Circulating Tight-Junction Proteins Are Potential Biomarkers for Blood-Brain Barrier Function in a Model of Neonatal Hypoxic/Ischemic Brain Injury

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Research

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Abstract

Background

Neonatal hypoxia-ischemia often leads to lifelong disabilities with limited treatments currently available. The brain vasculature is an important factor in many neonatal brain pathologies but there is a lack of diagnostic tools to evaluate the brain vascular health of neonates in a clinical setting. Measurement of blood-brain barrier tight-junction proteins have shown promise as biomarkers for brain injury in the adult. Here we tested the biomarker potential of tight-junctions in the context of neonatal brain injury.

Methods

The levels of TJ-proteins (occluding, claudin-5, and zonula occludens-1) in both blood plasma and cerebrospinal fluid (CSF) as well as blood-brain barrier function were measured in a clinically relevant hypoxia/ischemia model in neonatal rats.

Results

Temporally acute elevated levels of occludin and claudin-5 could be measured in blood and CSF after hypoxia/ischemia with males generally having higher levels than females. The levels of claudin-5 in CSF correlated with the severity of the brain injury at 24h post- hypoxia/ischemia. Simultaneously, we detected early increase in blood-brain barrier-permeability at 6 and 24h after hypoxia/ischemia.

Conclusions

Levels of circulating claudin-5 and occludin are increased after hypoxic/ischemic brain injuries and blood-brain barrier-impairment and have promise as early biomarkers for cerebral vascular health and as a tool for risk assessment of neonatal brain injuries.

Impact Statement

BBB dysfunction following neonatal hypoxia/ischemia is likely in part due to the loss of tight-junction proteins from cerebral blood vessels. BBB breakdown release tight junction proteins and BBB function may be assessed by measuring these proteins in the circulation. This is the first study which investigates tight-junction proteins in the CSF and correlate to levels in circulation in a neonatal animal model of brain injury. Elevated levels of blood-brain barrier-derived tight-junction proteins Claudin-5 and Occludin can be detected in the circulation at several time-points in a rat-model for neonatal HI, signifying the proteins potential as biomarkers for the brain vascular health in neonates.

Background

Neonates that are diagnosed with neonatal hypoxic-ischemic encephalopathy (HIE) are, if they survive, at high risk of developing life-long and permanent neurodevelopmental disabilities(1). Although

hypothermia has been shown to be a beneficial treatment under certain circumstances, since it has to be initiated within six hours and can only be implemented in some of the neonates, with a number needed to treat of around eight, new or add-on treatments are needed(2). One of the key steps in the development of new treatments is diagnostic tools enabling early diagnosis and/or monitoring of injury progression. The aetiology underlying brain injury in the newborn is complex and is likely to involve many factors, making early diagnosis very difficult. The most commonly used early clinical tools to assess neonatal HIE includes cord blood gas analyses(3), Apgar and Sarnat scoring systems(4). However, these methods have a low predictive value for the subsequent brain injury(5). The resulting brain injuries are typically visualised by advanced imaging methods such as MRI and CT-scans(6), techniques that are both expensive and have a relatively low availability, in terms of both equipment and skilled personnel, in many countries.(7) This is important to note as it has been estimated that as many as 96% of neonates affected by HIE are born in low- and middle-income countries(8). There is a clinical need for better and affordable diagnostics of neonatal brain injuries that would enable early risk assessment and intervention as well as monitoring of injury development. Putative biomarkers for neuronal damage following HIE would be a valuable diagnostic tool to predict long-term outcomes but has the drawback that they are usually measurable only after the injury has manifested(9).

The brain vasculature is a central factor in many neonatal brain pathologies and the blood-brain barrier (BBB) is disrupted early in the disease process(10). A biomarker that reflects the state and health of the neonatal brain vasculature could be a powerful predictor for brain injury, considering that studies in rats have shown that the dysfunction of the BBB mirrors the severity of HI-injuries(10, 11). Additionally, assessment of BBB function is of importance for the success of treatments that are directed at the brain. The endothelial cells of the cerebrovasculature are connected by complexes of tight-junction (TJ) proteins which restrict passage into the brain thus maintaining the integrity of the BBB. Previous studies in adults have shown that TJ proteins are shed from the BBB and cerebral vasculature and enters the circulation in a model of adult stroke in rats(12), in humans following stroke(13) as well as after intracranial haemorrhage(14). Furthermore, studies in cultured cerebral endothelial cells and in vivo adult rats have shown that TJ:s are disrupted after hypoxia(15-17) and that a functional TJ-barrier is formed early in development(18).

