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Blood–brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders

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Abstract

Background: Autism spectrum disorders (ASD) are complex conditions whose pathogenesis may be attributed to gene–environment interactions. There are no definitive mechanisms explaining how environmental triggers can lead to ASD although the involvement of inflammation and immunity has been suggested. Inappropriate antigen trafficking through an impaired intestinal barrier, followed by passage of these antigens or immune-activated complexes through a permissive blood–brain barrier (BBB), can be part of the chain of events leading to these disorders. Our goal was to investigate whether an altered BBB and gut permeability is part of the pathophysiology of ASD.

Methods: *Postmortem* cerebral cortex and cerebellum tissues from ASD, schizophrenia (SCZ), and healthy subjects (HC) and duodenal biopsies from ASD and HC were analyzed for gene and protein expression profiles. Tight junctions and other key molecules associated with the neurovascular unit integrity and function and neuroinflammation were investigated.

Results: Claudin (*CLDN*)-5 and -12 were increased in the ASD cortex and cerebellum. *CLDN*-3, *tricellulin*, and *MMP*-9 were higher in the ASD cortex. *IL*-8, *tPA*, and *IBA*-1 were downregulated in SCZ cortex; *IL*-1*b* was increased in the SCZ cerebellum. Differences between SCZ and ASD were observed for most of the genes analyzed in both brain areas. *CLDN*-5 protein was increased in ASD cortex and cerebellum, while *CLDN*-12 appeared reduced in both ASD and SCZ cortices. In the intestine, 75% of the ASD samples analyzed had reduced expression of barrier-forming TJ components (*CLDN*-1, *OCLN*, *TRIC*), whereas 66% had increased pore-forming *CLDN*s (*CLDN*-2, -10, -15) compared to controls.

Conclusions: In the ASD brain, there is an altered expression of genes associated with BBB integrity coupled with increased neuroinflammation and possibly impaired gut barrier integrity. While these findings seem to be specific for ASD, the possibility of more distinct SCZ subgroups should be explored with additional studies.

Keywords: Blood–brain barrier, Autism spectrum disorders, Gut–brain axis, Gut permeability, Schizophrenia, Neuroinflammation, Postmortem brain, Duodenal biopsies

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Background

Autism spectrum disorders (ASD) are neurodevelopmental diseases with complex symptoms and whose neurobiological basis remains poorly understood. Research suggests that a combination of genetic, autoimmune, environmental, and perhaps in utero risk factors leading to neuroinflammation can contribute to the pathogenesis of ASD [1–6] as well as other neurobehavioral and/or neuropsychiatric disorders including schizophrenia (SCZ) [7–13]. Over-expression of acute phase proteins in the serum, brain, and cerebrospinal fluid of ASD as well as SCZ individuals suggests that inflammation is involved in the pathophysiology of these diseases [4, 6, 14–21]. Autoimmune diseases have been associated with increased risk of ASD and SCZ [10, 22–28] suggesting altered or inappropriate immune responses.

Furthermore, many ASD patients experience gastrointestinal (GI) symptoms and/or dysfunctions [29–38]. Clinical observations describe increased intestinal permeability in ASD [39–42], and permeability to food antigens derived from the partial digestion of wheat (gliadorphins) and cow's milk (casomorphins) has been reported in both ASD and SCZ [41, 43–46].

Despite research efforts, there are no defined explanations of how environmental triggers can lead to these neurobehavioral conditions. One hypothesis, based on the interconnectivity of the gut–brain axis, suggests that inappropriate antigen trafficking through an impaired intestinal barrier, followed by passage of antigens or activated immune complexes through a permissive blood–brain barrier (BBB), can be part of the chain of events leading to neuroinflammation and thereby subsequent disease.

Table 1 List of primers used for RT-qPCR

Gene	Accession number	5' OLIGO	3' OLIGO	Gene function in the brain/gut context
<i>CLDN-1</i>	NM_021101	GTGATAGCAATCTTTGTGGC	ACTAAAATAGCCAGACCTGC	Barrier-forming claudin
<i>CLDN-5</i>	NM_001130861	GAACCTCCTGAAGTGGTGTC	CCAGACCTCTCAATCTTCAC	Barrier-forming claudin; endothelial cell-specific
<i>CLDN-12</i>	NM_001185072	GAGAAGCAGGCTCAGATTAT	AGATTCAGAACTTCCTGTG	Function at BBB unknown
<i>OCLN</i>	U53823	CACACCACCTACACTC	TCCAAGATAACCAATCTGCT	Barrier-forming component of the TJs
<i>VE-Cad</i>	X79981	AGTTCCTCCGAGTCACAAAA	TCAGGTTATACCAGGGGTAG	Main integral membrane protein of endothelial AJs; controls endothelial cell survival, stabilization of blood vessel assembly, and vascular permeability
<i>MMP-9</i>	NM_004994	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT	Metalloprotease involved in local proteolysis of the extracellular matrix and in leukocyte migration
<i>MMP-2</i>	NM_004530	TACAGGATCATTGGCTACACACC	GGTCACATCGCTCCAGACT	Metalloprotease involved in local proteolysis of the extracellular matrix and in leukocyte migration
<i>tPA</i>	NM_001319189	CCGGCTACGGCAAGCA	AGCGGCTGGATGGGTACA	Serine protease involved in the synthesis of MMPs and BBB damage
<i>18S</i>	X03205	AGAAACGGCTACCACATCCA	CCCTCCAATGGATCCTCGTT	Ribosomal RNA (control gene for qPCR)
<i>CLDN-2</i>	NM_001171092		QIAGEN	Pore-forming claudin; regulates paracellular ion and water flow
<i>CLDN-3</i>	NM_001306		QIAGEN	Barrier-forming component of the TJs
<i>CLDN-10</i>	NM_001160100		QIAGEN	Pore-forming claudin; regulates paracellular ion flow
<i>CLDN-15</i>	NM_001185080 NM_014343 NM_138429		QIAGEN	Pore-forming claudin; regulates paracellular ion flow
<i>TRIC</i>	NM_001038603		QIAGEN	Barrier-forming component of the TJs
<i>IL-1b</i>	NM_000576		QIAGEN	Pro-inflammatory cytokine involved in increased BBB permeability
<i>IL-6</i>	NM_000600		QIAGEN	Pro-inflammatory cytokine
<i>IL-8</i>	NM_000584		QIAGEN	Pro-inflammatory cytokine
<i>IL-10</i>	NM_000572		QIAGEN	Anti-inflammatory cytokine
<i>TSPO</i>	NM_000714		QIAGEN	Mitochondrial protein expressed on reactive glial cells; biomarker for inflammation in the brain
<i>tTG6</i>	NM_001254734		QIAGEN	Marker of gluten-related neuroinflammation
<i>MSFD2A</i>	NM_001136493		QIAGEN	Key regulator of BBB function; required for the establishment of a functional BBB
<i>IBA-1</i>	NM_001623		QIAGEN	Marker of microglia activation

Table 2 Demographic and clinical characteristics of subjects analyzed in this study

