Impaired intestinal permeability in patients with multiple sclerosis

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Background. A number of recent studies have shown that the intestinal microbiome, part of the brain-gut axis, is implicated in the pathophysiology of multiple sclerosis. An essential part of this axis, is the intestinal barrier and gastrointestinal disorders with intestinal barrier dysregulation appear to be linked to CNS demyelination, and hence involved in the etiopathogenesis of multiple sclerosis (MS).
Objective. The aim of this study was to evaluate the integrity of the intestinal barrier in patients with clinically definite multiple sclerosis (CDMS) and clinically isolated syndrome (CIS) using two serum biomarkers, claudin-3 (CLDN3), a component of tight epithelial junctions, and intestinal fatty acid binding protein (I-FABP), a cytosolic protein in enterocytes.
Methods. Serum levels of CLDN3 in 37 MS patients and 22 controls, and serum levels of I-FABP in 46 MS patients and 51 controls were measured using commercial ELISA kits. Complete laboratory tests excluded the presence of gluten-related disorders in all subjects. Thirty MS patients received either disease-modifying drugs (DMD), immunosuppression (IS) or corticosteroid treatment.
Results. CLDN3 levels were only significantly higher in the MS patients treated with DMD or IS compared to the control group (*P*=0.006). There were no differences in I-FABP serum levels between the groups. Serum CLDN3 levels did not correlate with serum I-FABP levels in CDMS, in CIS patients or controls.
Conclusions. In multiple sclerosis patients, the intestinal epithelium may be impaired with increased permeability, but without significant enterocyte damage characterized by intracellular protein leakage. Based on our data, CLDN3 serum levels appear to assess intestinal dysfunction in MS patients but mainly in treated ones.

Key words: multiple sclerosis, clinically isolated syndrome, claudin-3, I-FABP, intestinal permeability

Received: April 3, 2023; Revised: July 10, 2023; Accepted: July 13, 2023; Available online: August 11, 2023
https://doi.org/10.5507/bp.2023.033

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INTRODUCTION

Multiple sclerosis (MS) is a serious chronic neurological disease with the characteristics of autoimmunity, inflammation and neurodegeneration leading to progressive disability[1].

Its etiology is believed to be multifactorial. Besides immunological mechanisms, epigenetic and environmental factors are being investigated[1-5]. Several studies have reported on the role of a disturbed intestinal barrier and intestinal microbiome as a trigger or a consequence of MS (ref.[6,7]). Tlaskalová-Hogenová et al. for example, postulate that activation of the innate immune system by dietary proteins or components of the gut microbiota could participate in the impairment of intestinal barrier function and in the development of inflammatory and autoimmune diseases[8].

Our preliminary data showed higher levels of intestinal permeability markers in clinically isolated syndrome (CIS) which is a first episode of neurologic symptoms like MS compared to a healthy population[9].

Various options are available for assessing intestinal barrier alterations. One group of tests uses oral administration of various substances such as polyethylene glycols 400 Da or suitable monosaccharides or disaccharides and their determination in urine[10-12]. A more practical possibility is to use biomarkers that can be determined in serum/plasma or feces. These include for example, fatty acid binding proteins (FABP), occludins, claudins, zonulin, glucagon-like peptide (GLP)-2, and others[13,14].

Claudins are transmembrane proteins that form the major structural and functional component of tight junctions[15]. Twenty-seven mammalian claudin genes have thus far been described, 26 of which have been found in humans[16,17]. Claudin-3, a ubiquitous protein, is expressed in epithelia and endothelia of various tissues[16]. In the mammalian intestines, high expression is described in the large intestine, but its expression has also been found in the

small intestine[15]. Decreased expression of predominantly claudin-3 has been found in Crohn's disease and acute colitis, while both decreased and elevated expression has been reported in celiac disease (CD)(ref.[15,18]).

