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Plasma Exosomes Disrupt the Blood–Brain Barrier in Children with Obstructive Sleep Apnea and Neurocognitive Deficits

To the Editor:

Obstructive sleep apnea (OSA) is a prevalent condition in children and is associated with a significant constellation of morbidities, including neurocognitive, cardiovascular, and metabolic dysfunction (1–3). Activation and propagation of multiple inflammatory pathways, altered lipid metabolism, and oxidative stress mechanisms have all been implicated in end-organ morbidity (4, 5). In two recent papers, Lim and Pack and our group have proposed that disruption of the blood–brain barrier (BBB) may underlie the cognitive impairments associated with OSA (6, 7). As corroborative evidence, studies in mice exposed to intermittent hypoxia have shown increases in brain parenchymal water, along with alterations in aquaporin expression, indicating increased BBB permeability (8, 9). BBB permeability changes have also been inferred in adult patients with OSA (10). In this setting, it has been reported that endothelial cells secrete exosomes, and several reports show that endothelial cells can also be targeted by exosomes derived from different cell types. The tight junction complex is critically involved in the exchange of ions, solutes, and cells that travel across BBB paracellular spaces and tight junctions. Zonula occludens-1 (ZO-1) is one of several protein families that are essential for tight junction formation (11), and it is now established that stressful conditions can disrupt brain endothelial tight junctions and affect cognition via exosome-related biological activities affecting the BBB (12).

To examine the potential contribution of circulating exosomes to BBB disruption in the context of pediatric OSA, we explored, using an *in vitro* BBB system (13), the effect of plasma-derived exosomes from children with polysomnographically determined OSA with evidence of neurocognitive deficits (NC^+; $n = 12$); age-, sex-, ethnicity-, body mass index *z*-score–, apnea–hypopnea index–matched children with OSA and no evidence of cognitive deficits (NC^-; $n = 12$); and control children without OSA or cognitive deficits (CO; $n = 6$). The characteristics of the subjects are shown in Table 1. All subjects underwent overnight polysomnography, which was scored as per current American Academy of Sleep Medicine guidelines, and in the morning after the sleep study, fasting blood was drawn into ethylenediaminetetraacetic acid tubes, and plasma was immediately separated by centrifugation and stored until assay, immediately followed by cognitive test batteries (1). The presence of cognitive deficits (NC^+) was defined as the presence of two or more cluster subtests that were more than 1 SD below the mean, as previously described (14). Plasma exosomes were isolated and purified as previously described (2) and fulfilled all the required criteria as specified by the current consensus of the International Society for Extracellular Vesicles (15).

Using an immortalized human brain microvascular endothelial cell model (hCMEC/D3; Cat# SCC066, EMD Millipore), the effects of equivalent numbers of exosomes from each subject on transcellular electrical impedance of a hCMEC/D3 monolayer were evaluated by electric cell-substrate impedance-sensing arrays. As previously described (2), exosomes were added in duplicate wells and changes in impedance across the hCMEC/D3 monolayer were continuously monitored in the electric cell-substrate impedance sensing instrument (Applied Biophysics Inc.) for up to 48 hours. Appropriate internalization of the exosomes by human brain microvascular endothelial cells was verified in a preliminary set of experiments using time-lapse confocal microscopy. Of note, the resistance across the hCMEC/D3 monolayer at confluence was measured at more than 800 $\Omega \cdot cm^2$ (13). In addition, immunofluorescence staining of confluent hCMEC/D3 endothelial cell monolayers that were grown on 12-well cover slips for 24 hours in Dulbecco's modified Eagle medium containing 10% fetal bovine serum were also performed. Isolated exosomes from subjects were added individually to cover slips for 24 hours. Cells were fixed with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature and then washed again with PBS. The cell membranes were permeabilized by incubation with 0.25% (vol/vol) Triton-X-100 in PBS for 10 minutes at room temperature. After washing with PBS, the samples were blocked with 3% (wt/vol) bovine serum albumin in PBS for 45 minutes at room temperature, followed by overnight incubation at 4°C with ZO-1 antibody (1:400; Life Technologies). Alexa 488 was used as secondary antibody (1:400; Life Technologies), and nuclear staining

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Author Contributions: A.K. performed experiments, analyzed data, and drafted components of the manuscript; D.G. participated in the conceptual framework of the project, provided critical input in all phases of the experiments, analyzed data, and edited versions of the manuscript; L.K.-G. provided the conceptual framework of the project, analyzed data, drafted components, and finalized the manuscript and is responsible for the financial support of the project and the manuscript content; and all authors have reviewed and approved the final version of the manuscript.