						Cortex and CBL	
MBC #	Age	Race	Sex	Diagnosis	PMI	Cause of death	Additional clinical information
585	42	AA	M	SCZ	16	Pulmonary embolism	Tox (at death): doxepin
612	45	W	M	SCZ	24	Suicide, doxepin intoxication	N/A
628	59	W	M	SCZ	13	Pulmonary thromboembolia	Tox (at death): ETOH, diphenhydramine
664	53	W	M	SCZ	11	ASCVD	Tox (at death): olanzapine
705	47	W	M	SCZ	16	Deep vein thrombosis	N/A
737	55	W	M	SCZ	12	ASCVD	N/A
741	40	W	M	SCZ	14	Motor vehicle accident	Tox (at death): cocaine
751	28	W	M	SCZ	34	Suicide, GSW to chest	Tox (at death): olanzapine
783	53	W	M	SCZ	19	Manner undetermined	N/A
831	53	W	M	SCZ	14	ASCVD	N/A
4334	11	H	M	ASD	27	Acute hemorrhagic tracheobronchitis	History of epilepsy. Brain edema, acute, mild. Otherwise normal brain
4999	20	W	M	ASD	14	Cardiac arrhythmia	PDD, autism, and severe mental retardation. Naltrexone
5027	37	AA	M	ASD	26	Obstruction of bowel due to adhesion	Risperdal and Luvox
5115	46	W	M	ASD	29	Complications of pseudomyxoma peritonei	Adult brain with moderate atherosclerosis
5144	7	W	M	ASD	3	Cancer, complication of	Rhabdomyosarcoma
5176	22	AA	M	ASD	18	Subdural hemorrhage	Risperdal
5308	4	W	M	ASD	21	Skull fractures	Struck by a car. Multifocal subarachnoid hemorrhage
5403	16	W	M	ASD	35	Cardiac arrhythmia	Developmental delays. GI evaluations
1464	42	W	M	Control	19	Pulmonary embolism	Chest and abdominal pain, pale, and diaphoretic
1829	25	W	M	Control	21	Electrocution	N/A
1831	44	W	M	Control	17	CO ₂ inhalation complicating ASCVD	History of hypertension. Complained of headaches
1936	46	W	M	Control	13	ASCVD	History of HBP. Had been experiencing chest pain
5024	56	W	M	Control	10	ASCVD; diabetes mellitus	Smoker, no drinking of ETOH. History of prescription drug abuse. On insulin, metformin, oxycodone, Levaquin, and Lyrica
5185	21	W	M	Control	26	Car accident	Drink ETOH prior to death
5189	40	W	M	Control	14	Cardiac arrhythmia complicated by drowning	Severe stomach pains, chest palpitations, and nausea
5276	22	W	M	Control	18	Heroin intoxication	Asthma and drug abuse; smoker
5237	52	W	M	Control	13	HASCVD	ETOH and cocaine abuse. Clean for 30 days and not feeling well
5349	24	W	M	Control	16	Combined drug intoxication	Drug use
5553	54	W	M	Control	17	Hypertensive atherosclerosis heart disease	History of HTN and daily ETOH intake
5615	50	W	M	Control	19	N/A	N/A
4337	8	AA	M	Control	16	Blunt force neck injury. Car accident	Asthma. Brain petechiae, fresh
4670	4	W	M	Control	17	Comotio cordis (struck by a ball in the sternum)	N/A
5334	12	H	M	Control	15	Hanging/suicide	N/A
						Duodenum (biopsies)	
Study #	Age	Race	Sex	Diagnosis		GI symptoms	Additional clinical information
GBA #2	8	W	M	ASD		Non-apparent	Food allergies, GFCF diet, probiotics
GBA #3	11	W	M	ASD		GERD, vomiting, bloating	Asperger's. No milk diet
GBA #6	19	W	M	ASD		Regurgitation after each meal, constipation	History of aggressive behaviors

Table 2 Demographic and clinical characteristics of subjects analyzed in this study (*Continued*)

GBA #7	21	W	F	ASD	GERD, erosive esophagitis, IBS, severe constipation, occasional diarrhea	GFCF diet
GBA #10	9	W	M	ASD	EoE, GERD, constipation, vomiting	Asthma, somewhat loose stools. Dairy-free diet
GBA #12	6	AA	M	ASD	Non-apparent	Eats only pureed food
GBA #13	5	AA	M	ASD	Severe constipation, GERD, abdominal pain, runny stools	Asthma, food allergies
GBA #14	3	W	M	ASD	Chronic constipation	History of obesity, restricted diet (yogurt, milk, juice)
GBA #15	11	W	F	ASD	GERD, constipation	Macrocephaly, sleep disorder
GBA #16	6	W	M	ASD	Gastroesophageal reflux, constipation	Food and seasonal allergies, asthma
GBA #18	16	W	F	ASD	Constipation, abdominal pain, heartburn, regurgitation	Acute mono, weight loss
GBA #20	6	AA	F	ASD	GERD with esophagitis, constipation	ADHD, asthma. Vegetarian
GBA #5	10	HL	M	HC	Abdominal pain, nausea, vomiting, sometimes diarrhea	Lactose-free diet
GBA #8	16	W	F	HC	Constipation, abdominal pain, burping, bloating	GFCG diet, some FODMAP diet
GBA #9	16	W	F	HC	Dysphagia, abdominal pain, regurgitation	
GBA #11	15	W	F	HC	Abdominal pain, GERD	Seasonal allergies, lactose intolerance, dairy-free and nut-free diet, ADHD, obesity
GBA #17	21	W	F	HC	Nausea, vomiting, upset stomach, GERD. Abdominal pain	Previous duodenitis and gastritis, hepatic adenoma
GBA #19	6.5	N/A	M	HC	GERD, constipation	Hydronephrosis, nephrocalcinosis, clubfoot, seasonal allergies, asthma
GBA #21	15	W	M	HC	Dysphagia, esophagitis	Asthma
GBA #23	15	W	M	HC	Abdominal pain, IBS-like constipation	N/A
GBA #24	4	W	M	HC	Reflux	History of constipation. Dairy-free diet

W white, AA African-American, HL Hispanic-Latino, M male, F female, HC healthy control, ASD autism spectrum disorder, GBA gut-brain axis study; GERD gastroesophageal reflux disease, GFCF gluten-free casein-free, PMI postmortem interval, ADD/ADHD attention-deficit/hyperactivity disorder, ASCVD atherosclerotic cardiovascular disease, HASCVD hypertensive arteriosclerotic cardiovascular disease, PDD/PDD-NOS pervasive developmental disorder/not otherwise specified, OCD obsessive-compulsive disorder

The BBB plays a critical role in the central nervous system (CNS) defense through limiting the access of circulating solutes, macromolecules, and cells that could negatively impact neuronal activity. Dysfunctions of the BBB have been associated with numerous neurological disorders, such as stroke, epilepsy, multiple sclerosis, and Parkinson's and Alzheimer's disease [47–56].

The overall goal of this study was to assess whether a dysfunctional BBB or gut barrier could contribute pathophysiologically to ASD. To address this, we conducted an in-depth molecular analysis of the components associated with the BBB and gut barrier integrity in postmortem brain tissue and small intestinal biopsies obtained from ASD subjects. In addition, we assessed changes in the BBB integrity of patients with SCZ as SCZ is a psychiatric illness in which the involvement of inflammation, immunity and altered BBB integrity have been postulated [22, 23, 57, 58].

Methods

Postmortem tissues

Human frozen postmortem brain tissue blocks (1 to 2 cm³) from the frontal cortex (Brodmann's area 45) and

cerebellum (CBL) of 15 HC and eight ASD subjects were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA, which is a Brain and Tissue Repository of the NIH NeuroBioBank. Additionally, the Maryland Brain Collection of the Maryland Psychiatric Research Center at the University of Maryland School of Medicine, Baltimore, USA, provided ten SCZ samples from each of the two aforementioned brain areas of interest.

Duodenal biopsies

Biopsy specimens were taken from the second portion of the duodenum from ASD ($n = 12$) and control ($n = 9$) patients with GI symptoms undergoing esophagogastroduodenoscopy (EGD) for clinically indicated reasons. All study subjects provided written informed consent for the collection of additional biopsies, for the purpose of this study, during the procedure. This study was approved by the Partners Human Research Committee (PHRC) at Massachusetts General Hospital and performed in accordance with the ethical standards and regulatory requirements set forth by the Declaration of Helsinki.

Gene expression profile analysis

Total RNA was isolated from samples (~100 mg) using Trizol (Life Technologies, Carlsbad, CA) and/or Direct-zol RNA miniprep spin columns (Zymo Research, Irvine, CA) following the manufacturers' instructions. RNA concentrations and A260/A280 and A260/A230 ratios were measured with the NanoDrop spectrophotometer (Thermo Scientific) before 2 µg total RNA were reverse transcribed using random hexamer primers and Maxima universal first strand cDNA synthesis kit #1661 (Thermo Fisher Scientific, Waltham, MA). Real-time quantitative PCR (qPCR) was used to measure gene expression levels and was performed in an iCycler96X detection system (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. In brain tissues, we analyzed the expression level of genes associated with the formation, integrity, and function of the BBB and neuroinflammation, as follows: Claudin(*CLDN*)-1, *CLDN*-3, *CLDN*-5, and *CLDN*-12; occludin (*OCLN*); tricellulin (*TRIC*); vascular endothelial cadherin (*VE-Cad*); interleukin (*IL*)-1b, *IL*-6, *IL*-8, and *IL*-10; the microglia activation marker ionized calcium-binding adaptor molecule (*IBA*)-1; the translocator protein 18 kDa (*TSPO*); matrix metallo-protease (*MMP*)-2 and *MMP*-9; tissue transglutaminase (*tTG*)-6; tissue plasminogen activator (*tPA*); and

the major facilitator super family domain containing 2A (*MFS2A*). Duodenal biopsies were evaluated for the expression of *CLDN*-1, *CLDN*-2, *CLDN*-10, *CLDN*-15, *TRIC*, and *OCLN*. *18S* housekeeping gene was chosen as internal control. *CLDN*-2, *CLDN*-3, *CLDN*-10, *CLDN*-15, *TRIC*, *IL*-1b, *IL*-6, *IL*-8, *IL*-10, *tTG*-6, *IBA*-1, *TSPO*, and *MFS2A* primers were purchased from Qiagen (RT² qPCR or Quantitect Primer Assay). The other primer sets used were purchased by IDT (Integrated DNA Technologies, Coralville, IA), and their sequences together with gene function in the context of the BBB and/or gut are listed in Table 1. The sequence specificity of synthesized primers (IDT) were determined by the online Blast program from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and compared with the GenBank database. Differences in gene expression profiles were evaluated applying the 2^(-ddCt) method [59].

