Original Article

Gut Barrier Dysfunction—A Primary Defect in Twins with Crohn’s Disease Predominantly Caused by Genetic Predisposition

Åsa V. Keita*, Carl Mårten Lindqvistb, Åke Östc, Carlos D. L. Maganaa, Ida Schoultzb, Jonas Halfvarsond

*Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden  
bDepartment of Medical Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden  
cDepartment of Pathology and Cytology, Aleris Medilab, Täby, Sweden  
dDepartment of Gastroenterology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

Corresponding author: Åsa V. Keita, PhD, Department of Clinical and Experimental Medicine, Division of Surgery, Orthopedics & Oncology, Medical Faculty, Linköping University, 581 85 Linköping, Sweden. Tel: 46-101-038-919; Email: asa.keita@liu.se

Abstract

Background and Aims: The aetiology of Crohn’s disease is poorly understood. By investigating twin pairs discordant for Crohn’s disease, we aimed to assess whether the dysregulated barrier represents a cause or a consequence of inflammation and to evaluate the impact of genetic predisposition on barrier function.

Methods: Ileal biopsies from 15 twin pairs discordant for Crohn’s disease [monozygotic n = 9, dizygotic n = 6] and 10 external controls were mounted in Ussing chambers to assess paracellular permeability to 51Chromium [Cr]-EDTA and tracer cellular passage to non-pathogenic E. coli K-12. Experiments were performed with and without provocation with acetylsalicylic acid. Immunofluorescence and ELISA were used to quantify the expression level of tight junction proteins.

Results: Healthy co-twins and affected twins displayed increased 51Cr-EDTA permeability at 120 min, both with acetylsalicylic acid [p < 0.001] and without [p < 0.001] when compared with controls. A significant increase in 51Cr-EDTA flux was already seen at 20 min in healthy monozygotic co-twins compared with controls [p≤0.05] when stratified by zygosity, but not in healthy dizygotic co-twins. No difference in E. coli passage was observed between groups. Immunofluorescence of the tight junction proteins claudin-5 and tricellulin showed lower levels in healthy co-twins [p < 0.05] and affected twins [p < 0.05] compared with external controls, while ELISA only showed lower tricellulin in Crohn’s disease twins [p < 0.05].

Conclusion: Our results suggest that barrier dysfunction is a primary defect in Crohn’s disease, since changes were predominantly seen in healthy monozygotic co-twins. Passage of E. coli seems to be a consequence of inflammation, rather than representing a primary defect.

Key Words: Crohn’s disease; barrier function; genetics
1. Introduction

Crohn’s disease (CD) is a chronic inflammatory bowel disease (IBD) of unknown aetiology that is characterized by a disruption of the intestinal barrier. The inflammation is deemed to arise at the intersection of genetic predisposition and factors related to the exposome. Mounting evidence indicates that the mucosal barrier plays a key role in maintaining gut homeostasis and that CD is characterized by a disruption of the barrier. An enhanced urinary secretion of ingested probe markers, reflecting increased paracellular permeability in CD, was reported by Bjarnason et al. as far back as 1983. In line with these data, an altered expression of the tight junction complex and redistribution of its constituents, including occludins, claudins and junctional adhesion molecules, have been reported more recently. Ex vivo studies of epithelial function, using the Ussing chamber technique, have identified an augmented mucosal uptake of non-pathogenic Escherichia coli, possibly involving both transepithelial and intercellular passage routes, in the follicle-associated epithelium from patients with longstanding CD.