Given that TJ proteins have been suggested as potential biomarkers in adult brain injury models, we hypothesised that BBB related proteins can be detected in blood and CSF after neonatal HI. In this study we focused on three of the key TJ:s in the brain endothelium, claudin-5 (CLDN5), occludin (OCLN) and zonula occludens-1 (ZO-1)(19). CLDN5 is an integral transmembrane TJ protein expressed by brain vascular endothelial cells adhering neighbouring cells together with structural support from transmembrane OCLN and the intracellular scaffold ZO-1 protein(20). In order to study the role of TJ proteins in neonatal brain injury we used a well-established model wherein a HI-injury is induced in post-natal day (PND) 7 rats(21). Circulating TJ:s in blood plasma and cerebrospinal fluid (CSF), brain injury and BBB function after injury were thoroughly investigated. We found dynamic long-term changes of BBB function following the insult, first an opening of the barrier early after injury followed by an apparent lower BBB permeability at later time points. During the initial opening of the barrier we could detect raised

levels of TJ-proteins in both CSF and plasma of injured animals where the levels of CLDN5 in CSF correlated with the severity of the brain injury. We found a sex difference in the levels of some TJ-proteins in analysed fluids with higher levels in males.

Methods

Animals

PND7 Wistar rat pups were bred in-house at the Laboratory for Experimental Biomedicine of Gothenburg University (parents were sourced from Janvier Labs, Le Genest-Saint-Isle, France) and maintained under normal housing conditions with a 12h light/dark cycle and free access to water and standard laboratory fodder. Animals of both sexes and different litters were used for the experiments and care was taken to minimise the number of animals used and to maintain an even sex-balance in all experimental and control groups. All experiments were approved by the Gothenburg Committee of the Swedish Animal Welfare Agency (Application nos. 663/17) and performed in accordance with the ARRIVE guidelines. A total of 104 animals were used throughout the study.

Hypoxia-ischemia (HI)

Postnatal day 7 Wistar rat pups were anaesthetised with isoflurane (3.5-5%, Vetmedic, Stockholm, Sweden) in a 50/50 oxygen/nitrogen mixture, placed on their backs and a small incision was made in the neck to gain access to the carotid artery. A suture was placed permanently around the left carotid artery and the incision sealed with Vetbond tissue adhesive (3M, MN, USA). Surgery typically lasted for 3-5 min. Following surgery, pups were allowed to recover in their home cage together with their mother for 1h. Subsequently, operated pups were placed in a 36 °C chamber. The chamber was perfused first with humidified air for 10 min followed by 8% oxygen for 1h and then by humidified air for 10 min. After the hypoxic exposure, pups were returned to their home cages. Control animals was subjected to sham-surgery (anaesthesia and incision) but no hypoxia. During all procedures, animals were monitored for vitals (i.e. breathing and skin-colour); every animal in the study survived the surgery and hypoxia.

Sample collection and processing

For all time-points after HI (i.e. 6h, 24h and 5 days) injured and control animals were euthanized with a lethal overdose of pentobarbital. Cerebrospinal fluid was collected from the cisterna magna through glass capillaries as described previously(22) and blood was collected by cardiac puncture with EDTA-treated syringes. CSF was checked for blood contamination as previously described(23) and samples discarded when contamination detected (detection limit about 0.2%). Blood samples were centrifuged at 2000 *g* for five min to separate the plasma. Samples were placed on dry ice after collection and long-term stored in -80 °C freezer until analysed. Whole brains (excluding the cerebellum and brain stem) were collected and immersed in cold 6% buffered formaldehyde (Histofix; Histolab, Gothenburg, Sweden) at 4 °C for 24h before processing for paraffin embedding.