I-FABP (intestinal acid binding protein) is a small soluble intracellular protein located in the enterocytes and serves as the transporter of fatty acids within cells. I-FABP mRNA is detectable along the entire length of the small intestine[19]. On enterocyte damage, I-FABP is quickly and readily released from cells into the circulation. I-FABP has suitable properties for use as a biomarker of enterocyte damage due to its tissue specificity, low molecular weight and high content within cells[14,20]. I-FABP is considered to be a biomarker of intestinal epithelium injury[21-23]. I-FABP plasma/serum levels increase significantly in various intestinal diseases, including celiac disease[24-26] and, ischemia and inflammation[27,28].

A possible link between neurological diseases and increased intestinal permeability has been studied (both in human and animal models) in some neurodegenerative and psychiatric conditions[29-31]. However, the results of sporadic clinical trials are not conclusive[32-34].

In this study, we focused on a group of patients with clinically definite multiple sclerosis (CDMS) and clinically isolated syndrome (CIS) or the first episode of neurological symptoms which look like MS and which have a high probability of conversion to CDMS. We hypothesized that MS may be related to an altered intestinal barrier both in the early stage of the disease, and in CDMS, and that the early treatment of MS can also alter the barrier. The role of the pharmacotherapy (as a potential disruptor of intestinal permeability) in early CDMS or CIS was taken into consideration.

Enterocyte damage in our patients was examined by I-FABP serum levels, and increased intestinal permeability was evaluated by CLDN3 in serum. The subjects were additionally tested for celiac disease-associated auto-antibodies and anti-gliadin antibodies to identify the presence of gluten-related disorders, which may also affect intestinal permeability.

PARTICIPANTS AND METHODS

Participants

Ninety-seven participants examined in the Department of Neurology and Department of Internal Medicine, 3rd Faculty of Medicine of Charles University, Prague, Czech Republic, were recruited into our study. Subjects were divided into two groups. One group were patients with CDMS or CIS (n=46, age: 34±6 years, mean±SD; sex: 38 females, 8 males; CDMS n=30, CIS n=16). Experienced neurologists made the diagnosis. All patients met the criteria for MS (ref.[35,36]). Patients with CIS had experienced one episode of MS like symptoms with objective clinical evidence.

The CDMS and CIS group included two subgroups of patients differentiated on the basis of their pharmacotherapy. One group were patients who were receiving no current treatment but who had received i.v. methylprednisolone treatment which had been discontinued for at least one month (n=16). These patients were mostly at the disease onset stage prior to the start of specific treatment.

In the treated subgroup (n=30), 25 patients received glatimer acetate or interferon beta, and 5 patients received immunosuppression (azathioprin) or oral corticosteroids.

The patients underwent a baseline laboratory and clinical examination focused on objective neurological findings, including the Expanded Disability Status Scale (EDSS).

The control group were 51 healthy subjects (age: 33±7 years, mean±SD; sex: 32 females, 19 males).

In all subjects, possible celiac disease and/or gluten sensitivity using total IgA, anti-t-TG (anti-tissue transglutaminase antibodies) IgA, anti-t-TG IgG, EMA (anti-endomysial antibodies) IgA, EMA IgG, AGA (anti-gliadin antibodies) IgA and AGA IgG were excluded.

The CLDN3 and I-FABP biomarkers were determined in the serum of the participants to assess the permeability and integrity of the intestinal barrier.

Sera were obtained in a standard manner. Serum aliquots were stored at -80°C until laboratory analyses. Subjects signed an informed consent to participate in the study. The study was approved by the Ethics Committee of the Kralovske Vinohrady University Hospital, Charles University, Prague (EK-VP/16/0/2014).

Immunochemical analyses

CLDN3 and I-FABP serum levels were measured using commercial ELISA kits. The ELISA kit for CLDN3 (Cloud-Clone Corp., Houston, TX, USA) with a 0.112 ng/mL sensitivity and the Human I-FABP ELISA kit (HycultBiotech, Uden, the Netherlands / Wayne, PA 19087, USA) with 47 pg/mL limit detection were used for CLDN3 and I-FABP determination. ELISA assays were performed according to the manufacturer's instructions.