Protein detection

Western blot analysis was performed as routinely described. Brain tissues (~100 mg) were homogenized on ice in lysis buffer [RIPA Buffer (Sigma, S. Louis, MO)] containing protease inhibitor cocktail (Complete Mini,

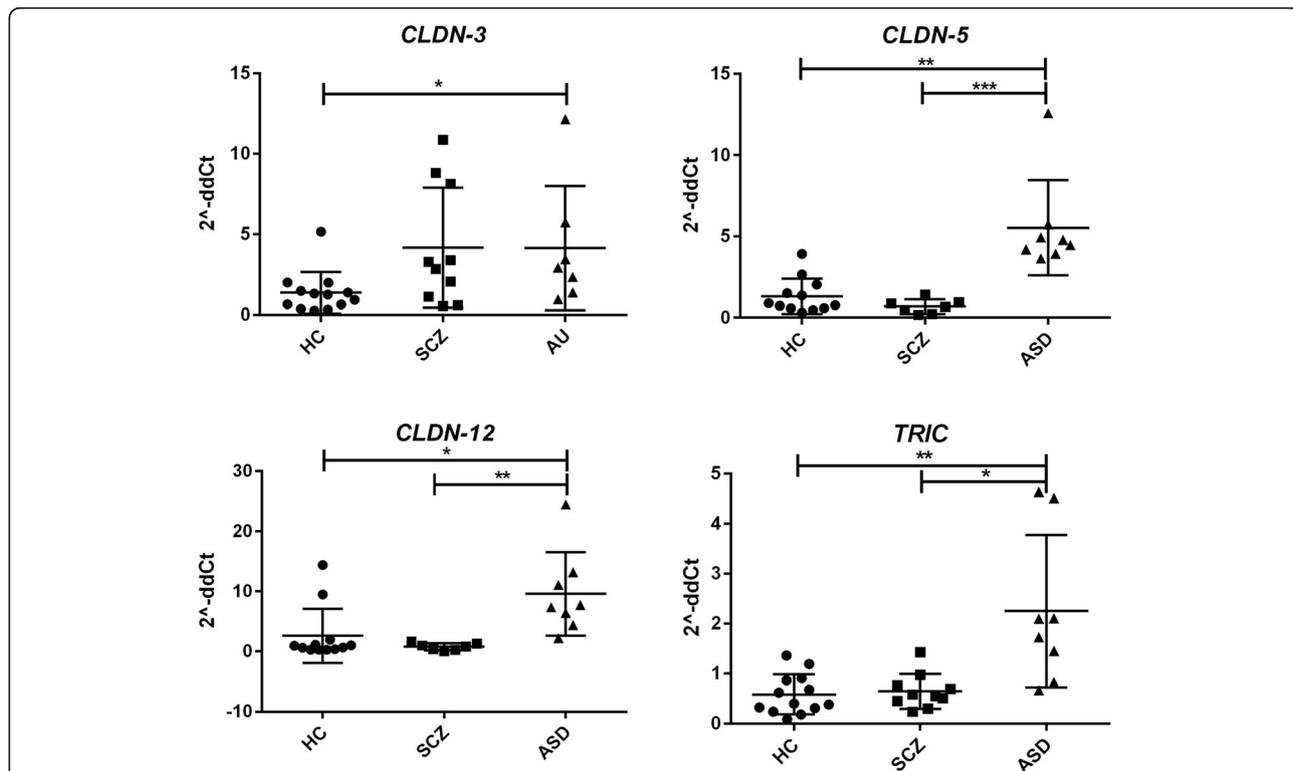


Fig. 1 Altered gene expression level of TJ components in the cortex of ASD subjects. Each dot represents data from a single subject. Gene expression level is reported as 2^{-ddCt} with normalization of mRNA expression to the endogenous control *18S*. Mean ± SEM are reported for each group. One-way ANOVA test has been used to evaluate statistical significance. **p* < 0.05; ***p* < 0.01; ****p* < 0.001

Roche). Homogenates were centrifuged at 14 K rpm for 30 min at 4 °C in a benchtop centrifuge. The supernatant, representing total protein lysate, was collected, and protein concentration was determined by Lowry protein assay (DC, BioRad, Hercules, CA) using bovine serum albumin (BSA) as a protein standard. Protein lysates were mixed with loading buffer and reducing agent (both from Life Technologies, Carlsbad, CA) and heated at 99 °C for 5 min. The lysates were then loaded on 4–20% Tris-Glycine gels (Life Technologies, Carlsbad, CA) and after gel electrophoresis transferred onto PVDF membranes. The membranes were blocked with 5% BSA

in Tris buffered saline with 0.1% Tween 20 (TBST), and incubated with primary antibody overnight at 4 °C. Actin was used as loading control while ACTA2, also known as alpha-smooth muscle actin (in this paper reported as SMA), was used for quantitative analysis normalization for tight junction (T) proteins. The membranes were washed three times with TBST and incubated with secondary antibody (diluted 1:5000 in 5% BSA-TBST) for 1 h at room temperature. Western blot signal was visualized using a LI-COR Odyssey infrared scan (LI-COR Biosciences, Lincoln, NE). Results were quantified with the ImageJ 1.47 software (NIH). The protein levels were

