Novel pathomechanism for spontaneous bacterial peritonitis - Disruption of cell junctions by cellular and bacterial proteases

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Supplements

Supplementary Material and Methods

Chemicals and reagents *E. coli* O55:H5 lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate, MMP inhibitors BB-94, BB-2516 and proteasome inhibitor clasto-lactacystin β-lactone (LA) were purchased from Sigma-Aldrich, Taufkirchen, Germany. DMSO served as control for LA, BB-94 and BB-2516, PBS for LPS and EtOH for phorbol 12-myristate 13-acetate.

Crystal violet assay Cells were washed with PBS, fixed with 80% EtOH and stained with crystal violet (0.5% crystal violet, 20% EtOH) (Sigma-Aldrich, Taufkirchen, Germany) for 10min. Afterwards, cells were washed with water and air dried.

Transwell experiments To characterize bacteria-to-cell interactions, *E. coli* were added at MOI 5 onto transwell inserts (0.4µm pore size, Corning, Wiesbaden, Germany) on top of Caco-2 cells for 4 h. As a control, Caco-2 cells were directly co-cultured with *E. coli*.

RNA isolation and reverse transcription Cells were harvested in RLT buffer (Qiagen, Hilden, Germany). RNA isolation and reverse transcription were performed according to the manufacturer's protocol of RNeasy Kit and QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Quantification of gene expression Expression of the following genes was analyzed by qRT-PCR using a Light Cycler 480 RT-PCR System (Roche, Mannheim, Germany): *CDH1*, *OCLN*, *MMP-1, -3, -7, -9, -10, -13*, -14, tissue inhibitor of MMPs (*TIMP)-2*, *MUC2, MUC5AC* and *GAPDH*. *GAPDH* served as internal standard. Primers for *MMP-7* and *MMP-9* were purchased from Qiagen (Hilden, Germany). All other primers were purchased from Metabion (Planegg, Germany) and corresponding sequences are provided in **supplementary table 7**. Data were analyzed based on the double cycle threshold method using the average cycle threshold values of triplicates.

Western blot analysis Cells were washed with PBS three times, lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail (cOmplete ULTRA, phosSTOP, Sigma-Aldrich, Taufkirchen, Germany). Samples were

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incubated on ice for 1h, spun at 16,000xg for 1h at 4°C and supernatant was collected. 10-30µg protein were separated by Western blot. Anti-ALPI (TA809178, OriGene Technologies, Rockville, USA), anti-CDH1, anti-GAPDH (3195/2118; Cell Signaling Technology, Danvers, MA, USA), anti-OCLN (NBP1-87402, Novus Biologicals, Abingdon, UK), anti-ubiquitin (05-1307, Millipore, Darmstadt, Germany), anti-β-actin (A3854, Sigma-Aldrich, Taufkirchen, Germany), anti-rabbit IgG and anti-mouse IgG (A0545/ A9044, Sigma-Aldrich, Taufkirchen, Germany) were used. Densitometry was performed using Image Lab Software (Bio-Rad Laboratories Inc., Munich, Germany) and Fusion Pulse TS (Vilber Lourmat, Eberhardzell, Germany) or Image Studio Lite Version 5.2 (Lincoln, NE, USA).

Immunofluorescence and microscopy Cells were grown on chamber slides (Thermo Fisher Scientific, Waltham, MA; USA), washed with PBS, fixed with 4% formaldehyde and permeabilized with 2% saponin, each for 15min. Cells were blocked in 5% BSA and stained using the following antibodies or isotype controls for 1h: mouse anti-occludin-488 (OC-3F10, Invitrogen, Carlsbad, CA, USA) or rat anti-E-cadherin-594 and mouse/rat 488/594-labeled isotype controls (DECMA-1/MOPC-21/RTK2071, BioLegend, London, UK). Cells were washed and labeled with 300nM DAPI for 3min.

To stain colon biopsies of patients with SBP and control patients, mouse anti-occludin-594 antibody (OC-3F10, Thermo Scientific, Waltham, MA, USA), rat anti-E-cadherin-594 and 594-labeled mouse/rat isotype control (MOPC-21, Biolegend, London, UK; DECMA-1/MOPC-21/RTK2071, BioLegend, London, UK) were applied.