Serological markers of CD (total IgA, anti-t-TG, anti-EMA, anti-DGP and AGA antibodies) were analyzed using a commercially available ELISA kit routinely used in the hospital immunology laboratory.

Statistical methods

The normal distribution of the variables was checked using the Shapiro-Wilk test. Since not all variables showed a normal distribution, we used nonparametric methods for statistical analyses. The 10^{th} and 90^{th} percentile medians were used to describe the basic statistical characterization of the data. The Kruskal-Wallis test was used to analyze the differences among more than two groups. The Mann-Whitney test was used in the case of 2 groups, or as a post-hoc comparison if the p-value in the Kruskal-Wallis test was <0.05. The relationships between variables were assessed by Spearman coefficients. The level of significance was established at 0.05.

Statistical analyses were performed using MedCalc statistical software (MedCalc Software Ltd, Ostend, Belgium).

Table 1. CLDN3 and I-FABP concentrations in patients with clinically definite multiple sclerosis and clinically isolated syndrome and in controls.

Patients (CDMS and CIS)	Groups divided based on therapy		Groups divided based on disease courses		Controls[a,b]
	Patients with therapy	Patients without therapy	CIS	CDMS	
CLDN3 (ng/mL)					
(n=37)	(n=24)	(n=13)	(n=13)	(n=24)	(n=22)
17 (12–23)	18 (13–25)	15 (11–19)	18 (12–22)	17 (12–25)	15 (10–20)
I-FABP (pg/mL)					
(n=46)	(n=30)	(n=16)	(n=16)	(n=30)	(n=51)
111 (0–366)	137 (0–363)	58 (0–430)	58 (0–256)	177 (0–381)	84 (0–380)

Data are presented as median (10^{th} to 90^{th} percentiles).
CLDN3, claudin-3; I-FABP, intestinal acid binding protein; CDMS, clinically definite multiple sclerosis; CIS, clinically isolated syndrome; n = number.
[a]*P*=0.02 vs. patients (CDMS and CIS).
[b]*P*=0.006 vs. patients with therapy.

RESULTS

The basic demographic and clinical characteristics of the participant

The basic demographic characteristics of the participants are presented in Table 2. There were no statistically significant differences in age between CDMS and CIS patients in comparison to the control group. The clinical characteristics of the patients are summarized in Table 3. The neurological disability (EDSS) of the patients ranged from 0 to 4.

CLDN3 and I-FABP serum levels in patients with CDMS + CIS and controls

The levels of claudin-3 and I-FABP in the different MS patient groups and controls are summarized in Table 1.

CLDN3 serum levels were significantly higher in CDMS + CIS patients than the controls (*P*=0.02). There were no statistically significant differences between the CIS, CDMS and control groups (*P*=0.07). The lowest CLDN3 levels were found in the control group, while increased CLDN3 levels were observed in the CIS and CDMS groups (Table 1).

When we compared the control group with the subgroup of treated MS patients, we found statistically significantly increased (*P*=0.006) CLDN3 levels in the treated subgroup, while no significant differences in CLDN3 levels were found between the control group and MS patients without treatment (Fig. 1). The difference between the treated MS patient group and untreated MS patient group was not significant at *P*=0.05 but the p-value was borderline (*P*=0.06) (Fig. 1).

I-FABP serum levels did not differ between the CDMS + CIS group and the controls, although the median in the patient group was higher. No other significant differences were found between controls and treated or untreated patients or among the CIS, CDMS and control groups (Fig. 2).