Caspase-3 activity assay

The activity of cleaved caspase-3 at 6h (n=7) and 24h (n=7) after HI was measured using a fluorometric assay based on an earlier study(24). Whole brain hemispheres where homogenised in cold RNase free PBS and sonicated in cold RNase free PBS containing 2% protease inhibitor cocktail (Sigma-Aldrich, MO, USA) and 10 mM EDTA. Aliquots were centrifuged at 10 000 G for 15 min in 4 °C and some supernatant were used for BCA concentration measurements. For caspase-3 activity, 20 µl supernatant were incubated with 80 µl extraction buffer composed of a buffer base (50 mM Tris, 100 mM NaCl, 5nM EDTA, 1 mM EGTA, pH 7.3) and 0.2% CHAPS, 1% protease inhibitor cocktail, and 1 mM PMSF (Sigma-Aldrich) on a 96 well plate for 15 min in RT. 100 µl assay buffer made up of buffer base plus 4mM DTT, 1 mM PMSF and 25 µM caspase-3 substrate (Peptides International, KY, USA) were added to the wells before the plate was read for 1h at 37 °C with 2 min intervals on a SpectraMax Gemini EM microplate reader (Molecular Devices, CA, USA) set to excitation wavelength 380 nm and emission wavelength 460 nm. Endpoint readings were made before and after 10 µl of 10 µM free AMC (Peptides International) and the V_{max} was calculated from the linear part of the curve, caspase-3 activity was expressed as pmol AMC/min*mg caspase-3.

Enzyme-linked immunosorbent assay (ELISA)

Plasma- and CSF-samples were analysed using pre-coated ELISA kits for tight-junction proteins CLDN5 (Nordic BioSite. Stockholm, Sweden), OCLN (Cusabio, Wuhan, China), and ZO-1 (Cusabio) as per the manufacturer's instructions. Plasma was diluted 20 times and CSF 10 times. In short, standards, CSF and plasma-samples were diluted in sample diluent buffer and incubated on ELISA-plates pre-coated with the antibody. After incubations with a biotinylated secondary antibody, HRP-avidin, TMB substrate, and a stop-solution the optical density was determined with a Spectramax Plus microplate reader (San Jose, CA, USA) set to 450 nm with 540 nm wavelength-correction (OCLN and ZO-1) or 450 nm (CLDN5). The protein concentration was determined from the resulting standard-curve. CSF and plasma from the same animals were analysed for both CLDN5 and OCLN, n=7-8 for all time-points. Due to some differences between ELISA-plates all data were normalised to the median of time-matched sham controls analysed on the same plate.

Blood-brain barrier assessment

The blood-brain barrier permeability was measured using radiolabelled sucrose as described by our group earlier(22). For all time points after HI; 6h (n =9), 24h (n=8), 5 days (n=9), injured and control animals (n=12) were injected i.p. with two µCi ^{14}C-sucrose (American Radiolabelled Chemicals, MO, USA) in saline (100 µl injection volume). Thirty min later, they were euthanized with a lethal overdose of pentobarbital. Blood was collected through cardiac puncture using a heparinised syringe and centrifuged at 2000 *g* for five min to separate plasma. Choroid plexuses were removed and whole cerebellum and brain stem as well as left and right hippocampus, cortex and striatum/thalamus were dissected and collected into pre-weighed scintillation vials and then re-weighed. 500µL of Solvable (PerkinElmer, MA, USA) was added to

all samples and they were incubated overnight in a 40 °C oven to dissolve the tissue. After checking that all tissues were solubilised, samples were left to cool down to RT, mixed with 10 mL Ultima Gold scintillation cocktail (PerkinElmer) and left for 60 min in darkness. The radioactivity in each sample was determined by liquid scintillation counting in a Tri-Carb 4910TR (PerkinElmer) and calculated as cpm/mg sample after background corrections. Brain/plasma sucrose concentration ratios were used as a measurement of BBB-permeability as previously described after correcting for residual blood space in brain.(25) These concentration ratios were calculated as a measure of BBB permeability in each region and ratios in the left (injured) hemisphere was compared to the right hemisphere as previously outlined(10).

BBB-disruption after HI was confirmed using injections of Evans blue (EB) dye, a dye that binds to albumin in the blood and thus should be regarded as a high-molecular marker opposed to sucrose(26). 4% EB dissolved in PBS were injected i.p. (4 µl per g body weight) 6h post-HI (n=3). After 1h animals were euthanized with a lethal overdose of pentobarbital and transcardially perfused with saline and 6% buffered formaldehyde. Whole brains (excluding the cerebellum and brain stem) were collected and immersed in cold 6% buffered formaldehyde at 4 °C for 24h before they were embedded in 4% agarose and cut in 100 µm thick sections in a Leica 1200 VT vibratome (Leica Biosystems, Wetzlar, Germany). Sections were mounted in water-based CC/Mount (Sigma) and imaged at 680 nm, the wavelength in which EB fluoresce(27). The visualised fluorescence were confirmed to stem from EB-labelling by imaging of brains from naïve control animals that received EB-injections and were or were not perfused (n=6) as well as animals that did not receive EB (n=3; no signal).