Electron microscopy Cells, cultured on Thermanox cover slides (Thermo Fisher Scientific, Waltham, MA; USA), were fixed with 0.1 M cacodylate-buffered Karnovsky solution (2% paraformaldehyde and 2.5% glutaraldehyde) followed by enclosure in 4% low-melting agarose and post-fixation with 1% cacodylate-buffered osmium tetroxide for 2 h. Cells were dehydrated in graded ethanol and embedded in Embed812 epoxy resin (= EPON, all reagents from EMS/Science Services Munich, Germany). After heat polymerization for 2 days at 60°C, semithin sections (0.8µm thickness) were obtained by means of ultramicrotome and stained with toluidine blue and basic fuchsine. Ultrathin sections (80 nm thickness) were cut with a diamond knife (DIATOME, Biel, Switzerland) mounted on grids (100 and 200 mesh) and double contrasted with aqueous uranyl acetate (2%) and lead citrate (Ultrostain II LEICA, Wetzlar, Germany) for 10min each. Sections were examined by a LEO912AB electron microscope. Findings were documented with a side-mounted 2k x 2k CCD camera (TRS, Moorenweis, Germany) with iTEM software (OSIS, Münster, Germany).

Immunoprecipitation and detection of ubiquitination of occludin and E-cadherin NP40 lysis buffer (150mM NaCl, 1% NP40, 50mM Tris pH 8.0 containing protease and phosphatase inhibitor cocktail) was used for cell lysis. Protein-A-agarose (P-9262, Sigma-Aldrich, Taufkirchen, Germany) was coupled with 200µg lysate in NP40 lysis buffer containing 2% BSA for at least 1.5h at 4°C. Simultaneously, E-cadherin (5296, Cell Signaling Technology, Danvers, MA, USA) and occludin (711500, Invitrogen, Carlsbad, CA) antibodies at 2–2.5µg were incubated with protein-A-agarose beads for 1.5 h at 4°C. Precleared supernatant and coupled antibodies were incubated for 2h at 4°C. The precipitated pellet was

washed with NP40 lysis buffer and analyzed by Western blot. 20µg cell lysate represented the input, the pellet of precleared supernatant (clear) was the control.

Flow Cytometry For toll like receptor (TLR) analysis (shown in **supplementary figure 3**) Caco-2 cells were blocked with PBS supplemented with 10% fetal bovine serum for 10min. Staining was performed in the dark for 20min using anti-TLR2-PE and anti-TLR4-FITC (12-9922-42/53-9917-42, eBioscience, Vienna, Austria) with corresponding isotype controls (mouse IgG2a-PE, 12-4724-81, Invitrogen, Carlsbad, CA, USA; mouse IgG2a-488, 53-4724-80, eBioscience, Vienna, Austria) in staining buffer (554657, BD, Heidelberg, Germany).

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Supplementary tables

Supplementary table 1 Characteristics of healthy controls and patients with liver cirrhosis shown in **figure 1**.

	non-cirrhotic controls (n = 19)	liver cirrhosis (n = 14)
median age	58.6 (35-83)	57.0 (48-75)
sex M/F	11/8	8/6
etiology of cirrhosis (alcohol-related/toxic/PSC/Caroli disease)		10/2/1/1
Child-Pugh class A/B/C		7/2/5
model for end-stage liver disease (MELD)-score		12.9 (7-32)
diagnosis of hepatocellular carcinoma (HCC)		3
portal hypertension*		14
spontaneous bacterial peritonitis (SBP)		5
ascites		9
antibiotic therapy	2 (piperacillin/tazobactam, clarithromycin)	6 (piperacillin/tazobactam, vancomycin, rifaximin)
use of non-steroidal anti-inflammatory drugs	4 (ibuprofen, acetylsalicylic acid, etoricoxib)	1 (acetylsalicylic acid)
use of corticosteroids	3 (prednisolone, beclomethasone)	1 (prednisolone)
use of proton pump inhibitors (PPI)	9 (omeprazole, pantoprazole)	7 (pantoprazole)

*portal hypertension was defined by either vascular alterations, ascites, hepatic transjugular intrahepatic portosystemic shunt (TIPSS), hepatopulmonary syndrome (HPS), hypersplenism, portal hypertensive gastropathy (PHG), esophageal varices, esophageal variceal bleeding, hepatic encephalopathy (HE), thrombocytopenia or postsurgical decompensation.[1,2]

Supplementary table 2: Characteristics of the PPI-negative control patients, depicted in **figure 1**.

