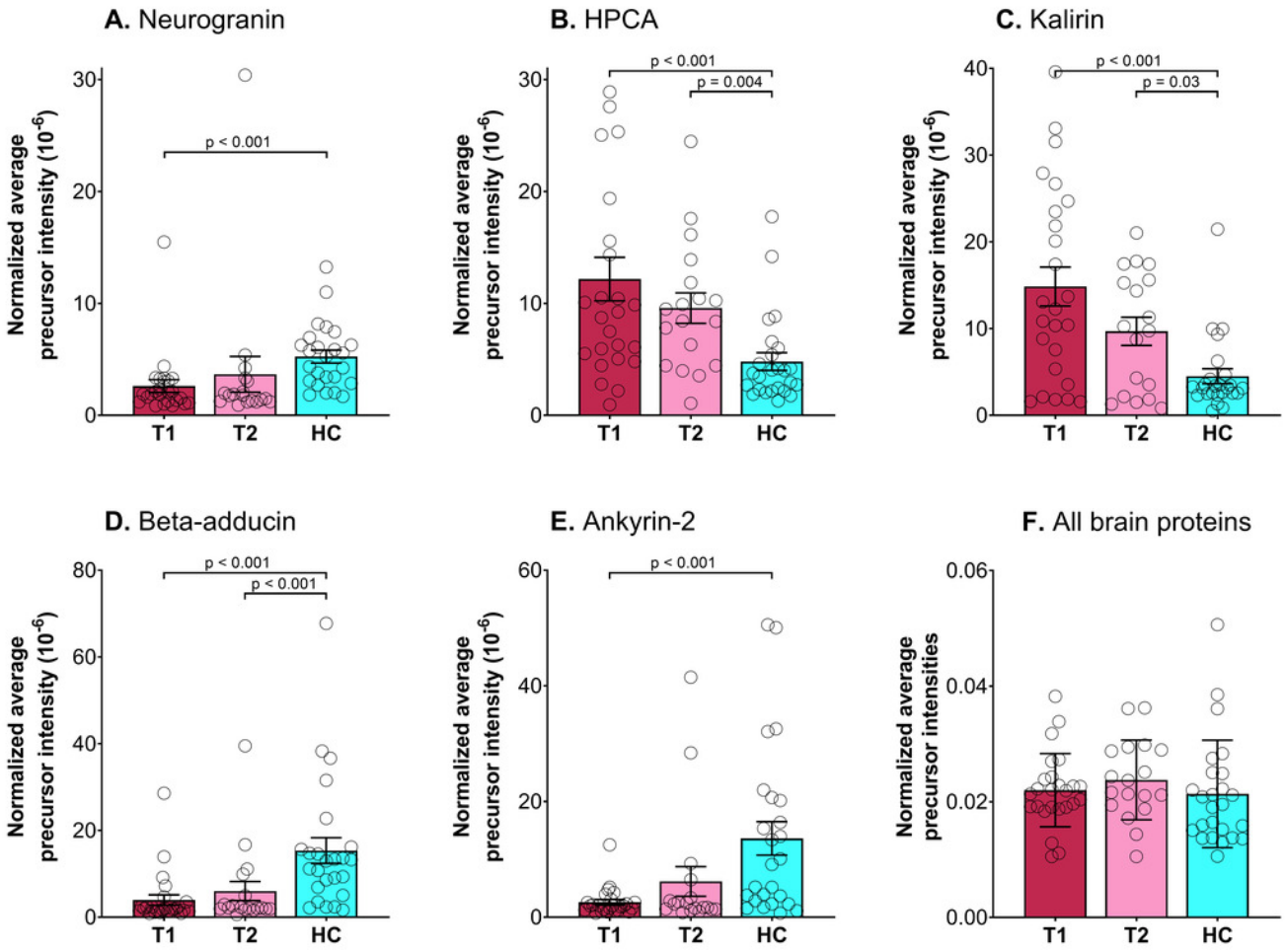


Table 1(on next page)

Main characteristics of EVs

A) Main characteristics of EVs in patients at first sampling point (T1) and controls. Values given as mean (SD). B) Main characteristics in EVs from the 18 patients with complete data at both sampling time points (T1 and T2). Values given as mean (SD).C) Main characteristics of EVs according to time since debut of psychosis at T1. Values given as mean (SD).