The observations that augmented permeability is associated with increased risk of relapse in patients with CD, and that intensified medical therapy seems to normalize the permeability and relapse rate, might indicate that an abnormal barrier function is a consequence of inflammation. On the other hand, reports of altered paracellular permeability in quiescent disease without any signs of macroscopic inflammation, and in healthy first-degree relatives of CD patients, and identification of IBD-specific genetic variants related to barrier function, point to barrier regulation as a primary cause of CD. This hypothesis is supported by data from the IL-10 knockout and the SAMPI/YitFc mouse models, where epithelial defects seem to precede the inflammation and may cause intestinal inflammation, even in the presence of normal gut microbiota and immunity. To what extent this can be applied on humans is largely unknown, but the gut barrier represents one of the domains that will be explored in the international GEM cohort. This study aims to determine possible causes for CD by following healthy individuals who are at a higher risk of developing the disease over time. An interaction between environmental and hereditary factors affecting barrier function in patients with CD has also been proposed. Söderholm et al. showed that baseline permeability is determined by environmental factors, whereas permeability provoked by acetylsalicylic acid (ASA) is a function of the genetically determined state of the mucosal barrier. Similar to the effect of the stimuli that derange mitochondrial activity, such as toxins and psychological stress, in vitro data have shown that ASA induces metabolic and oxidative stress, and is accompanied by mitochondrial dysfunction. Thus, whether mucosal barrier dysfunction is the cause or the consequence of inflammatory activity in CD remains unknown. Similarly, the relative contribution of the genome and of factors related to the exposome to mucosal barrier function is yet to be explored.

Twin studies can be of great help in this respect. Monozygotic twins are genetically identical and share external environmental exposure during childhood. In contrast, dizygotic twins have on average only half of the genes in common but share environmental exposure during childhood and adolescence to the same extent as monozygotic twins. Thus, an increased permeability in healthy monozygotic twin siblings but not in healthy dizygotic twin siblings in discordant twin pairs with CD would reflect a genetically determined permeability in CD. On the other hand, if the observed increased permeability in healthy first-degree relatives is due to environmental factors, an increased permeability would be observed in both monozygotic and dizygotic healthy twin siblings in discordant twin pairs with CD.

The aims of this study were to assess whether intestinal barrier dysfunction is a primary defect or a consequence of inflammation, and to evaluate the influence of genetics and environmental exposure on paracellular and transepithelial uptake in twins discordant for CD.

2. Material and methods

2.1. Twins

Twins were identified from a previously described population-based cohort of twins with IBD. In short, twin pairs where at least one twin in each pair had been hospitalized for IBD were identified by linking the Swedish Twin Registry with the Swedish Hospital Discharge Register. Identified twins responded to a questionnaire regarding general gastrointestinal symptoms including a possible diagnosis of IBD. All medical notes were scrutinized, after written consent from each twin, for verification of diagnosis of IBD and for determination of disease phenotype, according to the Montreal Classification.

Zygosity was determined by the Swedish Twin Registry based on questionnaire data regarding intra-pair similarities in childhood, being of opposite sex, or DNA analyses. The questionnaire has been validated previously, and intra-pair similarities have ≥98% accuracy when compared with DNA analyses. Discordant twin pairs of same sex with CD, under the age of 75 years, living within the middle part of Sweden, and who had approved further contact, were invited to take part in the study and to undergo colonoscopy. Twins with previous extensive CD-related surgical intervention, i.e. colectomy or extensive small bowel resection, were excluded. The Harvey Bradshaw Index was employed to classify clinical disease activity. Endoscopic remission was defined as absence of aphthoid lesions, superficial ulceration, and deep ulceration, except in twins who had undergone previous ileal resection where a Rutgeerts score <2 was used as the definition.

2.2. External controls

Similarly, non-related individuals with macroscopically normal ileal mucosa undergoing colonoscopy for polyp surveillance functioned as external controls. Exclusion criteria were disease history of any chronic gastrointestinal disease, including IBD, and/or any first-degree relatives with IBD.