Correlation analyses

As the I-FABP serum levels were not increased, CLDN3 serum levels did not correlate with I-FABP serum

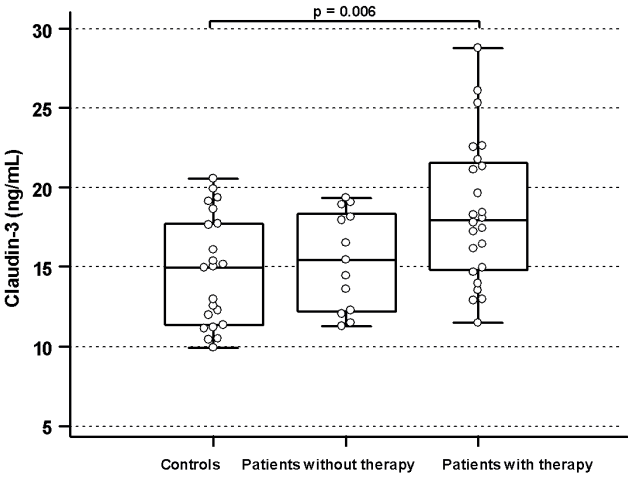

Fig. 1. Serum levels of claudin-3: a comparison between patients with therapy, patients without therapy and controls.
The central box defines the values from the 25^{th} to 75^{th} percentiles. A horizontal line is drawn at the median (valid for all graphs).

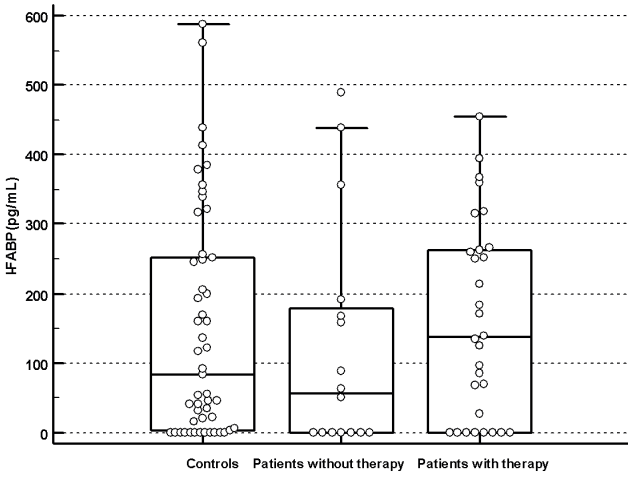

Fig. 2. Serum levels of I-FABP: a comparison between patients with therapy, patients without therapy and controls.

Table 2. Demographic and therapeutic characteristics of the control group and patients with clinically definite multiple sclerosis, and with clinically isolated syndrome including therapy.

Group	Patients CDMS+CIS		Controls	p
Number	46		51	
Age				
Median (years)	34		32	n.s.
10^{th} to 90^{th} percentile (years)	25–41		26–42	
Sex:				
Female/Male (n)	38/8		32/19	0.04*
Therapy	With	Without		
Number	30	16	0	

CDMS, clinically definite multiple sclerosis; CIS, clinically isolated syndrome; p, probability of a difference, n.s., not significant.
*Fisher's exact test

Table 3. Clinical characteristics of patients with clinically definite multiple sclerosis and with clinically isolated syndrome including treatment subdivision.

Group	CDMS	CIS	CDMS+CIS treated	CDMS+CIS untreated
number	30	16	30	16
Mean EDSS±SD	1.8±0.73	1.66±0.55	1.78±0.69	1.69±0.62
Number of OCB in CSF				
2 and more	20 (66.7%)	14 (87.5%)	20 (66.7%)	14 (87.5%)
Less than 2	3 (10.0%)	1 (6.3%)	3 (10.0%)	1 (6.3%)
Unknown	7 (23.3%)	1 (6.3%)	7 (23.3%)	1 (6.3%)
MRI findings				
DIT	21 (70.0%)	1 (6.3%)	18 (60.0%)	5 (31.3%)
DIS	28 (93.3%)	10 (62.5%)	25 (83.3%)	12 (75.0%)

CDMS, clinically definite multiple sclerosis; CIS, clinically isolated syndrome; EDSS, expanded disability status scale; SD, standard deviations; OCB, oligoclonal bands; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; DIT, dissemination in time; DIS, dissemination in space.

levels either group (CDMS + CIS patients, controls) or subgroups of patients (DMD treated, DMD untreated). No correlations between EDSS and CLDN3 or I-FABP levels were found.