Measurement of brain blood-vessel area

Entire hemispheres of CLDN5-stained fluorescent paraffin-sections of brains collected five days after HI and naïve controls (n=5 per group) were imaged with a tiling and stitching function. Two levels (700 µm apart) at mid-hippocampal level were imaged per animal and analysed with an in-house developed macro for the Fiji-build(28) of ImageJ(29) that utilises difference of Gaussian to eliminate all background while preserving all vessel information to accurately measure the area of blood vessels in an image. Briefly, entire CLDN5-stained brain hemispheres were imaged in a fluorescent microscope (Additional file 1a); the process is shown in a smaller selection of the image (Additional file 1b) for clarity. A copy of the image was subjected to Gaussian blurring with sigma = 10 (Additional file 1c). The blurred image was subtracted from the original and threshold applied with Fiji's "analyse particle"-tool to filter out any eventual debris so only marked blood vessels remained (Additional file 1d). The resulting vessel-image were then superimposed on the original image (Additional file 1) to confirm accurate vessel labelling. Blood vessel area was quantified in both injured and uninjured hemispheres by first outlining a ROI (delineating the entire cortex and hippocampus) and measure the total tissue area. Then the area of marked blood vessels within the ROI was determined and the percentage of blood vessel area of the total area in the hemisphere was calculated. Averages were calculated from the two mid-hippocampal levels per animal. Investigators were blinded to treatment groups during analysis.

Immunohistochemistry and microscopy

Paraffin-embedded brains were cut in seven µm thick coronal sections at six levels and 40 sections apart with a microtome, starting at what corresponds to approximately -2.5 mm from bregma in an adult rat. For DAB IHC, sections were deparaffinised by 30 min incubation at 65 °C followed by xylene, and decreasing gradients of ethanol (100% to 70%), and rinsed in dH_2O. Antigens were retrieved by boiling in citric buffer (10 mM, pH 6) before endogenous peroxidases were blocked with 3% H_2O_2. Unspecific binding was blocked by incubating sections in serum-free protein block (Aglient Dako, CA, USA) for 1h in room-temperature (RT) followed by 4 °C overnight incubation with primary antibodies (the used antibodies were directed against platelet endothelial cell adhesion molecule (CD31) microtubule-associated protein-2 (MAP-2), CLDN5, and OCLN, diluted in PBS/0.05% Tween20 (see Table 1). After incubation with the appropriate biotinylated secondary antibodies (Vector Laboratories CA, USA) for 1h at RT, the staining was enhanced by treatment with Vectastain Elite ABC HRP kit (Vector Laboratories). Finally, sections were dehydrated in gradients of ethanol (70-100%) followed by xylene and mounting in Pertex xylene-based mounting media (Histolab). For fluorescent IHC; deparaffinization, antigen retrieval, blocking, and antibody-incubations were performed as described above before mounting with ProLong Gold Antifade with or without DAPI (ThermoFisher, MA, USA). Between all staining steps, sections were washed three times with PBS/0.05% Tween20 (except for after blocking).

Table 1. The antibodies used for immunohistochemistry.

Primary antibodies	Secondary antibodies	Manufacturer (primary antibody)
Mouse-anti-MAP2 (1/1000)	Biotinylated horse-anti-mouse (1/250)	Sigma-Aldrich, M4403
Mouse-anti-rat CLDN5 (1/1000)	Goat-anti-mouse AF 594 (1/250)	ThermoFisher, 4C3C2
Rabbit-anti-rat OCLN (1/500)	Goat-anti-rabbit AF 488 (1/250)	ThermoFisher, 71-1500
Rat-anti-mouse CD31 (1/100)	Donkey-anti AF 594 (1/250)	BD Pharmingen, MEC 13.3

DAB-stained sections were imaged and photographed with a BX60 microscope equipped with a TH4-200 light-source using the cellSens software (Olympus, Tokyo, Japan) and fluorescently stained sections were examined with a Zeiss Axio Imager .Z2 equipped with Colibri 7 LED-light-source and a MRc AcioCam using the ZEN Blue software (Zeiss, Oberkochen, Germany).