Nr.	age	gender	diagnosis	indication for colonoscopy	portal hypertension*	SBP	PPI	antibiotic	anti-inflammatory drugs	mucus thickness (µm)
1	67	m	diverticulosis	diarrhea	no	no	no	no	no	15.67
2	50	m	abdominal pain	diagnostic colonoscopy	no	no	no	no	no	10.11
3	36	m	perianal pain	tumor exclusion	no	no	no	no	no	23.29
4	57	m	bacterial overgrowth	diarrhea	no	no	no	yes	yes (beclomethasone)	10.5
5	82	f	polymyalgia rheumatica	tumor exclusion	no	no	no	no	yes (prednisolone)	23.0
6	40	m	diagnostic colonoscopy	tumor exclusion	no	no	no	no	no	19.5
7	80	m	amyloidosis	rectal biopsy	no	no	no	no	no	18.43
8	68	m	history of heart transplant	tumor exclusion	no	no	no	no	yes (mycophenolic acid, etoricoxib, prednisolone, everolimus)	12.13
9	76	f	abdominal pain	tumor exclusion	no	no	no	no	no	12.56
10	83	f	coronary heart disease	tumor exclusion	no	no	no	no	yes (acetylsalicylic acid)	13.33

*portal hypertension was defined by either vascular alterations, ascites, hepatic transjugular intrahepatic portosystemic shunt (TIPSS), hepatopulmonary syndrome (HPS), hypersplenism, portal hypertensive gastropathy (PHG), esophageal varices, esophageal variceal bleeding, hepatic encephalopathy (HE), thrombocytopenia or postsurgical decompensation.[1,2]

Supplementary table 3: Characteristics of the PPI-positive control patients, depicted in **figure 1**.

Nr.	age	gender	diagnosis	indication for colonoscopy	portal hypertension*	SBP	PPI	antibiotic	anti-inflammatory drugs	mucus thickness (µm)
11	61	m	screening colonoscopy	screening colonoscopy	no	no	yes	no	yes (ibuprofen)	10.14
12	51	f	evaluation liver donation	tumor exclusion	no	no	yes	no	no	12.44
13	62	f	evaluation kidney donation	tumor exclusion	no	no	yes	no	no	9.75
14	46	m	weight loss	tumor exclusion	no	no	yes	no	no	35.88
15	42	f	familial renal cystic disease	tumor exclusion	no	no	yes	no	no	11.86
16	46	m	evaluation heart transplant ischemic cardiomyopathy	tumor exclusion	no	no	yes	no	yes (acetylsalicylic acid)	17.5
17	72	f	iron deficiency anemia	tumor exclusion	no	no	yes	yes	no	15.33
18	35	m	infectious colitis	diarrhea	no	no	yes	no	no	14.75
19#	60	f	diverticulosis	diarrhea	no	no	yes	no	no	17.29

*portal hypertension was defined by either vascular alterations, ascites, hepatic transjugular intrahepatic portosystemic shunt (TIPSS), hepatopulmonary syndrome (HPS), hypersplenism, portal hypertensive gastropathy (PHG), esophageal varices, esophageal variceal bleeding, hepatic encephalopathy (HE), thrombocytopenia or postsurgical decompensation.[1,2]

patients who donated colonic biopsies and are shown in **figure 1C**

Supplementary table 4 Characteristics of the PPI-negative patients with liver cirrhosis depicted in **figure 1**.

Nr.	age	gender	diagnosis	indication for colonoscopy	Child–Pugh score	MELD	portal hypertension*	SBP	PPI	antibiotic	anti-inflammatory drugs	mucus thickness (µm)
1	75	m	alcohol-related liver disease	tumor exclusion	A	15	yes (vascular alterations, ascites, HPS, HE)	no	no	no	no	14.00
2	60	m	alcohol-related liver disease	tumor exclusion	A	16	yes (vascular alterations, hypersplenism, PHG, esophageal, varices)	no	no	no	no	7.43
3	49	f	drug-induced liver disease	tumor exclusion	A	18	yes (thrombocytopenia)	no	no	yes	no	6.86
4	52	f	alcohol-related liver disease	tumor exclusion	B	8	yes (vascular alterations, TIPSS, hypersplenism, PHG, HCC)	no	no	no	no	6.56
5#	57	m	liver cirrhosis caused by Caroli disease	tumor exclusion	B	12	yes (ascites, TIPSS with dysfunction, HPS, splenomegaly, HE, postsurgical decompensation)	yes (*E. coli)* (**supplementary table 6**)	no	yes	yes (ASS)	10.87
6#	48	f	primary sclerosing cholangitis	tumor exclusion	C	17	yes (vascular alterations, ascites, HPS, hypersplenism, PHG, HCC)	no	no	yes	no	20.39
7	57	m	alcohol-related liver disease	tumor exclusion	C	32	yes (ascites, HPS, hypersplenism, PHG, esophageal varices, postsurgical decompensation)	no	no	yes	no	10.68