2.3. Biopsies

Biopsies from the terminal or neo-terminal ileum were obtained with biopsy forceps [CBE 2.5–230, Cook Sweden AB, Askim, Sweden] during ileocolonoscopy. In twins, biopsies for Ussing chamber studies were immediately placed in ice-cold oxygenated Krebs buffer [115 mM NaCl, 1.25 mM CaCl2, 1.2 mM MgCl2, 2 mM KH2PO4 and 25 mM NaHCO3, PH 7.35] transported from Örebro, Sweden, to Linköping, Sweden, and delivered to the laboratory within 120 min. External controls for barrier function experiments were recruited from the University Hospital of Linköping, and biopsies were prior Ussing experiments put in ice-cold Krebs buffer for 120 min to mirror the situation for twin biopsies. For studies of tight junctions, biopsies were obtained from external controls at Örebro University Hospital and directly put in 4% paraformaldehyde for immunofluorescence, or snap frozen in liquid nitrogen for enzyme-linked immunosorbent assay (ELISA) measurements.
2.4. Ussing chamber experiments

The details of the Ussing chamber experiments have been described in detail previously. In short, ileal biopsies were cautiously stretched out with the mucosa up in a petri dish, and evaluation under dissection microscope ensured that only non-squeezed biopsies of high quality were used. Biopsies were mounted in modified 1.5 ml Ussing chambers [Harvard Apparatus Inc., Holliston, MA, USA], with an exposed tissue area of 1.76 mm² according to the previously described technique. Cold 10 mM mannitol in Krebs was put into the mucosal compartments, and the serosal compartments were filled with 10 mM glucose in Krebs buffer. Chambers were continuously oxygenated at 37˚C with 95% O₂/5% CO₂ and circulated by a gas flow. To enable achievement of steady-state conditions in transepithelial potential difference (PD), tissues were equilibrated for 40 min before the chamber experiments started, by changing the mannitol or glucose buffer after 20 min. After 40 min, mucosal compartments were replenished with Krebs–mannitol with and without 5.55 mg ASA/ml, and permeability markers were added as described below. The PD, short-circuit current (ISC) and transepithelial resistance (TER) across the tissues were monitored throughout the experiments to ensure tissue viability.

2.5. Paracellular permeability

To measure paracellular permeability, 34 µCi/ml of the inert probe ⁵¹ᵐCr-EDTA [Perkin Elmer, Boston, MA, USA] was added to the mucosal side of each chamber. Serosal samples were collected at 0, 20, and 120 min and replaced with an equal volume of Krebs. ⁵¹ᵐCr-EDTA was measured by gamma-counting [1282 Compugamma, LKB, Sweden], and permeability was calculated during the 0–20 and 0–120 min period; the results are presented as P₅₁ [apparent permeability coefficient; cm/s × 10⁻⁵].

2.6. Bacterial passage

After equilibration, chemically killed fluorescein isothiocyanate [FITC]-conjugated E. coli K-12 BioParticles were added to the mucosal compartments to a final concentration of 1 × 10⁶ CFU/ml. Samples were collected from the serosal side at 0 and 120 min and analysed at 480 nm in a fluorimeter [Cary Eclipse, Varian, Victoria, Australia], in which 1 unit refers to 3.2 × 10⁶ CFU/ml. Results are given as passage 0–120 min.

2.7. Tight junction protein expressions

Immunofluorescence was performed to study the expression of tight junction proteins in the epithelial cell lining. To assess the overall expression of tight junction proteins in the entire biopsy, potential proteins that had been distributed away from the tight junction complex, complementary analyses with ELISA were performed.