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Table 1. Demographic and Polysomnographic Findings among Children with OSA with and without Cognitive Deficits and Control Subjects

	OSA–NC^+ ($n = 12$)	OSA–NC^- ($n = 12$)	CO ($n = 6$)
Age, yr	6.5 ± 1.4	6.3 ± 1.2	6.3 ± 1.7
Sex, male, %	50.0	50.0	50.0
Ethnicity, African American, %	66.7	66.7	66.7
Body mass index *z*-score	1.04 ± 0.26	1.06 ± 0.25	1.02 ± 0.29
Total sleep duration, min	478.8 ± 67.1	474.2 ± 66.3	473.2 ± 75.1
Stage 1, %	7.0 ± 3.8	7.2 ± 4.1	4.8 ± 3.7
Stage 2, %	40.1 ± 8.8	38.6 ± 8.9	35.4 ± 9.5
Stage 3, %	35.5 ± 12.8	37.1 ± 11.4	41.6 ± 14.2
REM sleep, %	18.2 ± 8.1	17.9 ± 9.5	20.2 ± 10.2
Sleep latency, min	22.4 ± 15.6	20.7 ± 13.9	24.6 ± 15.2
REM latency, min	117.7 ± 49.5	119.5 ± 52.4	118.7 ± 67.4
Total arousal index, events/h of TST	22.8 ± 11.4	20.9 ± 10.3	12.5 ± 8.6*
Respiratory arousal index, events/h of TST	7.9 ± 4.2	8.2 ± 4.7	0.4 ± 0.2*
Obstructive apnea–hypopnea index, events/h of TST	19.9 ± 7.1	19.4 ± 6.9	0.5 ± 0.3*
Sp_{O_2} nadir, %	80.3 ± 8.6	82.1 ± 9.0	94.1 ± 1.3*
ODI3%	18.4 ± 7.7	19.0 ± 8.4	0.4 ± 0.2*
NEPSY cognitive test battery			
Design copying	9.07 ± 3.89 (2)	9.69 ± 3.97 (0)	9.88 ± 4.22
Phonological processing	8.26 ± 3.75 (8)	9.16 ± 3.94 (2)	9.66 ± 4.17
Tower	9.48 ± 3.92 (7)	11.01 ± 3.76 (0)	11.16 ± 4.56
Speed naming	8.44 ± 3.65 (8)	9.22 ± 3.83 (1)	9.96 ± 4.58
Arrows	8.37 ± 3.87 (5)	10.22 ± 3.67 (0)	11.66 ± 3.84
Visual attention	10.16 ± 3.53 (1)	10.47 ± 3.89 (0)	10.76 ± 3.87
Comprehension	9.43 ± 3.78 (3)	10.72 ± 3.66 (0)	10.96 ± 4.04
Differential Ability Scales			
Verbal	92.18 ± 14.88 (9)	97.88 ± 15.61 (1)	100.25 ± 17.37
Nonverbal	95.63 ± 12.88 (5)	100.08 ± 15.12 (1)	103.55 ± 17.31
Global	93.91 ± 11.93 (8)	99.23 ± 15.32 (1)	102.82 ± 16.14

Definition of abbreviations: CO = control subjects; NC^+ = with neurocognitive deficits; NC^- = without neurocognitive deficits; NEPSY = Developmental Neuropsychological Assessment; ODI3% = oxyhemoglobin desaturation index 3%; OSA = obstructive sleep apnea; Sp_{O_2} = oxygen saturation as measured by pulse oximetry; TST = total sleep time.

All data are expressed as mean ± SD unless otherwise indicated. For NEPSY cognitive tests and Differential Ability Scales, the number of children who had test performance at least 1 SD below the mean is shown in parentheses.

*OSA versus CO ($P < 0.001$).

with DAPI (1:1000; Life Technologies) was performed. Appropriate controls and preadsorption experiments were performed to ascertain the specificity of the staining. Images were captured with a Leica SP5 Tandem Scanner Spectral 2-photon confocal microscope (Leica Microsystems, Inc.) with a 63× oil-immersion lens. For quantitative data comparisons, unpaired *t* tests were applied and a *P* value less than 0.05 was considered as statistically significant.

Plasma-derived exosomes from both OSA groups elicited significant declines in BBB transendothelial impedance compared with CO (Figure 1A; $P < 0.001$). Furthermore, the declines in impedance induced by NC^+ exosomes were significantly larger than those of NC^- ($P < 0.01$). In addition, ZO-1 immunostaining revealed significant and consistent disruption continuity of this tight junction protein in hCMEC/D3 cells treated with exosomes from NC^+, but not when exosomes from the other 2 groups were added (Figure 1B).