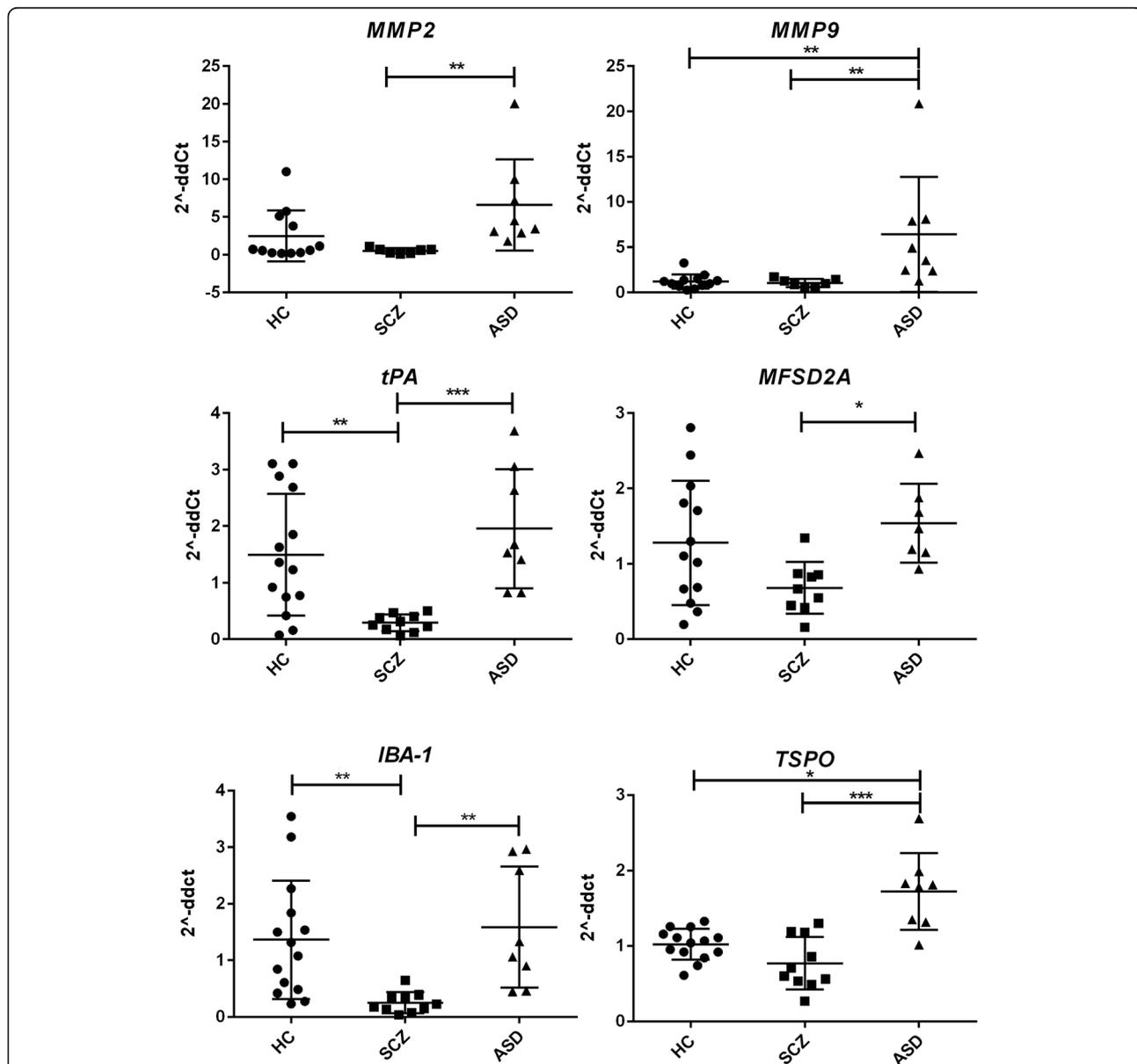


Fig. 2 Gene expression profile of BBB function associated components in the cortex of HC, ASD, and SCZ subjects. Each dot represents data from a single subject. Gene expression level is reported as 2^{-ddCt} with normalization of mRNA expression to the endogenous control 18S. Mean \pm SEM are reported for each group. One-way ANOVA test has been used to evaluate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

normalized as ratio to actin or SMA, as internal controls. Data represent the average of a minimum of two independent experiments.

Antibodies

Mouse anti-claudin-5 (cat # 35–2500; 1:500) antibody was purchased from Life Technologies, (Carlsbad, CA). Mouse anti-actin protein antibody (cat # 82353; 1:2000) was purchased from Thermo Fisher Scientific (Waltham, MA). Rabbit anti-claudin-12 (cat # NBP1-87450; 1:500) was purchased from Novus Biologicals (Littleton, CO). Rabbit anti-alpha-smooth muscle actin (SMA) antibody (#Ab5694; 1:1000) was purchased from Abcam (Cambridge, MA). LI-COR IRDye800 anti-rabbit (LI-COR Biosciences, Lincoln, NE; cat # 92632211) and Alexa Fluor 680 goat anti-mouse (Thermo Fisher Scientific, Waltham, MA; cat # A21057) were used as secondary antibodies in WB analysis.

Statistics

Data were analyzed by using the GraphPad (San Diego, CA) software. Comparisons among groups were made by the one-way ANOVA (Kruskal–Wallis *H* test; Dunn’s test for multiple comparisons). Differences between two groups were calculated by the unpaired *t* test (Mann–Whitney). All data with *p* < 0.05 were considered significant.

Results

Subjects

A total of 33 brain sections from the three subject groups were studied. No drug abuse or gunshot wounds were reported for ASD subjects. Two of the ASD individuals were on therapy with one antipsychotic and/or antidepressant. Those ASD cases whose cause of death was drowning were not considered for the brain study to eliminate the damaging effects on brain and vasculature caused by hypoxia/anoxia [60, 61]. Of the ten SCZ individuals, no medical records were available. The only existing information was collected at autopsy: the post-mortem toxicological analysis showed that three had used antipsychotics and/or antidepressants, one had used cocaine and one had ingested alcohol prior to death. Similar to ASD patients, no gunshot wounds at the head region were reported for SCZ subjects. The control cases had no known neurological disorder or known neuropathology. Three HC out of 15 had a history of drugs and/or alcohol abuse. Biopsies from the second portion of the duodenum were collected from the ASD (*n* = 12) and control (*n* = 8) patients with gastrointestinal symptoms. The available clinical characteristics of the analyzed subjects, together with demographic data are summarized in Table 2.

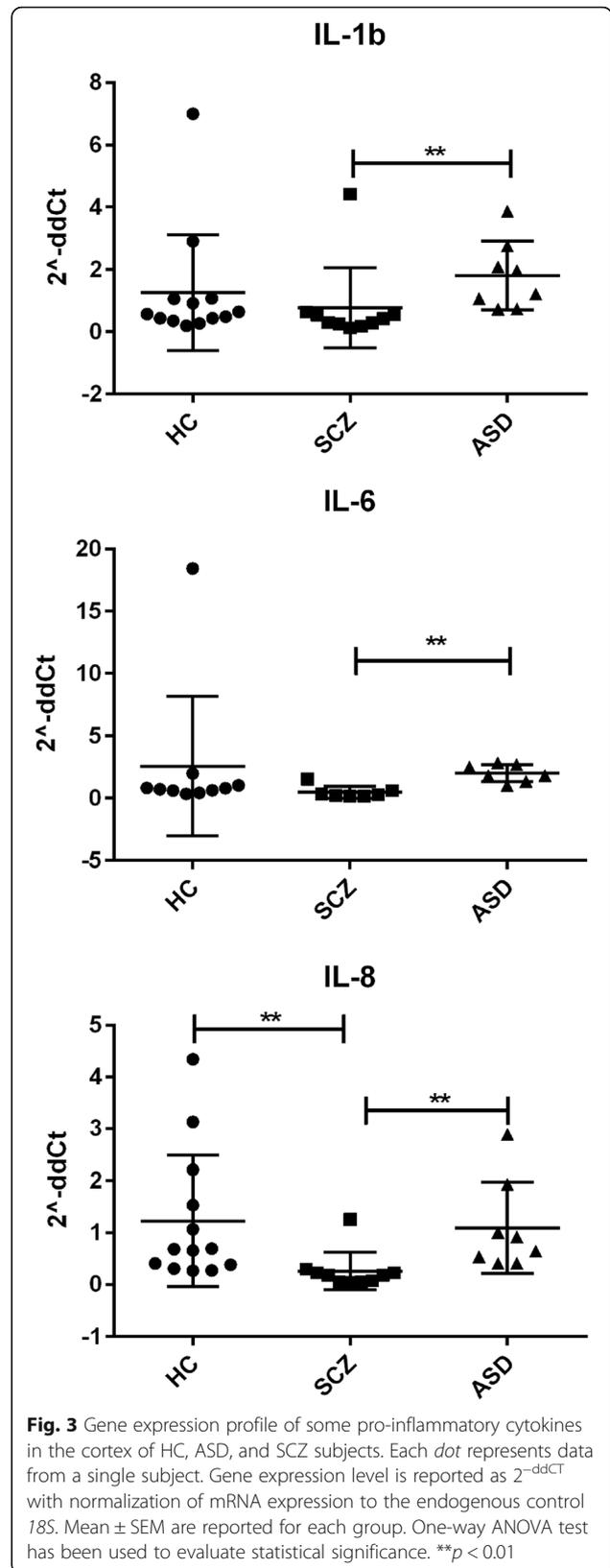


Fig. 3 Gene expression profile of some pro-inflammatory cytokines in the cortex of HC, ASD, and SCZ subjects. Each dot represents data from a single subject. Gene expression level is reported as $2^{-\Delta\Delta Ct}$ with normalization of mRNA expression to the endogenous control 18S. Mean \pm SEM are reported for each group. One-way ANOVA test has been used to evaluate statistical significance. *******p* < 0.01

Genes associated with BBB integrity and function are differentially regulated in the brain of ASD subjects

To assess the integrity of the BBB, we measured the gene expression levels of the major components of the brain microvascular endothelial tight junctions (TJs) by qRT-PCR, in the cortex and CBL of HC ($n = 15$), ASD ($n = 8$), and SCZ ($n = 10$) subjects. In the cortex, *CLDN-3*, *-5*, and *-12* and *TRIC* were upregulated in ASD subjects compared to controls (Fig. 1). Neither *CLDN-1*, *OCLN*, nor *VE-Cad* were differentially expressed in the cortex in any of the groups analyzed (not shown). Gene expression profiles of *tPA*, *IBA-1*, *TSPO*, *MFSD2A*, pro-inflammatory cytokines, and members of the matrix metalloprotease (MMP) family, described to be linked to BBB function disruption and/or neuroinflammation, were also analyzed. Results showed that in the cortex of ASD subjects, *MMP-9* and *TSPO* were significantly upregulated compared to those of the HC, whereas *tPA* and *IBA-1* appeared downregulated in SCZ compared in HC (Fig. 2). None of the pro-inflammatory cytokines analyzed were differentially regulated in ASD compared in HC. While *IL-8* appeared significantly reduced in SCZ vs. HC, all cytokines evaluated were expressed at a lower level in SCZ compared to ASD (Fig. 3). *IL-10* and *tTG6* expressions were below detection levels. In the CBL, similar to our findings in the cortex, *CLDN-5*, *-12* and

TSPO were found to be elevated in ASD subjects when compared to HC (Fig. 4). Among the other genes analyzed, *IL-1b* appeared significantly higher in SCZ vs. HC (Fig. 4). As a general observation, unlike ASD, SCZ subjects clustered well together for most of the genes analyzed in both brain areas. The gene expression fold changes relative to HC (=1) and the corresponding p values are summarized in Table 3.