*portal hypertension was defined by either vascular alterations, ascites, hepatic transjugular intrahepatic portosystemic shunt (TIPSS), hepatopulmonary syndrome (HPS), hypersplenism, portal hypertensive gastropathy (PHG), esophageal varices, esophageal variceal bleeding, hepatic encephalopathy (HE), thrombocytopenia or postsurgical decompensation.[1,2]

patients who donated colonic biopsies and are shown in **figure 1C**

Supplementary table 5 Characteristics of the PPI-positive patients with liver cirrhosis depicted in **figure 1**.

Nr.	age	gender	diagnosis	indication for colonoscopy	Child–Pugh score	MELD	portal hypertension*	SBP	PPI	antibiotic	anti-inflammatory drugs	mucus thickness (µm)
8	52	f	alcohol-related liver disease	tumor exclusion	A	7	yes (vascular alterations, TIPSS with slight dysfunction, hypersplenism, PHG, esophageal varices 0-I)	no	yes	no	no	10.00
9	58	f	nutritional-toxic liver cirrhosis	tumor exclusion	A	10	yes (hypersplenism, PHG, esophageal varices)	no	yes	no	no	9.38
10#	61	m	alcohol-related liver disease	tumor exclusion	A	7	yes (ascites, HPS)	no	yes	no	no	17.86
11	53	f	alcohol-related liver disease	tumor exclusion	A	7	yes (ascites, PHG, HCC, HE)	yes	yes	no	no	8.33
12#	61	m	alcohol-related liver disease	tumor exclusion	C	10	yes (ascites, thrombocytopenia, HE)	yes	yes	no	no	11.22
13#	60	m	alcohol-related liver disease	tumor exclusion	C	14	yes (ascites, TIPSS with dysfunction, HPS, hypersplenism, HE, postsurgical decompensation)	yes	yes	yes	yes (prednisolone)	15.14
14#	55	m	alcohol-related liver disease	tumor exclusion	C	21	yes (vascular alterations, ascites, hypersplenism, PHG, esophageal varices)	yes (*E. coli*) (**supplementary table 6**)	yes	yes	no	17.38

*portal hypertension was defined by either vascular alterations, ascites, hepatic transjugular intrahepatic portosystemic shunt (TIPSS), hepatopulmonary syndrome (HPS), hypersplenism, portal hypertensive gastropathy (PHG), esophageal varices, esophageal variceal bleeding, hepatic encephalopathy (HE), thrombocytopenia or postsurgical decompensation.[1,2]

patients who donated colonic biopsies and are shown in **figure 1C**

Supplementary table 6 Antibiograms of (patient-derived) bacteria (compare **supplementary table 4, 5**).

E. coli O6:Hnt , O16:H5 (PDEC 1), Ont:H7 (PDEC 2) and *P. mirabilis* were used for the *in vitro* studies.

antibiotics	*E. coli* patient-derived Child-Pugh C patient (14) shown in **figure 1C**	*E. coli* patient-derived Child-Pugh B patient (5) shown in **figure 1C**	*E. coli* ATCC25922 O6:Hnt (*in vitro* studies)	*E. coli* O16:H5 (PDEC 1) patient-derived (*in vitro* studies)	*E. coli* Ont:H7 (PDEC 2) patient-derived (*in vitro* studies)	*P. mirabilis* patient-derived (*in vitro* studies)
ampicillin or amoxicillin	resistant	resistant	sensitive*	resistant	sensitive	sensitive*
amoxicillin/clavulanic acid	intermediate	sensitive*	sensitive*	resistant	sensitive	sensitive*
piperacillin		resistant				sensitive
piperacillin/tazobactam	sensitive	sensitive		sensitive	sensitive	sensitive
cefuroxime (intravenous)	sensitive	sensitive*	sensitive*	resistant	sensitive	sensitive*
ceftriaxone or cefotaxime	sensitive	sensitive		resistant	sensitive	sensitive
ceftazidime	sensitive	sensitive		intermediate	sensitive	sensitive
cefepime	sensitive	sensitive		resistant	sensitive	sensitive
imipenem	sensitive	sensitive		sensitive	sensitive	
meropenem	sensitive	sensitive		sensitive	sensitive	sensitive
ertapenem	sensitive	sensitive			sensitive	sensitive
ciprofloxacin	resistant	resistant	sensitive	sensitive	sensitive	sensitive
levofloxacin	resistant	resistant			sensitive	sensitive
cotrimoxazole	resistant	resistant	sensitive	sensitive	sensitive	sensitive
gentamicin	sensitive	resistant			sensitive	sensitive
amikacin	sensitive	sensitive			sensitive	sensitive
tobramycin		resistant				sensitive
tigecycline	sensitive	sensitive				resistant
colistin						resistant
fosfomycin		sensitive				sensitive
pivmecillinam		sensitive				
nitrofurantoin	sensitive	sensitive			sensitive	
trimethoprim		resistant				resistant
aztreonam	sensitive	sensitive			sensitive	sensitive
ticarcillin/clavulanic acid		intermediate				sensitive