Serological gluten-related antibodies

The results of serum antibodies for CD and gluten sensitivity were negative for all subjects.

DISCUSSION

In this study, two proteins (CLDN3 and I-FABP) were measured in the serum of patients with CDMS or CIS to assess their intestinal barrier permeability or integrity. CLDN3 levels were significantly higher in CDMS + CIS patients compared to the healthy control groups, especially in the subgroup of treated patients. Based on these data, determination of CLDN3 appears to be valuable in evaluating the intestinal barrier in treated MS patients. However, I-FABP (a biomarker of intestinal integrity) levels in serum did not distinguish groups or subgroups. Celiac disease and gluten sensitivity were excluded in all cases. That is, our results were not influenced by gluten-mediated enterocyte disruption.

The intestinal barrier and gut microbiota are postulated to participate in the etiopathogenesis of MS (ref.[37]). In one study, an alteration of the intestinal permeability was reported in relapsing-remitting MS (ref.[10]). However, no changes in gut barrier using dimethyl fumarate treatment were observed: some cases improved, while others worsened during follow-up without a clear longitudinal pattern[12]. Claudins, as membrane proteins of tight epithelial junctions, are involved in the regulation of epithelial permeability. The tissue specificity of CLDN3 is lower than the intestinal-specific biomarker I-FABP. For example, in addition to the gastrointestinal epithelium, CLDN3 is also localized in the epithelia of the respiratory and urinary tracts[16]. Therefore, non-intestinal system disorders can contribute to increased CLDN3 levels. However, studies on CLDN3 plasma/serum or urinary levels are unclear. Elevated CLDN3 plasma and urinary levels have been found in patients with liver diseases and in children undergoing major non-abdominal surgery[38-40]. Our patients had no obvious signs of urinary tract or respiratory system disease, but they did not undergo any specific relevant examinations. This study showed significant elevation of CLDN3 serum in MS patients treated with either immunosuppressive or DMD drugs. This could be caused by the treatment itself, as well as by the progression of the

disease. There were more patients with CDMS, with a higher EDSS and signs of dissemination over time in the treated patient group. The design of our study does not allow us to draw conclusions as to whether therapy affects the intestinal barrier in MS patients. However, similar to our results, indirect evidence suggesting that DMD therapies may influence the status of the intestinal barrier has been widely reported[10,12,33,41]. The increased CLDN3 in the subgroup of treated patients compared to untreated patients was near statistical significance. Further, we found no difference in CLDN3 levels between patients with CIS and CDMS. The number of CIS patients was small and they were not differentiated with respect to the further development of the disease, and we thereby only consider this to be a preliminary result and further analysis is needed. Buscarinu et al.[12] found variable intestinal barrier alteration in MS patients treated with dimethyl fumarate.

Importantly, to evaluate serum/plasma levels in neurological diseases, it will be necessary to consider that CLDN3 is a constituent of tight choroidal plexus junctions and may play a crucial role in the integrity of the blood-cerebrospinal fluid barrier (BCSFB). This assumption has been verified in both animal and human models[42-46]. The expression of CLDN3 in the choroid plexus was decreased in MS patients in comparison to healthy controls. In animal models, mice lacking CLDN3 showed earlier onset and worse progression of autoimmune encephalomyelitis based on increased leukocyte transition through BCSFB in the choroidal plexus[44]. In another animal study, CLDN3 was found to be a part of the BCSFB but played no important role in the blood brain barrier[43,46]. Increased CLDN3 cerebrospinal fluid levels may also reflect BCSFB damage, however in our study we focused on and found increased levels of CLDN3 in sera of treated MS patients, therefore we cannot validate this potential connection. To our knowledge, neither CLDN3 cerebrospinal fluid nor serum levels have been studied to date.