Current findings show for the first time that circulating exosomes in children with OSA are capable of disrupting the integrity of the BBB, as illustrated by reduced impedance across the BBB, as well as increased discontinuity of ZO-1 along the cell membrane. Furthermore, the adverse effects of plasma exosomes on the BBB are accentuated in children with OSA who also manifest evidence of cognitive deficits. Although these studies are clearly descriptive in nature, and did not identify which elements of the exosome cargo underlie the functionally deleterious effects on the BBB, we postulate that differentially expressed cargo elements, such as microRNAs (2, 16), may play a mechanistic role in the emergence of such neurocognitive deficits by disrupting the BBB and by inducing the activation and propagation of pathophysiological cascades that ultimately foster astroglial and microglia inflammation and proliferation, increased reactive oxygen species formation, and ultimately increased neuronal cell losses, particularly in vulnerable brain regions (17). ■

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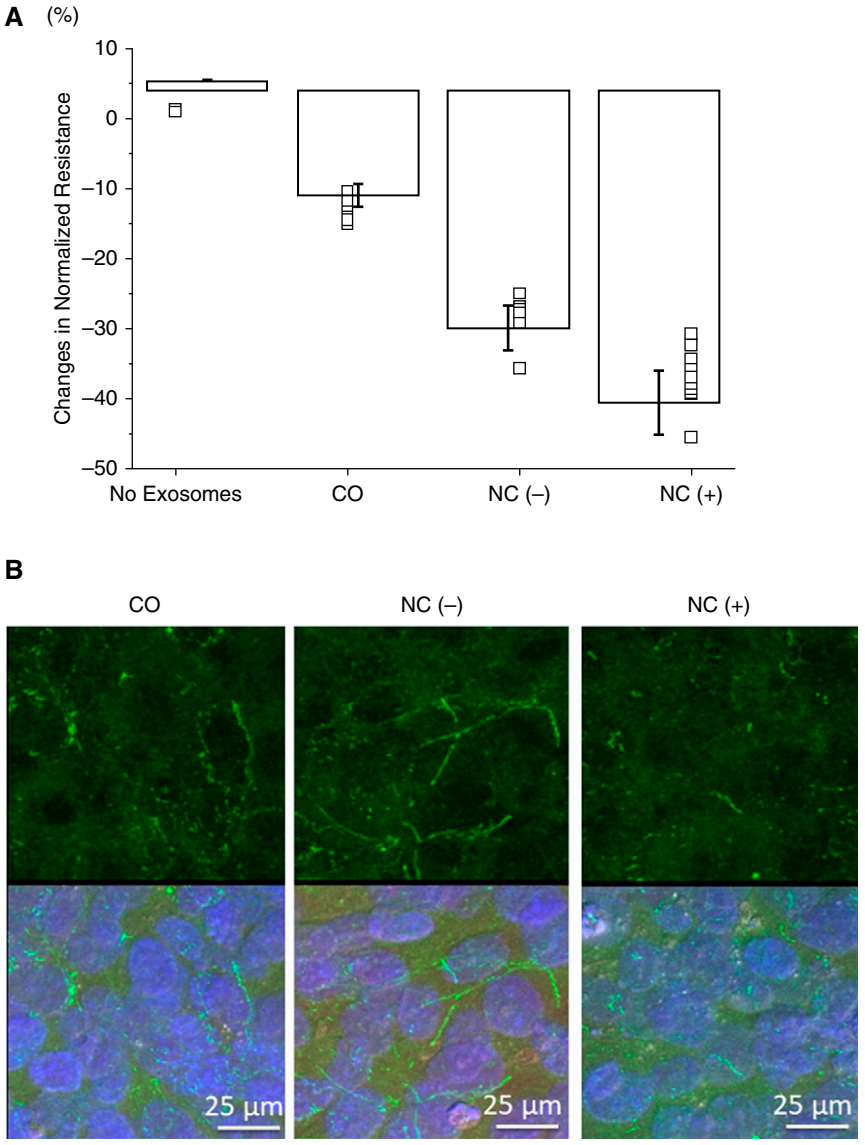

Figure 1. (*A*) Changes in human brain microvascular endothelial cell model/D3 monolayer cell impedance after *in vitro* administration of plasma exosomes from children with obstructive sleep apnea with (NC^+; $n = 12$), and without (NC^-; $n = 12$) neurocognitive deficits and control subjects (CO; $n = 6$). Data are shown as mean ± SD. (*B*) Representative confocal microscope images ($n = 6$/group) of ZO-1 (zonula occludens-1) immunoreactivity (green) in human brain microvascular endothelial cell model/D3 cells treated with exosomes from CO, NC^-, and NC^+ subjects for 24 hours. Cells were also stained with DAPI (blue). The upper panel shows ZO-1 staining alone; the lower panel shows ZO-1 and DAPI staining together. The continuity of ZO-1 in NC^- and CO is apparent but was always absent in NC^+ cells.