* sensitive* indicates that a higher dose of corresponding antibiotic agent should be used. PDEC = patient-derived *E. coli*.

Supplementary table 7 Sequences of primers applied for qRT-PCR.

gene	forward (5' – 3')	reverse (5' – 3')
CDH1	ATGAGTGTCCCCCGGTATCT	GGTCAGTATCAGCCGCTTTC
OCLDN	ACAAGCGGTTTTATCCAGAGTC	GTCATCCACAGGCGAAGTTAAT
MMP-1	GCTCATGAACTCGGCCATTCTCTTGGACT	CGGGTAGAAGGGATTTGTGCGXATGTA
MMP-3	CCGAGAAATGCAGAAGTTCCTTGGATTGG	TCGGGATGCCAGGAAAGGTTCTGAAGTG
MMP-10	TCTTGCATTCCTTGTGCTGTTG	ATTGCTGGGCAAGATCCTTGT
MMP-13	ACACCTACACCGGCAAAAGC	CATTTGTCTGGCGTTTTTGGA
MMP-14	GGCTGCCTACCGACAAGATT	GTACTCGCTATCCACTGCCC
MUC-2	CACCTGTGCCCTGGAAGGC	CGGTCACGTGGGGCAGGTTC
MUC5AC	CGGGTCCACGAGGAGACGGT	GCTTCTGCAGCCAGGCACGA
TIMP-2	CCCCAAGCAGGAGTTTCTCGACATCG	TGGACCAGTCGAAACCCTTGGAGGCT
GAPDH	CGACCACTTTGTCAAGCTCA	GCTGGTCCAGGGGTCTTACT

Supplementary figure legends

Supplementary figure 1 Characterization of the Caco-2 *in vitro* model. Caco-2 cells were cultured for 1 to 14 days and cell confluence was analyzed *via* microscopy (upper panel). In addition, dead cells were labeled with 1µg/ml DAPI and cell death was examined *via* fluorescence microscopy (lower panel).

Supplementary figure 2 Western blot analysis of cell-to-cell junction proteins E-cadherin and occludin in the Caco-2 *in vitro* model upon co-culture with ten patient-derived *E. coli* strains (**p**atient **d**erived **E**. **c**oli = PDEC). (A) Caco-2 cells were co-cultured with different patient-derived *E. coli* strains (PDEC 1 – 10) at MOI 10 for 4h. (B) E-cadherin and occludin protein levels were quantified by densitometry and relative changes to control were calculated. PDEC 1 = O16:H5 and PDEC 2 = Ont:H7 were chosen for further analyses.

Supplementary figure 3 Surface expression of toll-like-receptor (TLR)2 and TLR4 on Caco-2 cells was studied by flow cytometry. Caco-2 cells were incubated with *E. coli* O55:H5 LPS (10µg/ml) or phorbol 12-myristate 13-acetate (PMA) (10ng/ml) for 24h. Caco-2 cells with LPS or phorbol 12-myristate 13-acetate (PMA) stimulation are shown in yellow, isotype controls in grey. As positive controls for TLR2 and TLR4 expression HEK-blue IL-2 and U937 cells were used (red), isotype controls in blue**.**

Supplementary figure 4 *P. mirabilis*-induces caspase-independent cell death of Caco-2 cells. Flow cytometric DAPI exclusion analysis of Caco-2 cells stimulated with patient-derived *P. mirabilis* (MOI 10, 4 h). Prior to bacterial treatment, Caco-2 cells were stimulated either with 10 µM BB-94 or 50 µM of the pan-caspase inhibitor zVAD-FMK (Selleckchem, Munich, Germany) for 20min. Caco-2 cells co-cultured with *P. mirabilis* (i) in total (attached + detached), (ii) attached and (iii) detached were analyzed (n = 3). One representative flow cytometric blot of each sample is shown.

References for supplementary table 1 - 5

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