BBB tight junction proteins are altered in ASD and SCZ subjects

To understand whether our gene expression results correlated with levels of protein produced, we performed western blotting analysis of tissue lysates using commercially available antibodies against some TJ components in both the cortex and CBL of HC, ASD, and SCZ subjects. Consistent with the gene expression profile, we observed a significant higher level of *CLDN-5* in the cortex of ASD subjects, compared in HC (Fig. 5). Interestingly and in this case in opposition to gene expression results, *CLDN-12* was reduced in the cortex of ASD and also showed a reduced trend in SCZ subjects (Fig. 5).

In the CBL (Fig. 6), the production of *CLDN-5* in ASD appeared slightly higher than in HC ($p = 0.1$), whereas its level in SCZ was reduced compared to

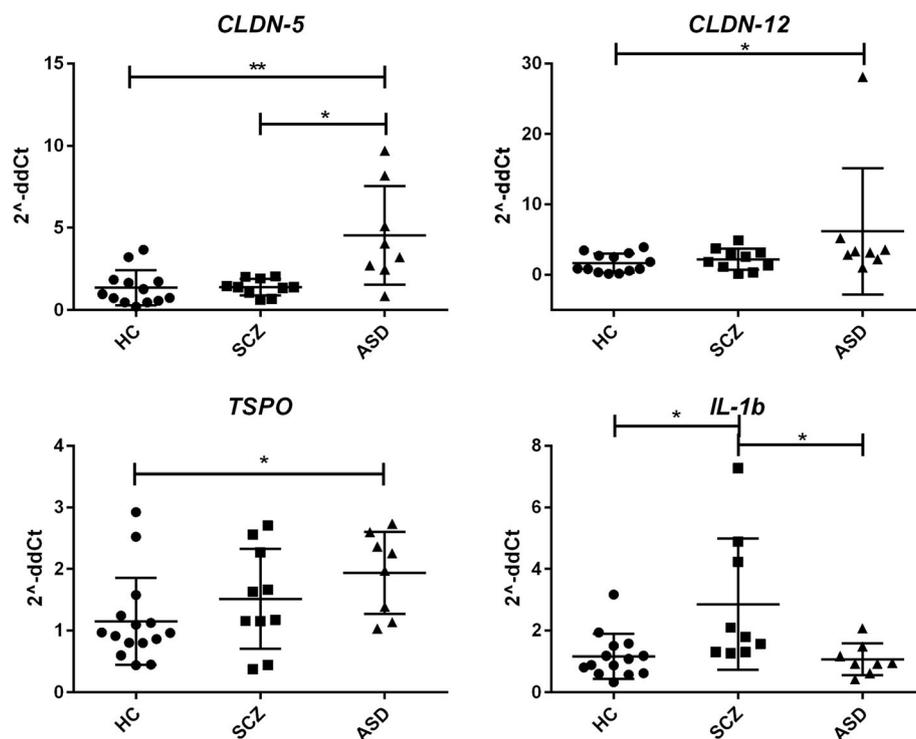


Fig. 4 Increased claudins and inflammatory markers gene expression levels in the cerebellum of HC, ASD, and SCZ subjects. Each dot represents data from a single subject. Gene expression level is reported as $2^{-\Delta\Delta Ct}$ with normalization of mRNA expression to the endogenous control 18S. Mean \pm SEM are reported for each group. One-way ANOVA test has been used to evaluate statistical significance. * $p < 0.05$; ** $p < 0.01$

Table 3 Summary of gene expression profiling by RT-qPCR

Gene	Fold change		<i>p</i> values		
	SCZ	ASD	Cortex HC vs. SCZ	HC vs. ASD	ASD vs. SCZ
<i>CLDN-1</i>	0.7	2.3	ns	ns	ns
<i>CLDN-3</i>	2.7	3.0	<0.05	<0.05	ns
<i>CLDN-5</i>	0.5	5.1	ns	<0.0001	<0.0001
<i>CLDN-12</i>	0.5	7.7	ns	<0.01	<0.001
<i>IBA-1</i>	0.2	1.2	<0.01	ns	<0.01
<i>IL-1b</i>	0.4	1.5	ns	ns	<0.01
<i>IL-6</i>	0.3	1.9	ns	ns	<0.01
<i>IL-8</i>	0.2	1.1	<0.01	ns	<0.01
<i>IL-10</i>	0.5	3.9	ns	ns	ns
<i>MMP-9</i>	0.9	4.5	ns	<0.001	<0.001
<i>MMP-2</i>	0.4	4.9	ns	<0.05	<0.001
<i>MSFD2A</i>	0.6	1.5	ns	ns	ns
<i>OCLN</i>	1.2	1.4	ns	ns	ns
<i>tPA</i>	0.2	1.7	<0.001	ns	<0.01
<i>TRIC</i>	1.3	1.8	ns	<0.001	<0.01
<i>TSPO</i>	0.7	1.7	ns	<0.05	<0.001
<i>VE-Cad</i>	0.8	1.3	ns	ns	ns
			Cerebellum		
<i>CLDN-1</i>	1.3	1.8	ns	ns	ns
<i>CLDN-3</i>	0.6	1.2	ns	ns	ns
<i>CLDN-5</i>	1.3	3.6	ns	0.01	<0.05
<i>CLDN-12</i>	1.5	3.7	ns	<0.05	ns
<i>IBA-1</i>	1.0	1.2	ns	ns	ns
<i>IL-1b</i>	2.3	1.0	0.01	ns	<0.05
<i>IL-6</i>	1.1	0.3	ns	ns	ns
<i>IL-8</i>	1.8	0.9	ns	ns	ns
<i>IL-10</i>	1.3	0.9	ns	ns	ns
<i>MMP-9</i>	1.0	1.9	ns	ns	ns
<i>MMP-2</i>	0.7	2.8	ns	ns	ns
<i>OCLN</i>	1.2	1.5	ns	ns	ns
<i>tPA</i>	1.2	1.5	ns	ns	ns
<i>TRIC</i>	0.6	0.9	ns	ns	ns
<i>TSPO</i>	1.3	1.8	ns	<0.05	ns
<i>VE-Cad</i>	1.4	1.3	ns	ns	ns
			Duodenum (biopsies)		
<i>CLDN-1</i>	N/A	1.7	N/A	ns	N/A
<i>CLDN-2</i>	N/A	2.3	N/A	ns	N/A
<i>CLDN-10</i>	N/A	4.0	N/A	ns	N/A

Table 3 Summary of gene expression profiling by RT-qPCR (Continued)

<i>CLDN-15</i>	N/A	1.6	N/A	ns	N/A
<i>OCLN</i>	N/A	1.2	N/A	ns	N/A
<i>TRIC</i>	N/A	1.6	N/A	ns	N/A

Gene expression levels are presented as fold change vs. HC (=1) using the formula $2(-\Delta\Delta Ct)$. Statistical difference among groups is calculated by the one-way ANOVA (Kruskal–Wallis *H* test). Differences between two groups are calculated by the Mann–Whitney unpaired *t* test. All data with *p* < 0.05 were considered significant
ns non-significant, *N/A* not applicable

that in HC (*p* = 0.07), although data did not reach statistical significance. No differences were observed for *CLDN-12* among the groups.

Increased pore-forming CLDNs and decreased barrier-forming TJ components expression in the intestine of ASD patients

To corroborate our hypothesis of a possible involvement of a dysfunctional intestinal barrier in the pathogenesis of ASD, we analyzed small intestinal biopsies of ASD and HC subjects for TJ defects (Fig. 7). Our results showed that 75% (9 out of 12) of the samples analyzed had a decreased expression of at least one barrier-forming TJ component (*CLDN-1*, *OCLN*, *TRIC*), whereas about 66% (eight out of 12) showed increased levels of pore-forming CLDNs, described to increase intestinal permeability (*CLDN-2*, *CLDN-10*, *CLDN-15*) ([62–65]).