Table 1A: T1 vs HC	Psychotic patients (T1, n=25)		Healthy Controls (HC, n=25)		p- value[1]
Diameter of EVs (nm)	195 (20)		180 (12)		0.002[2]
Concentration of EVs (particles/ml plasma)	$2.4x10^7$ ($1,1x10^7$)		$1.2x10^7$ ($5.0x10^6$)		<0.001[2]
Protein content EVs (µg/ml plasma)	28.2(15.4)		13.3(8.9)		<0.001[2]
Table 1B: T1 vs T2	**Acute phase (T1)**	**Improved (T2)**	**Change**	**95% CI**	**p-value[3]**
CGI score, median	7	4			
CGI score, mean	6.5 (0.65)	3.8 (1.23)			
Diameter of EVs (nm)	199 (18)	200 (25)	1	-17 – 13	0.798
Concentration of EVs (particles/ml plasma)	$2.2x10^7$ ($1.0x10^7$)	$2.3x10^7$ ($1.3x10^7$)	$9.5x10^5$	$-8.6x10^6 – 6.7x10^6$	0.796
Protein concentration in EV fraction (µg/ml plasma)	26.8(12.0)	21.4(13.1)	-5.4	-5.1 – 15.9	0.294
Table 1C: T1	**Psychotic patients (T1, n=25)**		**Mean**	**95% CI interval**	**p-value[1]**
Years since debut of psychosis	<1 (n=10)	≥ 1 (n=15)			
Months since debut of first psychosis	4.1(4.8)	102.4(83.7)			
Concentration of EVs (particles/ml plasma)	$2.0x10^7$ ($8.3x10^6$)	$2.7x10^7$($1.2x10^7$)	$-7.4x10^6$	$-1.6x10^7 – 1.7x10^6$	0.106
Diameter of EVs (nm)	193(14)	196(23)	-3	-20 – 13	0.678
Protein concentration in EV fraction (µg/ml plasma)	22.4(8.5)	32.0(17.9)	9.6	-22.2 – 3.0	0.130

[1]Two sample t-test. [2]Equal variance not assumed. [3]Paired t-test.

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Table 2(on next page)

Significantly changed brain proteins

Overview of the five brain-specific proteins identified as different between healthy controls (HC) and patients with psychosis (T1) and in improved state (T2). The table shows accession number in Uniprot, molecular weight (MW), number of validated peptides and spectra across all samples, and Average precursor intensity within each group given as mean ± standard deviation (number of samples where the protein is identified).

Uniprot Accession	Protein name	MW (kDa)	#Validated Peptides	#Validated Spectra	Average precursor intensity (Mean ± SD (n))		
					HC	T1	T2
Q92686	Neurogranin	7,6	2	76	146020 ± 80464 (20)	130421 ± 153699 (9)	195775 ± 403355 (10)
P84074	Neuron-specific calcium-binding protein hippocalcin	22,4	4	80	188914 ± 114734 (14)	281593 ± 145068 (21)	263648 ± 145611 (16)
O60229	Kalirin	340,0	11	106	229014 ± 142100 (9)	337939 ± 176321 (19)	330904 ± 115006 (11)
P35612	Beta-adducin	80,8	13	99	305652 ± 169514 (20)	165875 ± 63467 (3)	309466 ± 201639 (5)
Q01484	Ankyrin-2	433,4	6	53	295801 ± 111670 (16)	122702 ± 9911 (2)	556426 ± 745193 (4)

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