2.7.1. Immunofluorescence

Ileal biopsies from the 15 twin pairs and 10 external controls were used for immunofluorescence studies of claudin-1, claudin-5, and tricellulin expressions. Biopsies were obtained and directly put in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 µm. Slides were dehydrated according to standard procedures, and after conventional citrate buffer antigen retrieval, sections were blocked for 10 min with universal blocking serum [Background Sniper, Biocare Medical, Concord, CA, USA], washed in PBS, and incubated overnight at 4°C with primary mouse-anti-claudin-1 [1:100, Invitrogen, Sweden], rabbit-anti-claudin-5 [1:100, ThermoFisher, Fisher Scientific, Sweden], or rabbit-anti-MALD2 [1:100, ThermoFisher] antibodies. Three sections per CD twin, healthy twin, or external control were stained with each antibody, respectively. After incubation, sections were rinsed and secondary antibodies rabbit-anti-mouse IgG alexa fluor 594 [1:400, ThermoFisher] or donkey-anti-rabbit IgG alexa fluor 488 [1:400, Invitrogen] were added. After incubation for 1 h at room temperature, slides were rinsed and mounted with ProLong® Gold with DAPI [Life Technologies, Stockholm, Sweden]. Sections with no primary antibodies were used as negative controls in all experiments. Three sections per subject were evaluated in a blinded fashion for tight junction expressions in a Nicon Eclipse E800 fluorescence microscope equipped with a Nikon DS-R1i digital camera. In total, 10 pictures/section were obtained. Using ImageJ software [National Institutes of Health], the epithelial linings in each picture were encircled, and a mean fluorescence intensity from each of the three sections from each twin/control was calculated using the program. A mean value was calculated for all three sections, and a median value from all twins/controls was calculated. All pictures evaluated had the same parameter settings, and the fluorescence intensity was measured against a set background correction value.

2.7.2. ELISA

Frozen biopsies from the 15 twin pairs and 10 external controls were washed in cold PBS, weighed, and then we added 50 ml/mg cold RIPA-buffer [Pierce, Thermo Scientific, Sweden], inhibiting cOmplete Mini Protease inhibitor Cocktail [Roche, Germany], and 50 U/ml nuclease [Pierce]. Samples were homogenized in a TissueLyser II [Qiagen, Sweden], 30 frequency/s for 1 min, and then centrifuged for 15 min at 18000g at 4°C. Supernatants were redrawn, protein concentrations were measured according to the Bio-Rad DC protein assay [Bio-Rad, Sweden], and homogenates were diluted in Laemmli sample buffer [Bio-Rad] to a final concentration of 1 µg/µl, heated for 9 min at 95°C, and put in –80°C until analysed by ELISA. Three different ELISA kits were used—the human ELISA kits for Tricellulin [USCNK, Cloud Clone Corp, Houston, USA], Claudin-1 [Cusabio, Baltimore, USA], and Claudin-5 [Cusabio]—according to the manufacturer’s instructions. In brief, plates were pre-coated with secondary antibody and blocked for non-specific binding. Undiluted plasma or biopsy lysates, positive and negative controls, and standard points were added in duplicates. Primary biotinylated antibody, streptavidin–horseradish peroxidase, tetramethylbenzidine enzyme substrate, and 2 M HCL for stopping the reaction, were added subsequently. Absorbance was measured at 450 nm in a VERSAmax Tunable Microplate Reader [Molecular Devices, San Diego, CA, USA] using Softmax pro 5 [Molecular Devices]. The software generated a standard curve based on the standard points, from which the concentrations of the samples were calculated.

2.8. Histological assessment

Hematoxylin and eosin–stained sections were scored by a pathologist [AO] according to previous guidelines, and the evaluator was blinded to diagnosis.

2.9. Statistical considerations

Continuous data are presented as the median interquartile range [IQR]. The healthy co-twins and the CD twin group were compared with external controls using the Chi-square test for qualitative variables and the non-parametric Mann–Whitney U-test for continuous variables. Values of p < 0.05 were considered statistically significant. Degree of significance was indicated as follows: p < 0.05 [*], p < 0.01 [**].
[**], p < 0.001 [***] or ns [non-significant]. For each group, values outside ±3 IQR of the 1st and 3rd quartiles were judged as outliers and not included in the test.

2.10. Ethical statement
The Local Ethics Committee approved the study, and all twins as well as external controls gave their written informed consent.