Brain injury and tight-junction protein level

To test correlation of tight-junction protein levels and degree of brain injury, brains, CSF and blood plasma were collected from HI-animals (n = 12) 24 post-HI. The plasma and CSF were analysed for CLDN5 and OCLN with ELISA as described above while the brains were embedded in paraffin and sectioned to assess

the brain injury. Grey matter tissue loss in the injured hemisphere was determined in brightfield-micrographs of coronal brain-sections stained for the neuron- and dendrite-marker Microtuble-associated protein 2 (MAP2). The images were analysed in ImageJ by delineating regions of interests encompassing the entire injured or uninjured hemispheres and measuring the MAP2 positive immunoreactivity in each hemisphere by investigators blinded to which groups and animals the images belonged to. The percentage of tissue loss in each level were calculated from the MAP2-positive area with this formula: $(MAP2_{uninjured} - MAP2_{injured})/MAP2_{uninjured}$ X 100(30). In all animals, the analysis was performed at six levels encompassing the entire brain and the mean tissue loss of all levels was used in the correlation analysis.

Statistics and graphs

Statistical analyses were made using GraphPad Prism version 8.00 for Windows (GraphPad Software, CA, USA). We used one-way ANOVA with Dunnett's multiple comparison test, and Pearson's correlations. The Benjamin-Hochberg method (FDR 0.1) was used to control for multiple correction problems when multiple t-tests were conducted. Tests are stated in Figure legends. Principal component analysis was made using Qlucore Omics explorer software (Lund, Sweden) where the built-in statistics module was used to test differences between sexes on variables. Images were processed in the Fiji build(28) of ImageJ(29), figures were designed in Affinity Photo and Designer (Serif Europe, West Bridgford, United Kingdom).

Results

HI induced caspase-3 activation in the injured brain hemisphere

The combination of left carotid artery ligation and global hypoxia produces a brain injury and tissue loss in the left hemisphere. To confirm injury in all animals the activity of caspase-3, a hallmark of apoptosis, was measured in homogenates from both injured (left) and uninjured (right) hemisphere of HI and naïve control animals (Additional file 2). Virtually no caspase-3 activity was detected in neither the uninjured hemispheres of HI-animals nor in hemispheres of the naïve controls while a significant increase in caspase-3 activity was seen in the injured hemisphere of HI-animals 6h after HI compared to the uninjured hemisphere ($p=0.0344$) or naïve control animals ($p=0.028$). Caspase-3 activity was further increased in the left hemisphere at 24h after HI compared to 6h after HI ($p=0.0087$), controls ($p=0.0027$) as well as the uninjured hemisphere ($p=0.0060$). The range of caspase activity for the 6h and 24h post-HI groups were 3-77 and 10-950 pmol AMC/min x mg protein, respectively.

HI-injury resulted in temporally increased levels of circulating tight-junction proteins CLDN5 and OCLN in cerebrospinal fluid and plasma and ZO-1 in CSF.

Levels of CLDN5, OCLN and ZO-1 were measured in CSF and plasma with ELISA at 6, 24h and 5 days after HI and all time-points were compared with a naïve control group collected and analysed at the same time and on the same ELISA-plate (Fig. 1). We could detect CLDN5 and OCLN proteins in all samples with

levels ranging between ~41 to 1300 pg/ml (plasma), ~100 to 2400 pg/ml (CSF) for OCLN and ~2 to 30 ng/ml (plasma), ~30 to 53 ng/ml (CSF) for CLDN5. Elevated CLDN5-levels were detected in CSF (Fig. 1d) at 24h post-HI (p=0.0082), while plasma-concentrations (Fig. 1c) were higher than controls at 6h (p=0.0427). There were no difference at later times between HI and controls. Similarly, OCLN concentration in CSF (Fig. 1b) was raised at 6h (p=0.0026) after HI while the levels were higher in plasma (Fig. 1a) of HI-animals at 24h (p=0.0285). ZO-1 was only detectable in 47% of CSF samples. We found great variably in levels between plasma with as high as 2000pg/mL in one animal but no differences were measured across groups at any time point after HI (Additional file 3). Principal component analysis was performed (dimension reducing) as means of discriminate analysis with the input of all above measured variables for OCLN and CLDN5. HI and control animals were grouped into three distinct groups at 6h and 24h after HI (Fig. 2a). At 5 days post-HI (Fig. 2b), PCA could not discriminate between HI and controls.

To test effect of sex on levels of TJ-proteins we performed two-group comparison between male and female animals using all samples and time as eliminating factor. Male rats showed significantly higher levels of OCLN in plasma (p=0.035) and CLDN5 in CSF (p=0.036) than female rats (Fig. 2c). In HI-animals, the average amount of OCLN in plasma were ~700 pg/ml for males and ~400 pg/ml for females, for CLDN5 in CSF the numbers were ~36 ng/ml for males and ~26 ng/ml for females.