Discussion

To the best of our knowledge, this is the first study addressing the molecular signature of BBB dysfunctions in ASD and SCZ in human samples. In spite of the obvious small sample number, due to the nature of the samples and the consequent procurement difficulty, we observed statistically significant differences among the study groups. However, it is also worthwhile to acknowledge that the small sample number may have affected some otherwise possibly significant results, which could have emerged if a larger number of tissues had been available.

Our molecular analysis of the BBB integrity and function shows an altered BBB in the ASD subjects evaluated. Indeed, the increased gene expression of *MMP-9* we detected in ASD subjects supports our hypothesis of an impaired BBB, most likely associated with neuroinflammation. Several pieces of evidence indicate that *MMP-9* secretion induces BBB disruption and this is an important step in the development of inflammatory diseases of the nervous system [66–74]. Although we did not observe elevated gene expression levels of pro-inflammatory cytokines in the ASD subjects analyzed in this study, we found a significantly high expression of *TSPO* in the ASD brain. *TSPO* increased expression level in activated microglia and reactive astrocytes [75–81] is

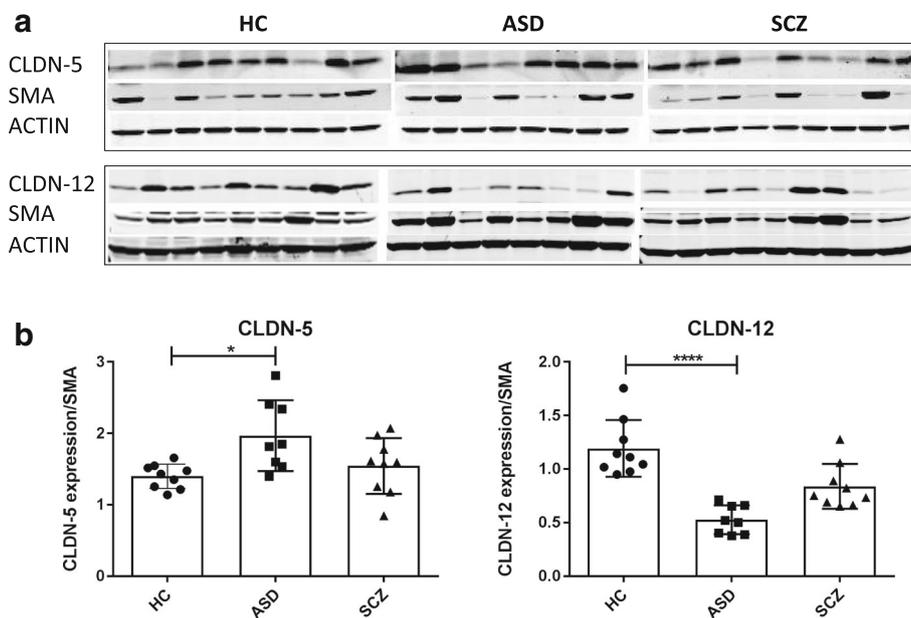


Fig. 5 Increased CLDN-5 and decreased CLDN-12 expression in the cortex of ASD subjects. **a** Brain tissues were lysed and immunoblotted with anti-claudin-5, anti-claudin-12, SMA or actin antibody. **b** Densitometry analysis of the results from the western blots is shown, where the data are normalized against SMA expression and are expressed as the mean ± standard error. Quantitative results represent the average of three independent experiments. * $p < 0.05$; **** $p < 0.0001$

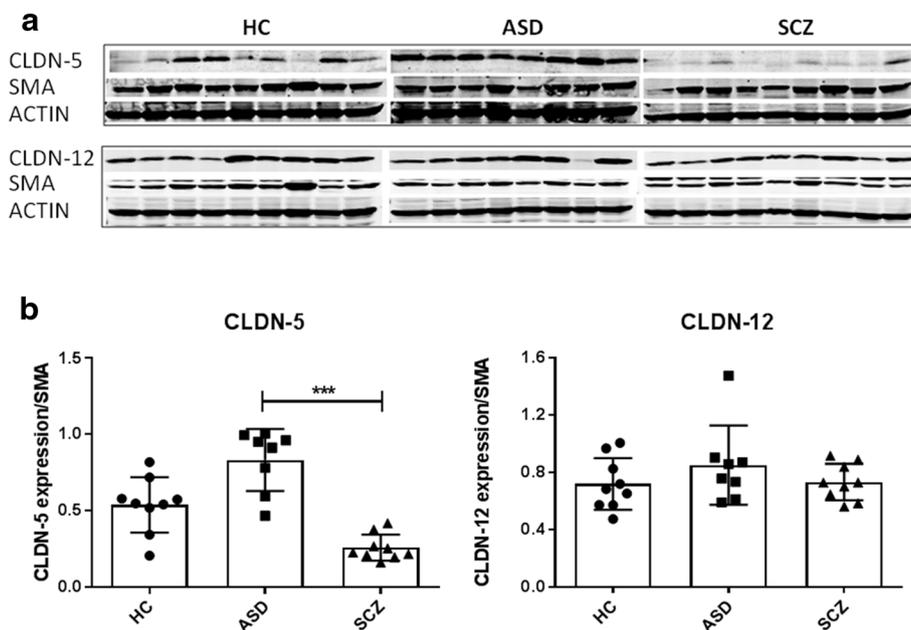


Fig. 6 Increased CLDN-5 expression in the cerebellum of ASD subjects. **a** Western blots of brain lysates immunoblotted with anti-claudin-5, anti-claudin-12, SMA or actin antibody. **b** Densitometry analysis of the results from the western blots is shown, where the data are normalized against SMA expression and are expressed as the mean ± standard error. Quantitative results represent the average of three independent experiments. *** $p < 0.001$

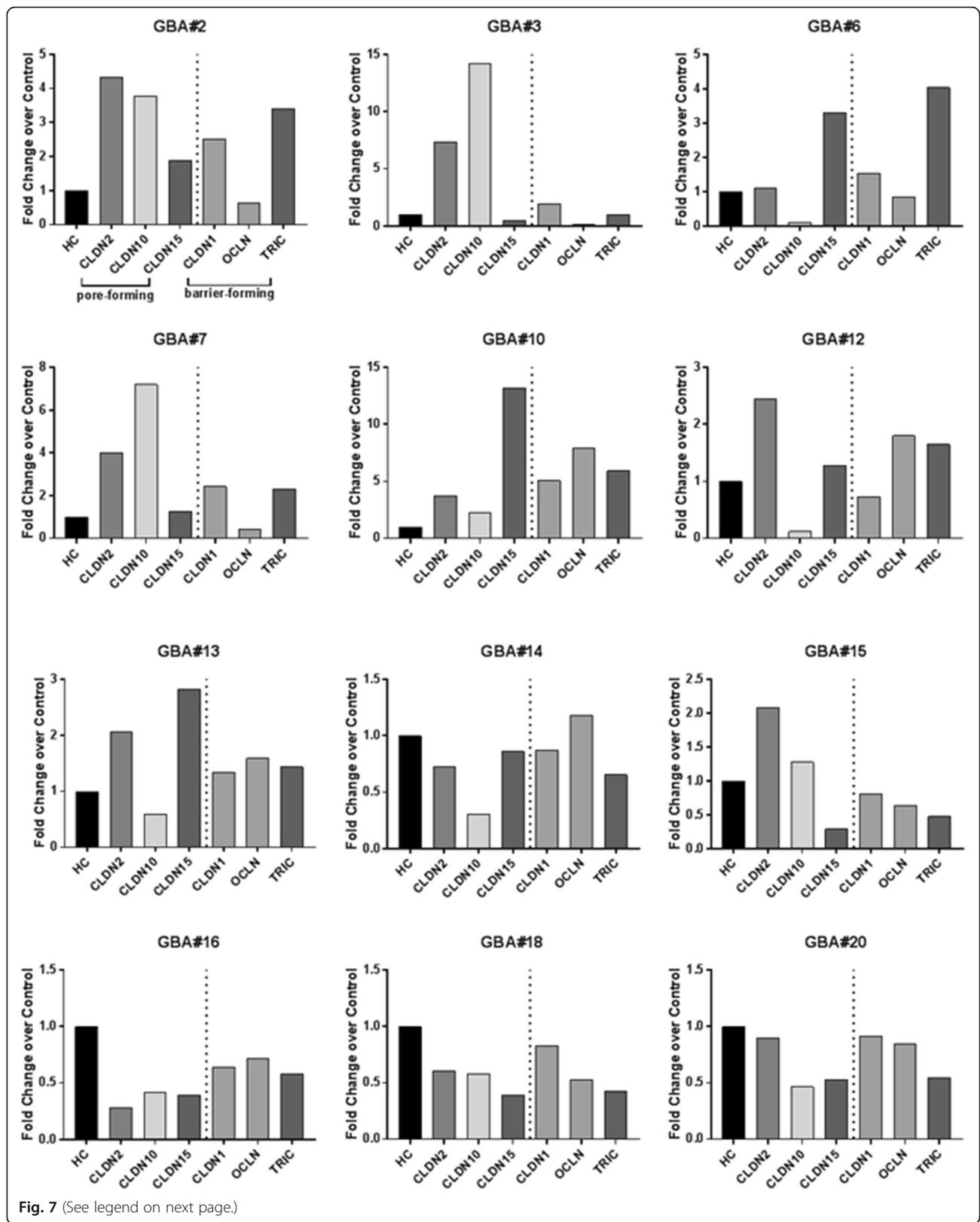


Fig. 7 (See legend on next page.)