3. Results
In total, 15 twin pairs discordant for CD were included in the study; clinical characteristics and basic demographics are shown in Table 1. In total, 6/15 [40%] of the twins with CD had undergone previous surgical resections. External controls for barrier function experiments numbered 10 individuals [median age 69 years, IQR: 60–76] and for studies of tight junctions, 10 individuals [median age 54, IQR: 47–74].

3.1. Electrophysiology
Using chamber experiments showed stable PD in all biopsies after equilibration and throughout the experiments [data not shown]. At 120 min from start, the active net ion transport assessed as ISC was similar in all groups; however, ASA-provocation increased ISC in all groups; CD twins, p < 0.001, healthy co-twins, p < 0.001 and external controls, p < 0.01 [Figure 1A]. In line with ISC, there were no significant differences in TER between groups at 120 min, while ASA-provocation decreased TER in all groups, p < 0.05 [Figure 1B]. The increase in ISC and decrease in TER indicates a weakening effect on the barrier by ASA in both CD twins and healthy co-twins.

3.2. Increased paracellular permeability
Increased paracellular permeability, assessed by 51Cr-EDTA flux, was observed after 120 min in CD twins compared with external controls, p < 0.001, and the difference was more pronounced with ASA-provocation, p < 0.001 [Figure 2]. A non-significant trend of increased 51Cr-EDTA passage was observed in the CD twins by 20 min [p = 0.094], but was not visible with ASA-provocation [Figure 2].

When healthy co-twins were compared with external controls at 120 min, there was a difference in 51Cr-EDTA permeability both with, p < 0.001, and without ASA-provocation, p < 0.001 [Figure 2A]. The increased 51Cr-EDTA passage was predominantly seen in the healthy monozygotic twins, when the analyses were stratified by zygosity. A difference between healthy monozygotic twins and external controls was observed at both 20 min [p = 0.05 with ASA, p < 0.05 without ASA] and at 120 min [p < 0.01 with ASA, p < 0.01 without ASA] [Figure 2B]. The corresponding comparisons for healthy dizygotic twins vs external controls were not significant at 20 min [p = 0.19 and p = 0.42, respectively] but significant at 120 min [p = 0.001 and p < 0.01, respectively]. In addition, a significantly increased 51Cr-EDTA passage was observed at 20 min when healthy monozygotic twins were compared with healthy dizygotic twins [p < 0.05].

3.3. No difference in passage of E. coli K-12
There were no significant effects on E. coli K-12 passage with or without ASA provocation at 120 min [Figure 3]. A trend towards augmented passage in CD twins vs external controls was observed after ASA provocation, p = 0.15, but no difference could be observed without ASA provocation, p = 0.55. There was no difference in passage when healthy co-twins were compared with external controls, and stratification by zygosity did not influence the results.

3.4. Influence of inflammation on paracellular permeability
To assess whether the observed increased paracellular permeability to 51Cr-EDTA in the CD twins and potentially their healthy co-twins was explained by histologic inflammation, ileal sections were scored. In total, 2 of the 15 CD twins displayed histologic activity. Correspondingly, all healthy co-twins had normal histology reports. No difference in passage of 51Cr-EDTA was observed between CD twins with ongoing histologic inflammation and CD twins with normal histology reports.

3.5. Altered tight junction expression
Based on the observed increased paracellular permeability in CD twins and their healthy co-twins compared with external controls, levels of the tight junction proteins claudin-1, claudin-5, and tricellulin were assessed by immunofluorescence and ELISA. Immunofluorescence revealed significantly lower levels of claudin-5 and tricellulin in both CD twins and healthy co-twins, and significantly higher levels of claudin-1 in CD twins, compared with external controls [Figure 4A–D]. There were no obvious differences in the localization of the tight junction stainings between CD twins, healthy co-twins, and external controls. ELISA revealed that CD twins demonstrated significantly lower levels of tricellulin compared with external controls, p < 0.05, while there were no differences in claudin-1 or claudin-5 expressions, and no differences at all between healthy co-twins and external controls for any of the tight junctions investigated [Table 2].