Using I-FABP, the status of the gut barrier was also evaluated in patients with various forms of MS. As in our study, non-elevated I-FABP levels have already been reported in animal models and MS patients[47,41]. In contrast, Saresella et al. observed significantly increased I-FABP levels in MS patients[48]. However, only patients with relapsing-remitting and secondary progressive MS were included in the latter, in contrast to our cohort involving not only patients with advanced stages, but also early stages of the disease. It can also be assumed that increasing I-FABP levels require more pronounced damage to the intestinal barrier than the CLDN3 levels.

Camara-Lemarroy et al.[49] believe that since the intestinal barrier is a complex multilayered structure, the use of only one biomarker may not be sufficient to assess it. This assumption is also supported by our study, in which we only demonstrated an increase in one intestinal barrier indicator. The same intestinal surrogate markers were used by Sikora et al. as in our study[50]. These authors showed in patients with psoriasis, another immunologically mediated extra intestinal disease, that the intestinal barrier was disrupted which was reflected in elevated I-FABP and CLDN3 serum levels. This was associated with gastrointestinal symptoms and signs of systemic inflammation.

As we found no difference in I-FABP serum levels between MS patients and the control group, we can conclude that intestinal damage in our CIS and CDMS patients was not characterized by substantial enterocyte membrane damage leading to intracellular protein leakage.

We consider the patient group heterogeneity as the main limitation of our study. The patient group included those with CDMS, as well as a small sample of patients with CIS. Different kinds of treatments have variable mechanisms of action, which can also influence the intestinal barrier function. Although statistical comparisons between the subgroups of patients did not indicate a significant difference in any of the examined proteins or antibodies, the results could be different in a larger sample of MS patient subgroups. The relatively small sample size of our serum results limits our ability to draw conclusions of these findings to the broader MS population. Future prospective studies involving a representative sample of MS patients are needed to further validate these findings.

CONCLUSION

Intestinal barrier damage may be involved in the etiopathogenesis of MS. Serum CLDN3 level was dysregulated in multiple sclerosis patients and this may be used for assessing intestinal permeability impairment in MS patients with disease specific treatment; however, the influence on blood-cerebrospinal fluid barrier (so called gut-brain axis) and a damaged blood-cerebrospinal fluid barrier may play a role. Based on these assumptions it would be possible to consider CLDN3 as a serum surrogate marker that overall reflects tight junction damage in the epithelium at both locations.

CLDN3 testing may by useful for identifying CDMS or CIS patients who could profit from intestinal permeability modification via food supplements (probiotics, prebiotics and synbiotics).

More research will be needed in a larger cohort with a detailed evaluation of the intestinal and BCSFB to determine the benefit of CLDN3 serum measurement in patients with CIS and CDMS.

Acknowledgements: The study was supported by Research Project Charles University in Prague Cooperation 38 Neurosience, SVVV 260648/SVV/2023 and Medical Diagnostics and Basic Medical Sciences, by MH CZ DRO VFN 64165 (given by the Czech Ministry of Health). Funding sources had no involvement in things such as study design, data collection, analysis and interpretation, in the writing of the report or in the decision to submit the article for publication. Help with grammar correction was performed by the certified Czech translation company, Skřivánek, s. r. o.

Author contributions: IS, IH, LF: study conceptualization, design, interpretation of the findings and writing of the manuscript; PB, LF, DZ, PK: provided clinical and

serological data; LF: performed statistical analysis; LN: performed the immunochemical analyses of CLDN-3 and I-FABP; MZ, PB: manuscript writing; AB: provided a critical review of the article and part of the serum samples.

Conflict of interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of University Hospital Kralovske Vinohrady under number EK-VP/16/0/2014.

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