Dynamic changes in BBB function following HI in neonates

To determine changes in BBB function over time after HI we performed measurements of BBB-permeability in different brain-regions (i.e. hippocampus, cortex, and striatum/thalamus) by quantifying the permeability for ^{14}C-labelled sucrose across the BBB. We previously showed (10) that the BBB in the uninjured hemisphere is not altered. We confirmed this also for the 5-day time-point (Additional file 4) corroborating with caspase-3 activation (Additional file 2) in current study.

Increased BBB permeability occurred at 6h post-HI in the cortex (1.10±0.05, p=0.0007), hippocampus (1.07±0.08, p=0.0277), and striatum/thalamus (1.05±0.05, p=0.0111) and was also significantly higher at 24h in both hippocampus (1.12±0.12, p=0.0486) and cortex (1.18±0.28, p=0.0474) while the striatum/thalamus appeared unaltered (1.01±0.07, p=0.7624) (Fig. 3a). Mentionable is that these concentration ratios are probably somewhat affected by the edema occurring in the injured hemisphere after the insult meaning that the magnitude of BBB permeability increase is likely marginally higher than what these ratios reflect. The concentration ratios 5d post-HI was significantly lower the injured cortex (0.88±0.12, p=0.0191) as well as the entire injured hemispheres (0.91±0.10, p=0.0303) (Fig. 3b).

BBB disruption following HI were confirmed via a qualitative assessment of EB-bound albumin extravasation into the brain parenchyma in brain sections. Animals injected with EB at 6h post-HI showed fluorescence in areas around the injury (fig. 3d) while the uninjured hemisphere showed weak fluorescence restricted to blood vessels (fig. 3e). Naïve control animals injected with EB and not perfused

showed distinct blood vessels-restricted fluorescence (fig. 3f) while naïve animals not injected with EB showed no signal.

The vascular density of the brain is not altered five days after HI

Since cerebrovascular area, which is the effective surface area for exchange between blood and brain, can affect our measurements of BBB-permeability we developed an in-house written macro for blood vessel analysis in CLDN5 (as a vascular marker) immunolabelled sections. We specifically wanted to estimate cerebrovascular area at later times after injury since there is loss of brain tissue and potentially blood vessels. Sections from brains 5 days post-HI were used to calculate the area of blood vessels in the brain. Two levels were analysed per animal and results averaged (Fig. 3c). For this analysis, the hippocampus and cortex results were combined in each hemisphere. Combining control animals and the uninjured hemisphere of HI-animals, 2.01±0.64% of the brain area was comprised of vessels while the density of vessels in the injured hemisphere was 2.05±0.61%. No significant differences were detected between control and injured hemispheres ($p>0.05$).

CLDN5 and OCLN immunoreactivity is not altered in the cerebral blood vessels of neonatal rats

Given that there are reports of changes in TJ-protein immunoreactivity following brain injuries, we performed double immunofluorescent labelling of CLDN5 (Fig. 4a) and OCLN (Fig. 4b) together with blood vessel marker CD31 (platelet endothelial cell adhesion molecule) in brain-sections collected 6h after HI (time chosen given BBB changes). In control animals, we found robust immunoreactivity of TJ-proteins in vessels in all brain regions examined including the cortex, hippocampus, and striatum/thalamus, while no labelling was detectable in parenchyma of the brain. Likewise, in animals after HI we found immunolabelling of blood vessels across all brain regions including infarct regions with no apparent changes compared to control animals.

CLDN5 levels in CSF correlates with brain-injury severity 24h after HI

The correlation between circulating TJ-levels and brain injury severity were investigated 24h after HI by analysing plasma- and CSF-levels of TJ:s and simultaneously quantifying the loss of grey matter in the brains of the same animals. This time-point was chosen based on the earlier ELISA-results. The average brain tissue loss in the HI-group ($n=12$) varied from 24.7 to 59.9% (one representative level is shown in Fig. 5b and d, respectively). By using the Pearson correlation on the levels of circulating TJ:s and the tissue loss percentages (Fig. 5a), the levels of CLDN5 in CSF was found to significantly ($p=0.016$) correlate with the severity of the brain injury ($r=0.702$) while no significant correlations was found with OCLN-levels (data not shown).