4. Discussion
A compromised intestinal barrier function, with augmented passage of non-pathogenic bacteria, is believed to play a key role in the pathophysiology of CD. However, at present a controversy exists as to
whether increases in epithelial permeability are a primary or secondary consequence of the disease. By analysing twin pairs discordant for CD, we demonstrated that a dysregulated intestinal barrier characterizes both CD twins and their healthy co-twins. These findings strongly support the hypothesis that intestinal barrier dysfunction is a primary defect in the pathogenesis of CD, rather than a secondary effect of the inflammatory process. Similar conclusions have been drawn from studies in animal models, where defects restricted to the epithelium have been observed to cause intestinal inflammation even in the presence of normal gut microbiota and immunity.

Intestinal permeability has been hypothesized to be a familial trait of aetiological importance in CD, because probe studies in the 1990s identified an augmented permeability in first-degree relatives of patients with CD. Since then, the initial hypothesis has been questioned because subsequent studies yielded conflicting results. Using the Ussing chamber technique, we demonstrated that the paracellular passage, defined as $^{51}$Cr-EDTA flux, is increased in CD twins as well as in their healthy co-twins, i.e. first-degree relatives, when compared with external controls. Histologic examination confirmed that none of the healthy co-twins displayed any histologic activity, which strengthened our conclusion that barrier dysfunction represents a primary defect in CD.

The clustering of disturbed intestinal permeability among families with CD, especially between siblings, points towards the influence of genetic predisposition or shared environment during childhood. The importance of environmental exposure is further emphasized by the finding that healthy spouses of CD patients display an elevated permeability. Söderholm et al. proposed that baseline permeability is determined by environmental factors, whereas ASA provocation reveals the genetically predisposed state of the mucosal barrier. To disentangle the relative contribution of genetic predisposition and environmental influence, we compared the permeability in twin pairs discordant for CD and stratified by zygosity. A significantly increased passage of $^{51}$Cr-EDTA was observed in healthy monozygotic co-twins after 20 min, compared with dizygotic co-twins without incubation with ASA. Furthermore, when subgroups of healthy twins were compared

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**Figure 1.** Electrophysiology measurements at 120 min from the start of the Ussing chamber experiments showed: [A] A similar short-circuit current [ISC] in ileal biopsies from Crohn’s disease (CD) twins, healthy co-twins, and external controls. Provocation with acetylsalicylic acid [ASA] significantly increased ISC in all groups. [B] A similar transepithelial resistance [TER] in all groups, and significantly lower TER after ASA-provocation. The intergroup comparisons were not statistically significant.
after 20 min with external controls, an increased passage was observed in healthy monozygotic twins only and not in healthy dizygotic twins. This suggests that paracellular permeability is primarily driven by genetic predisposition. However, the study is limited by the fact that we were not able to compare the presence of specific genetic risk loci specifically barrier-associated variants such as the $C1orf106$ IBD-associated risk variant$^{38}$ between the twins and the external controls.

To characterize the observed increased paracellular permeability further, we investigated the expression levels of components of the tight junction in the intestinal mucosa. Claudin-1, claudin-5, and tricellulin have been described as key components of the tight junction complex. We observed a decreased level of claudin-5 and tricellulin in CD twins as well as in their healthy co-twins when compared with external controls. Claudin-5 downregulation and redistribution have previously been observed in patients with active CD$^3$ and might also represent a link to the observed increased paracellular permeability, since it has been proposed to play an active role in antigen entry into the mucosa.$^4$ Similarly, tricellulin may represent a link to the observed increased permeability, since it has previously been associated with uptake of macromolecules in patients with UC.$^{39,40}$ The decreased levels of claudin-5 and tricellulin might be one of the mechanisms behind the observed increase in paracellular permeability in the healthy co-twins. Claudin-1 was found in increased levels in CD twins but not in healthy co-twins, compared with external controls. This finding may seem to be contradictory, since it has been proposed that claudin-1 should have barrier protective properties.$^{41,42}$ However, previous studies evaluating claudin-1 have obtained inconsistent results, with respect to CD.$^{43,44}$ Weber et al. reported increased levels of claudin-1 expression in patients with active CD, but no difference was observed between patients with inactive CD and control subjects.$^{44}$ This observation might point to a possible correlation between levels of claudin-1 and inflammatory activity in patients with CD, but no difference was observed between patients with active CD and control subjects.$^{44}$ This observation might point to a possible correlation between levels of claudin-1 and inflammatory activity in patients with CD, but no difference was observed between patients with active CD and control subjects.$^{44}$