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Early Identification of Bronchopulmonary Dysplasia Using Novel Biomarkers by Proteomic Screening

To the Editor:

Bronchopulmonary dysplasia (BPD) concerns up to 77% of all preterm infants and is notable for its significant long-term sequelae. Defined by the need for oxygen supplementation or ventilator support at term, early and quantifiable disease markers still remain elusive.

Our aim was therefore to identify and validate early plasma markers indicating BPD development with high sensitivity by the use of comprehensive protein screening.

Patients and Methods

Study population. Thirty-five preterm infants with informed parental consent and a gestational age below 32 weeks were prospectively included in the study (Table 1): exploration cohort, Perinatal Center of the Ludwig-Maximilians-University, Campus Grosshadern ($n = 18$; EC #195-07); independent confirmation cohort, Perinatal Center of the University Hospital Giessen ($n = 17$; EC #135/12). Mild, moderate, or severe BPD was diagnosed at 36 weeks (1), together with the days of invasive and noninvasive mechanical ventilation and oxygen treatment. Sepsis was defined by presence of both clinical and laboratory findings (temperature instability, metabolic acidosis, cardiorespiratory instability, hyperglycemia, capillary refill time >2 s, c-reactive protein, IL-6, total white cell count, and immature to total neutrophil ratio). Some cases were confirmed by pathogen detection from blood or cerebrospinal fluid. Chorioamnionitis was confirmed by placental histology (50%) or maternal/fetal signs of infection at birth.

Table 1. Patient Characteristics

	Exploration Cohort *(n = 18)*	Confirmation Cohort *(n = 17)*
Gestational age, wk PMA	26.2 (24.3–28.2)	26.2 (24.4–29.6)
Birth weight, g*	755 (510–1040	840 (340–1470)
IUGR*	4 (22.2%)	1 (5.9%)
Sex, female/male	11/7	12/5
pH, umbilical artery	7.33 (6.95–7.47)	7.35 (7.01–7.48)
ANCS	17 (94.0%)	14 (82.4%)
Chorioamnionitis (11)	13 (72.0%)	12 (70.6%)
Clinical sepsis (12, 13)	6 (33.3%)	4 (23.5%)
RDS ⩾grade 3 (14)	9 (50.0%)	4 (23.5%)
Days of mechanical ventilation*	54 (33–78)	24 (2–74)
Endotracheal mechanical ventilation, *n*/d	6 (1–41)	2 (0–32)
Pharyngeal ventilation/CPAP, *n*/d	40.5 (30–55)	20 (2–52)
PDA	10 (55.6%)	12 (70.6%)
Postnatal corticosteroids*	11 (61.1%)	1 (5.9%)
ROP ⩾grade 3*	0 (0.0%)	5 (29.4%)
IVH ⩾grade 3*	0 (0.0%)	2 (11.8%)
ICU stay, d*	78.5 (57–110)	38.0 (5–93)
BPD (1)		
None	4 (22.2%)	3 (17.6%)
Mild	8 (44.4%)	9 (52.9%)
Moderate	2 (11.1%)	1 (5.9%)
Severe	4 (22.2%)	4 (23.5%)

Definition of abbreviations: ANCS = antenatal corticosteroids (two doses of betamethasone >24 hours before and no later than 7 d before birth); BPD = bronchopulmonary dysplasia; CPAP = continuous positive airway pressure; IUGR = intrauterine growth retardation (birth weight <10th percentile); IVH = intraventricular hemorrhage; PDA = patent ductus arteriosus; PMA = postmenstrual age; RDS = respiratory distress syndrome; ROP = retinopathy of prematurity.
Data are given as median (range) or number (percentage of total in group).
*$P < 0.05$.

Biomarker analysis. Serial plasma samples generated from a whole-blood ethylenediaminetetraacetic acid specimen obtained in the first week of life (Days 0–4, $n = 16$; Days 5–7, $n = 16$) and at Day 28 ($n = 14$) were subjected to proteomic screening (SOMAscan; SomaLogic). Protein binding to 1,129 individual high-affinity molecules was quantified by custom Agilent hybridization array (2, 3) with high reproducibility even in low-amount samples smaller than 100 μl. Confirmation of protein expression in ELISA technique (SIGLEC-14 [sialic acid-binding Ig-like lectin 14], R&D Systems; BCAM [basal cell adhesion molecule], Thermo Fisher Scientific; ANGPTL3 [angiopoietin-like 3 protein], Raybiotech) used one to two samples from the first week of life.

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