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Fig. 7 Increased pore-forming claudins and decreased barrier-forming TJ components expression in the small intestine of HC and ASD subjects. Gene expression levels of TJ components in duodenal biopsies from HC ($n = 9$) and ASD patients ($n = 12$). *CLDN-2*, *-10* and/or *-15* levels are higher in eight out of 12 ASD samples, compared in controls. *CLDN-1*, *OCLN*, and/or *TRIC* levels are decreased in nine out of 12 ASD samples over controls. Each graph represents single patient results. Data are expressed as fold change over the averaged controls

associated with brain injury and neuroinflammation, making TSPO a reliable marker of brain inflammation.

Of the four claudins (i.e., *CLDN-1*, *-3*, *-5* and *-12*) that to date are thought to be incorporated in the BBB [82–85], we found that two were significantly more expressed in the ASD brain as compared in HC. *CLDN-5*, the major and more abundant cell adhesion molecule of TJs in brain endothelial cells, is expressed 5.1 and 3.6 times higher in the cortex and CBL of ASD subjects, respectively, than in those of HC (Table 3). Similarly, *CLDN-12* is expressed about 7.7 and 3.6 times more in the ASD cortex and CBL, respectively, than in the control group. *CLDN-3*, on the other hand, appears elevated by about 2.7 and 3.0 times in the cortex of both SCZ and ASD subjects, respectively, compared in that of HC. *TRIC*, another component of the TJs associated with increased epithelial tightness and decreased permeability to macromolecules [86], appears significantly elevated in the ASD cortex, suggesting either a more competent BBB or a compensatory mechanism for disrupted endothelial barrier integrity.

At the protein level, CLDN-5 is elevated in the ASD cortex, consistent with its mRNA expression, whereas, on the other hand, CLDN-12 is significantly reduced. An increased gene expression of sealing TJ components (*CLDN-5*, *-12*, and *-3* and *TRIC*) should imply a tighter BBB and a better control in the diffusion of macromolecules. However, our data on *MMP9* and *TSPO* expression compatible with ongoing neuroinflammation suggest a compensatory mechanism to repair a compromised BBB. Another plausible explanation for the CLDN-5 data is that, once produced, the protein is not capable of being incorporated into the TJ complex because of impaired trafficking to the endothelial cell–cell interface or because the protein has some mutations that prevent it from establishing homophilic and/or heterophilic protein–protein interactions, leading to a sustained compensatory gene expression and protein accumulation. On the other hand, the discrepancy between gene upregulation and decreased protein expression observed for CLDN-12 might suggest the existence of a compensatory mechanism for post-translational destruction/degradation of the protein itself. While the sealing function of CLDN-5 in BBB TJs is well established [83, 87], that of CLDN-3 and *-12* are less understood. CLDN-1 and *-3* sequences are highly homologous to CLDN-5, suggesting similar properties. Indeed, several studies strongly suggest that CLDN-3 contributes to BBB tightness [85, 88–90]. Conversely, CLDN-

12 sequence is quite dissimilar from those of CLDN-1 and *-3*, and this might indicate a different function. Piontek et al. [91] showed that CLDN-12 cannot form homophilic trans-interactions or homopolymeric strands in vitro, suggesting that “heterophilic cis- and/or trans-interactions with another TJ-protein are necessary for the incorporation of CLDN-12 in strands.” It may be hypothesized that the increased CLDN-5 we observed in ASD subjects might be compensatory to a decreased CLDN-12 in a heterophilic cis- and/or trans-interaction between the two claudins.

Our cohort of SCZ individuals did not show major changes in the genes analyzed compared to HC, except for an increased *IL-1b* in the CBL and significantly reduced levels of tPA and *IBA-1* in the cortex. Whereas we do not know the physiological relevance of downregulated *IBA-1*, our results are in agreement with data from other studies indicating activation of pro-inflammatory networks and reduced tPA activity in SCZ [92–102], this latter feature leading to a dysfunctional coagulation system and increased risk of thrombotic events in SCZ patients [101]. Other genes analyzed in SCZ appeared to follow a trend similar to ASD subjects (i.e., increased *CLDN-3* and reduced CLDN-12 protein level in the cortex). Furthermore, although non-statistically significant ($p = 0.07$), we did observe a trend toward reduced CLDN-5 protein level in the SCZ CBL as well, suggesting a possibly leaking BBB. Previous clinical and postmortem research in SCZ has suggested that an impaired BBB could be a contributing factor to the pathogenesis of the disease in only a subgroup of SCZ patients [103–105]. It is important to mention that the SCZ and ASD subjects studied were not selected on the basis of specific immune biomarkers and, therefore, may represent a mixed group of patients, which thereby limits the statistical significance of some of our findings. A sample stratification approach based on specific immunological features might be a better way of analyzing pathways of interest, and this may be particularly true for SCZ, a disorder characterized by marked clinical and neurobiological heterogeneity. Alternatively, SCZ physiopathology may lie in subtle changes that chronically affect brain function.

As for the intestine, due to the high heterogeneity intrinsic of ASD, our goal was to elucidate the TJ component profile from a functional perspective. Toward this aim, we have analyzed the expression level of TJ elements dividing them into two major functional groups: pore-forming (*CLDN-1*, *OCLN*, *TRIC*) and

barrier-forming components (*CLDN-2, -10, -15*). These results, showing increased expression levels of pore-forming (66% of the ASD samples) and decreased levels of barrier-forming (75% of the ASD samples) TJ components in the duodenal samples, suggest an impaired gut

barrier and serve as a proof of concept to support the hypothesis of a gut–brain axis dysfunction in a subgroup of ASD patients. According to this hypothesis, non-self antigens crossing a damaged intestinal barrier elicit a local and/or systemic inflammatory reaction that,