Figure 2. Paracellular permeability assessed by $^{51}$Chromium (Cr)-EDTA flux in ileal biopsies from healthy co-twins, twins with Crohn’s disease (CD), and external controls mounted in Ussing chambers. Twins were subdivided into monozygotic (filled squares) and dizygotic (filled circles). Permeability to $^{51}$Cr-EDTA was measured after 20 and 120 min, with and without ASA provocation. [A] Comparisons between CD twins, healthy co-twins, and external controls. [B] Healthy co-twins stratified on zygosity and compared between mono- and dizygotic healthy co-twins, and external controls. Comparisons were done using the Mann-Whitney U test. Grey points represent values ±3 IQR outside the 1st and 3rd quartiles, which were judged as outliers and not included in the comparison.
In contrast to claudin-1 and claudin-5, a change in expression of tricellulin was also observed with ELISA. This might, as mentioned before, reflect differences in the technique and how the protein of interest is displayed in the different assays. In the ELISA technique, the protein is separated from its original position in the tissue and a larger surface is displayed for detection. Immunofluorescence on the contrary shows the expression level of the protein still in the original position in the tissue, where fewer epitopes are displayed. Thus, immunofluorescence might be better suited for investigating tight junction protein expression in this study.

Even though some of the differences between healthy co-twins and external controls were observed in healthy monozygotic twins only, the observed increased permeability in both monozygotic and dizygotic healthy co-twins at 120 min indicates that environmental factors may also have some impact on paracellular passage. Given that the twins were separated at around the age of 20 years, our results suggest that environmental factors might be of extra importance in early life, i.e. during childhood and adolescence, and influence the mucosal barrier negatively. Exposure to environmental factors during childhood, such as repeated antibiotic use and frequent intake of fast food, have been linked to IBD onset.$^{48–50}$ Further supporting the notion that environmental factors might influence the intestinal barrier negatively.

There are several ways for solutes to cross the intestinal epithelium. Under normal conditions, the paracellular route is believed to be impermeable to protein-sized molecules and thus to constitute an effective barrier to antigenic macromolecules. In the present study, the passage of $E. coli$ K-12 was relatively low and no difference was observed between the groups. We have previously shown an augmented mucosal uptake and translocation of non-pathogenic $E. coli$ in patients with long-standing CD.$^5$ However, this process seems to primarily involve transepithelial passage, in which bacteria [up to several $\mu m$ in size] and bacterial products [such as lipopolysaccharides] are taken up by binding to the cell membrane via receptors. Normally, the intestinal barrier allows only small amounts of antigens and bacteria to pass the mucosa to interact with the immune system. If the control of the barrier is broken, it can implicate enhanced passage, which in turn may damage the mucosa and trigger inflammatory conditions such as CD.$^{51}$ Thus, the absence of increased passage of $E. coli$ K-12 in healthy co-twins concurs with previous observations of patients with long-standing CD explained as a consequence of inflammation rather than representing a primary defect. However, bacterial products that cannot pass through the cell membrane or the paracellular space because of their size can be taken up by the cell through invagination of the plasma membrane followed by vesicle formation, i.e. endocytosis. Hence, we cannot exclude an increased adherence of bacteria to the intestinal epithelia in the twins, since an analysis of intraepithelial translocation of $E. coli$ K-12 was not performed.