Discussion And Conclusions

Neonatal HI leading to HIE is still a major cause of child mortality and the development of severe disabilities. The brain vasculature is a central component in HI associated brain injuries that appears

damaged early in the injury process. Both preclinical and clinical studies indicate loss of BBB function in response to neonatal HI. However, diagnostic tools to evaluate brain vascular health in neonates in an early, affordable, accessible, and reliable manner are lacking. Given that TJ-proteins have shown promise as biomarkers in adult stroke, we wanted to test these in the setting of neonatal brain injury. We show that elevated levels of OCLN and CLDN5 in both CSF and plasma in response to loss of blood-brain barrier function and that CLDN5 levels in CSF correlates with brain-injury severity 24h after HI. Our data show that loss of BBB function is at least partially due to damage to endothelial tight-junctions after HI injury. The data suggest that the intermembrane TJ-proteins CLDN5 and OCLN are putative blood or CSF biomarkers for cerebral vascular health and brain damage in neonates.

The cerebrovasculature is specialised in that it harbours a range of barrier and transport mechanism not found in peripheral blood vessels, which compartmentalises the brain from the rest of the body so that brain cells can function in a controlled environment and provides protection for the brain from potential harmful blood solutes. These mechanisms are normally referred to as the blood-brain barrier and an essential part are the tight-junctions localised to the luminal side of the inter-endothelial cleft forming a physical barrier between blood plasma and brain.(31) The protein architecture of these junctions is complex with both inter- and intracellular elements. In this study we focused on two intracellular TJ-proteins, CLDN5, which are specific and essential for normal BBB function(32) and, OCLN, which plays a role in BBB modulation(33), as well as the intercellular scaffold protein ZO-1.

For the first time we made measurements of both BBB function together with measurements of TJ-proteins in both CSF and plasma in order to interpret data in an integrated manner. We found dynamic changes in both BBB function and TJ-proteins levels following HI.

Detection of Evans blue in the injured region of HI-injured animals confirmed BB-disruption following HI and the BBB permeability was acutely increased following HI in rat neonates agreeing with earlier rat studies(11, 34, 35) and with a similar time-line to what we have seen in mice(10).

Clinical data show about five times higher albumin CSF/blood ratios of babies diagnosed with HIE indicative of BBB damage also in human newborns (Kumar et al., 2008). Overall, the greatest changes in BBB-opening appeared to be in the cortex, the region most affected in this HI-model of rats(36). Thus, not surprisingly and likewise similar to the mouse, we see a correlation between severity of injury and BBB opening in the different brain regions. OCLN in CSF were significantly increased 6h after HI while levels in plasma increased at 24h. Levels of CLDN5 on the other hand were elevated in plasma at 6h post-HI, while levels in CSF were higher than normal at 24h. Levels of CLDN5 and OCLN appeared to be normalised in both plasma and CSF five days after HI. High levels of ZO-1 could only be measured in the CSF in some of the animals and was not detectable in plasma, thus it appears that ZO-1, being an intracellular protein, is not as readily released into the blood after BBB damage. These raised levels of TJ-proteins in both CSF and plasma indicate that BBB dysfunction after neonatal HI injury is likely to be at least partially due to direct damage to the endothelial TJ:s, increasing paracellular permeability. Intriguingly, the raised levels of circulating OCLN and CLDN5 did not occur at the same time after HI showing that these proteins

although normally intimately localised do not appear to be released into these biofluids in the same manner. Further studies will be needed to define the molecular alterations and/or damage to the tight-junctions to understand this paradigm. Our previous results in neonatal mice are in agreement with studies in both adult humans and rats after injuries related to the brain vasculature where raised levels of TJ-proteins were detected shortly after brain insults.(12, 13). The plasma results are similar with the report from Pan et al(12) that elevated levels of CLDN5 could not be detected in blood plasma until 4.5h after the induction of ischemic stroke via middle-cerebral artery occlusion (MCAO) in adult rats. Their levels were more markedly elevated than in our neonatal HI animals but it may be as a result of the MCAO model which more directly involves vascular damage. They also report loss of immunoreactivity of OCLN from brain blood vessels, which we could not detect, further indicating more severe BBB damage than in our model. Furthermore, we tested whether levels TJ proteins could reflect severity of injury, choosing the 24h time-point since our results indicated particularly raised levels of CLDN5 at this time. This showed that levels of CLDN5 in CSF correlated with the severity of brain injury with a correlation coefficient of 0.70 at 24 hours while OCLN levels showed no correlation. Taken together our study shows that even a moderate opening of BBB in this model results in raised levels of TJ-proteins at early time points after HI which suggests that TJ-proteins are released into the circulation in the early stages of BBB-damage and could act as biomarkers for vascular integrity and possibly also be useful as brain injury predictor. Our results also indicated a sex difference where males had higher levels of circulating OCLN in plasma and CLDN5 in CSF. This resonates well with previous studies that have shown that there is a tendency for male rats to have graver injuries than females after neonatal HI in an almost identical model to the one employed in this study(37) and it is also known that male human infants have a higher risk for HIE.(38) CLDN5 and OCLN showed different patterns of release into biofluids indicating that measuring both of them in tandem would give a better interpretation of injury to the brain vasculature after HI. We therefore performed discriminate analysis showing that HI-animals grouped together and were separate from controls up to 24h after HI when the levels of CLDN5 and OCLN in both plasma and CSF were analysed together.