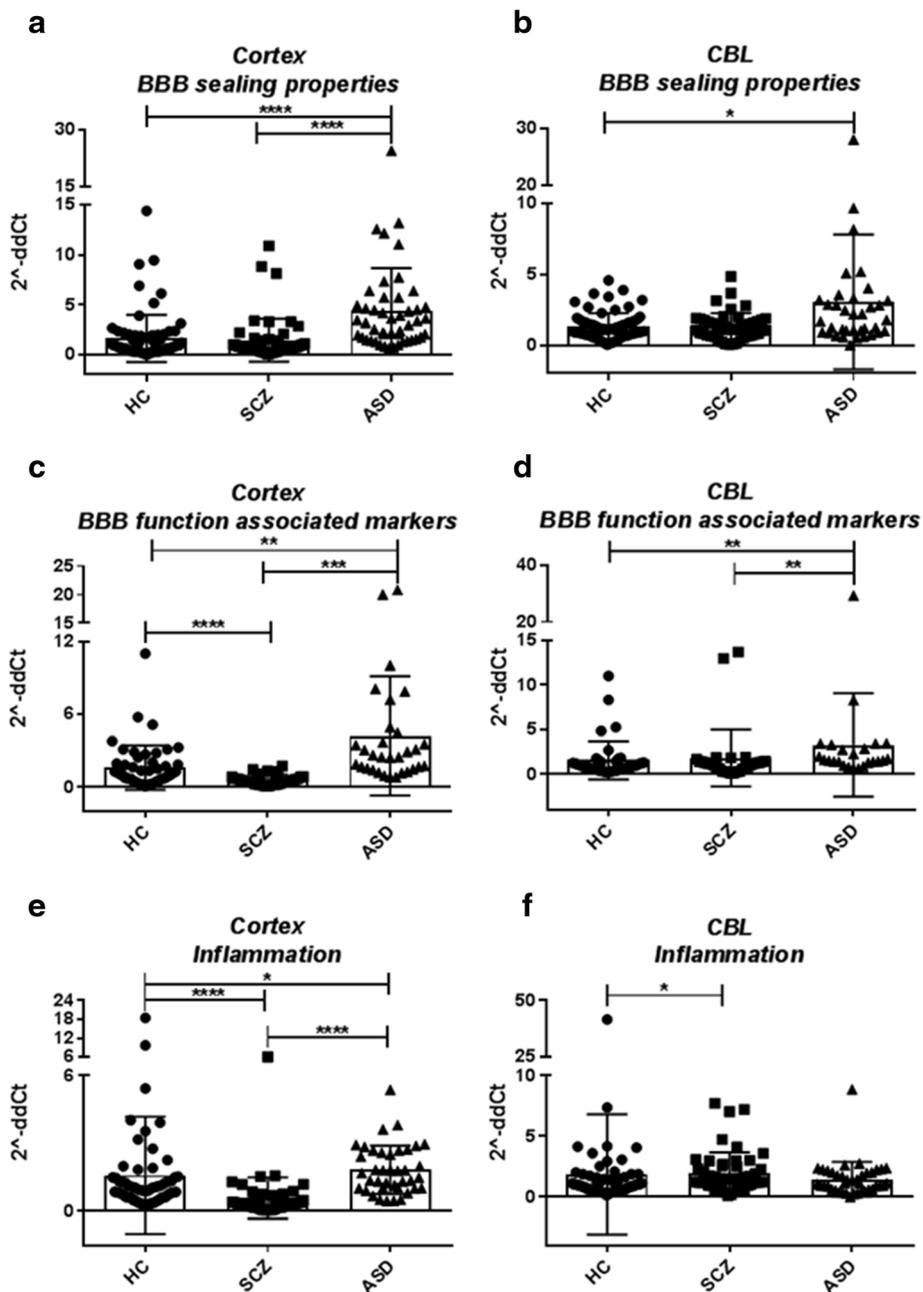


Fig. 8 Gene expression in the cortex and cerebellum (CBL) of HC, ASD, and SCZ subjects clustered by functional category. Results are represented as scatter plots where each *dot* represents data obtained from one subject sample. **a** Cortex “BBB sealing properties” group includes *CLDN-3, -5, and -12, TRIC, and OCLN*. **b** CBL “BBB sealing properties” group includes *CLDN-3, -5, and -12, TRIC, and OCLN*. **c** Cortex “BBB function associated markers” group includes *tPA, MMP2/9, and MSFD2A*. **d** CBL “BBB function associated markers” group includes *tPA and MMP-2/9*. **e** Cortex “Inflammation” group includes *IL-1b, IL-6, and IL-8; TSPO; and IBA-1*. **f** CBL “Inflammation” group includes *IL-1b, IL-6, and IL-8; TSPO; IBA-1*. Gene expression level is reported as 2^{-ddCt} with normalization of mRNA expression to the endogenous control *18S*. Mean \pm SEM are reported for each group. One-way ANOVA test has been used to evaluate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

associated with a breach of the BBB, may lead to ASD in genetically predisposed subjects.

This study addressed for the first time, at the molecular level, some relevant and still open questions regarding the gut–brain axis in neurobehavioral disorders and furthermore demonstrated that both the BBB and gut barrier may be affected in ASD. However, these findings present with a few limitations. The first lies in the postmortem nature of the samples with potential confounding factors deriving from tissue preservation and the personal history of each subject, including exposure to medications, health conditions, cause of death, and postmortem interval (PMI). Our statistical analysis of the PMI showed no differences among groups suggesting that PMI might not have affected our results. Similarly, data from medical records or postmortem toxicology analysis showed that about the same number of subjects in ASD and SCZ groups were on antipsychotics/antidepressants, so it is unlikely that the differences we observed among groups could be explained by the effects of the drugs. For clarity, a few studies addressing the impact of antipsychotics on the BBB function do suggest a disturbance effect on its sealing property [106, 107], whereas other studies on the blood–cerebrospinal (BCS) fluid barrier show divergent results [103, 108]. Furthermore, there are numerous studies reporting the suppressing effect of antipsychotics on the blood cytokine profile in SCZ and ASD [98, 109–118], as well as on microglia activation [119, 120], that could explain our data on *IBA-1* and the cytokine gene expression profiles. However, our *IBA-1* results are well in agreement with the published studies on postmortem brain tissues showing no significant changes in its expression in ASD [121].

The second limitation of this study is represented by its descriptive nature that does not allow us to draw conclusions on the causality of a breach of the intestinal/BBB integrity supporting neuroinflammation and ASD and SCZ symptoms. However, our data is in line with animal studies suggesting causality between loss of barrier function and neurobehavioral changes compatible with ASD [122–124]. Finally, as already mentioned, a third limitation is represented by the small number of samples analyzed due to limited availability which might impact on the statistical significance of some of our results. However, given the complexity of the studies performed and the difficulty associated with the procurement of these samples, our study represents a solid foundation to justify a more robust sample collection and analysis to further investigate the gut–brain axis in ASD.

Conclusions

In conclusion, our results seem to point to a dysfunctional gut–brain axis associated with neuroinflammation in ASD. When clustered together by functional groups

(barrier properties, pro-inflammatory markers, and enzymatic activity), our data support the notion that in ASD, there is a differential regulation of the pathways associated with our hypothesis of a gut–brain axis dysfunction involving the intestinal barrier, BBB integrity/function, and neuroinflammation (Fig. 8). Certainly, more in-depth molecular studies, possibly in animal or in vitro cell culture models, are necessary to understand the specific mechanisms behind the BBB disturbance we have herein reported. These studies will allow for patient stratification and personalized interventions (precision medicine) to target specific pathways involved in the pathogenesis of ASD.

Abbreviations

AJ: Adherens junction; ASD: Autism spectrum disorders; BBB: Blood–brain barrier; BCS: Blood–cerebrospinal; CBL: Cerebellum; CLDN: Claudin; CNS: Central nervous system; GBA: Gut–brain axis study; GI: Gastrointestinal; HC: Healthy controls; IBA-1: Ionized calcium-binding adaptor molecule 1; IL: Interleukin; INF: Interferon; MSFD2A: Major facilitator super family domain containing 2A; MMP: Matrix metalloprotease; NICHD: National Institute of Child Health and Human Development; OCLN: Occludin; PMI: Postmortem interval; SCZ: Schizophrenia; TJ: Tight junction; TNF: Tumor necrosis factor; tPA: Tissue plasminogen activator; tTG6: Tissue transglutaminase 6; TRIC: Tricellulin; TSPO: Translocator protein; VE-Cad: Vascular endothelial cadherin; ZO-1: Zonula occludens-1

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

MF, AS, AF, and NC conceived the study. MF and AS conceived and designed the experiments. MF, AS, and SS performed the experiments. MF and AS analyzed the data. MF drafted the manuscript. AF, NC, AS, SS, and DLK helped with the interpretation of the data. AF, NC, DLK, SSC, and SS assisted in writing and/or editing the manuscript. SSC, SMK, and TMB actively contributed to the procurement of the duodenal biopsies and to the critical reading of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Postmortem human tissues were collected under specific Biobank protocols, and family consent was collected for the tissue to be used for scientific purposes including publication. For duodenal biopsies, parental or patient (if older than 17 years old) consent was collected for the tissue to be used for scientific purposes, including publication.

Ethics approval and consent to participate

The human postmortem tissue collection, medical records, and family contact have been approved by the University of Maryland and Department of Health and Mental Hygiene Institutional Review Boards (IRBs). In addition,

the specific collection was approved by the Maryland Psychiatric Research Center (MPRC), Maryland Brain Collection Steering Committee, and the NIH NeuroBioBank Tissue Access Committee. The specimens were collected under specific protocols, and the family consent was collected for the tissue to be used for scientific purposes including publication. Study procedures for the collection of the duodenal biopsies were reviewed and approved by the Partners Human Research Committee (PHRC) at Massachusetts General Hospital and performed in accordance with the Declaration of Helsinki. The PHRC operates in full compliance with all applicable federal, state, and local laws and regulations to protect the rights and welfare of the human subjects. Written informed consent was obtained from all the subjects at the time of enrollment.

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