Based on the hypothesis that increased permeability is a primary phenomenon that might precede development of clinical manifestation
of CD, one might speculate that the healthy co-twins displaying increased intestinal permeability will develop CD later in life. However, the median age for evaluation of intestinal permeability was 52 years in the healthy co-twins, which by far is beyond the age-specific incidence peak of 15–25 years for CD.\textsuperscript{52,53} In addition, age at onset of disease has been observed to be highly similar in twin pairs concordant for CD.\textsuperscript{54} This observation indicates that the potential risk that any twin would have been erroneously classified...
Table 2. Tight junction expressions in ileal biopsies from Crohn’s disease [CD] twins, healthy co-twins, and external controls measured by ELISA.

| ng/mg tissue | Claudin-1 | Claudin-5 | Tricellulin |
|--------------|-----------|-----------|-------------|
| CD twins     | 4.6 [2.6–5.4] | 1.4 [0.9–3.1] | 3.4 [2.8–5.4]* |
| Healthy co-twins | 5.5 [3.8–6.8] | 2.1 [1.3–2.8] | 4.6 [2.7–6.7] |
| External controls | 4.4 [3.6–5.1] | 1.6 [1.4–3.1] | 6.1 [3.9–6.7] |

Levels of the tight junction proteins claudin-1, claudin-5, and tricellulin were measured in homogenized ileal biopsies from 15 twin pairs and 10 external controls by ELISA. Absorbance was measured at 450 nm, and the concentrations of the samples were calculated based on the standard curve and expressed as ng/mg tissue. Data are presented as median [interquartile range]. The CD twin group and healthy co-twins were compared with external controls using the Chi-square test for qualitative variables and the non-parametric Mann–Whitney U-test for continuous variables. *p < 0.05 tricellulin expressions in CD twins vs external controls.

as being healthy is rather low, since the median disease duration in the diseased twin was 17 years [12–23]

On the other hand, the long disease duration of CD before inclusion in the study may represent a limitation, since it questions the impact of the observed disruptive barrier on disease pathogenesis. Notably, the observed dysregulation alone does not cause the disease, since it was also observed in the healthy co-twins. The study is also limited by the low number of discordant twin pairs, and the absence of significant differences may reflect low statistical power rather than true negative findings. Crohn’s disease is a complex disease and different mechanisms may contribute to the pathogenesis of the disease in different patients. More than one-third of the twins with CD were diagnosed with colonic disease without any ileal involvement. This may challenge some of our findings, since regulation of barrier homeostasis differs between ileum and colon.38 However, no difference was observed when CD twins were stratified by disease location [ileal involvement versus colonic disease] and 51Cr-EDTA levels were compared. Consistently, claudin-1 as well as claudin-5 levels did not differ, although tricellulin levels seemed to be slightly higher in twins with ileal involvement compared with twins with colonic CD [data not shown].

In conclusion, we demonstrated an increased paracellular permeability in healthy co-twins in twin pairs discordant for CD. These results strongly suggest that barrier dysfunction is a primary defect in CD. The dysregulated barrier might be explained by the influence of genetic factors, because we observed a significant increase in paracellular permeability in healthy monozygotic co-twins compared with dizygotic co-twins. Passage of E. coli seems to be a consequence of inflammation, rather than representing a primary defect.

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Author Contributions

AVK: Study concept and design; performed parts of the Ussing chamber experiments; analysis and interpretation of data; drafting of manuscript; funding. ML: Analysis and interpretation of data; responsible for statistical analyses. AO: Performed histological assessment of biopsies. CDLM: Designed and performed the studies of tight junctions. IS: Performed parts of the Ussing chamber experiments; analysis and interpretation of data; participated in manuscript drafting. JH: Study concept and design; responsible for patient inclusion; analysis and interpretation of data; drafting of manuscript; funding. All authors read and approved the final manuscript.

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