While TJ proteins seem to have potential as standalone biomarkers for neonatal cerebral vasculature health many studies, as reviewed in Douglas-Escobar and Weiss(39), Chalak(40), and Lv(9) , have focused on biomarkers related to inflammation and brain injury. As the reviews state, the best course of action will most likely be to develop a panel of different markers in conjunction with diagnosis and other monitoring methods of neonatal brain injuries. Promising biomarker-candidates for assessing HIE-brain injuries includes neuronal injury markers such as Tau and neurofilament light proteins(41), brain injury marker protein S100(42), and inflammation-related cytokines like IL-6 and IL-8(43). From the data of our study, we believe the inclusion of markers for vascular health in combination with markers for inflammation and injury could increase such a panel's discriminatory and predictive power.

Intriguingly, our results indicated that BBB function does not normalise at later times after HI but instead there is an apparent decrease in permeability below normal levels five days after the insult, however, these measurements should be interpreted with caution. We first believed that this could be due to a loss of blood vessels in brain tissue, which would reduce the surface area for exchange and thus reduce flux

between blood and brain. We therefore estimated the cerebrovascular area at 5 days after HI, which showed that the tissue remaining in the injured hemisphere did not differ in vascular area to control animals, thus, this unlikely explains the difference in measured BBB permeability. From our previous study in neonatal mice we found an upregulation of both CLDN5 and OCLN genes following HI injury, possibly a response to normalise barrier function, that may underlie a decrease in barrier permeability. However, to understand this fully, future studies should investigate the functionality of the vessels that are present during this time of injury progression when both injury and reparative functions are ongoing. Overall however, the dynamic changes we detected in BBB exchange are likely to influence the efficacy of many treatments showing increased rate of entry into the brain in the acute phase followed by decreased entry rate in the later stages of the injury process.

A limitation of the study is inherent to this model-system of neonatal HI which is well known to have large inter-animal variability (44), evident in the measurements of activated caspase-3 in the injured hemispheres. Furthermore, the animals also only develop brain injury in the HI hemisphere while no tissue loss is seen in the hemisphere only subjected to hypoxia, unlike human infants which often develop more generalized brain injuries (45).

In summary, we found temporally elevated levels of circulating tight-junction proteins in blood and CSF following HI brain injuries in neonatal rats coupled with dysfunction of the BBB, likely in part due to the loss of tight-junction proteins from cerebral blood vessels. We also found dynamic changes in entry rate from blood to brain after HI-injury that may affect efficacy of neuroprotective treatments. The results signify the potential for detecting vascular proteins in general and BBB proteins specifically as biomarkers for cerebrovascular integrity and brain injury in the neonate.

Abbreviations

BBB – Blood-brain barrier

CLDN5 – Claudin-5

EB- Evans blue

HI – Hypoxia/ischemia

HIE – Hypoxic/ischemic encephalopathy

MAP2 - Microtubule Associated Protein 2

OCLN – Occludin

PND# - Post-natal day #

TJ – Tight-junction(s)

ZO-1 – Zonula occludens-1

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Gothenburg Committee of the Swedish Animal Welfare Agency (Application nos. 663/17) and performed in accordance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The data behind the conclusions of this study are available from the corresponding author upon reasonable request.

Competing interests

The Authors declares that there is no conflict of interest with respect to the research, authorship, or publication of this article.

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Authors' contributions

CJE and CM conceived and designed the study and supervised all aspects of the study. EAA and CJE performed animal experiments. EAA performed *ex vivo* experiments, image- and statistical- analyses. Results were interpreted by EAA, CJE, and CM. EAA wrote the initial draft of the paper with input, revisions, and approval from all authors. Correspondence to be addressed to CJE.

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