Confocal laser endomicroscopy reliably detects sepsis-related and treatment-associated changes in intestinal mucosal microcirculation

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Editor’s key points
- Confocal laser endoscopy was used both in patients with sepsis and a pig model.
- Microcirculation in different areas of the gut mucosal bed was imaged.
- Functional capillary density (FCD) in the duodenum was decreased in patients.
- In pigs, FCD decreased after sepsis and improved with volume therapy.
- Confocal laser endoscopy may be useful clinically.

Background. Microcirculatory alterations play a central role in the pathophysiology of sepsis. We investigated probe-based confocal laser endomicroscopy (pCLE) to assess alterations in mucosal microcirculatory perfusion in vivo in a porcine model of septic shock and in patients fulfilling consensus criteria for severe sepsis.

Methods. Septic shock was induced using a faecal peritonitis model in anaesthetized, mechanically ventilated pigs. Mucosal microcirculation was assessed using pCLE in the stomach, duodenum, terminal ileum, and rectum. Duodenal microcirculation was further evaluated in 10 patients with severe sepsis and in 8 healthy controls to quantify capillary diameter, capillary length, and functional capillary density (FCD).

Results. In the animal model, FCD was markedly decreased in duodenal (P<0.001), ileal (P<0.001), gastric (P<0.001), and rectal mucosa (P<0.005) 4 h after induction of sepsis. After volume therapy, FCD partially recovered to 90.0% (duodenum), 94.4% (ileum), 95.4% (gastric), and 97% (rectum) of baseline values, indicating decoupling of microvascular and macrovascular flow. In septic patients, the mean capillary diameter (P<0.01) and FCD (P<0.05) in duodenal mucosa were decreased compared with healthy controls.

Conclusions. pCLE reliably detected and quantified microcirculatory alterations in the gastrointestinal mucosa in a porcine model of sepsis and in patients with severe sepsis. Our data suggest that pCLE is a promising tool to assess the efficacy of therapeutic interventions on mucosal microcirculation in real-time, even in the clinical context.

Keywords: drug therapy; microscopy, confocal; perfusion; plasma substitutes; shock

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Microcirculatory alterations are common in sepsis and play an important role in the pathophysiology of sepsis-associated organ dysfunction. The mechanisms of sepsis-related microvascular haemodynamics are complex and involve local changes in blood flow and alterations of microvascular regulatory mechanisms because of the effects of cytokines on endothelium and vascular smooth muscle cells. The splanchnic bed and the gut mucosa, in particular, are prone to early injury in the course of septic shock.

Several experimental studies have confirmed the early occurrence of splanchnic hypoperfusion in the course of endotoxaemia, before the development of hypotension. Improvement of the microcirculation is an emerging novel therapeutic target in sepsis treatment, requiring bedside evaluation of microcirculation. Orthogonal polarization spectroscopy (OPS) and sidestream dark field (SDF) imaging techniques have been used to directly see the microcirculation in accessible mucosal surfaces in humans.

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mucosal beds accessible for imaging using these techniques remain limited. Therefore, studies using OPS or SDF in clinical settings have largely focused on the sublingual region. Although these studies have resulted in important observations on the prognostic implications of microcirculatory alterations in critically ill patients, it remains unknown whether microcirculatory events observed in the sublingual region adequately reflect microcirculatory changes in other vascular beds. Data on human intestinal microcirculatory alterations in sepsis are scarce and mainly derived from measurements in ileal stomas of patients with abdominal sepsis.

Confocal laser endoscopy (CLE) is a newly introduced endoscopic tool that allows the observation of not only living tissue but also the vascular networks in the bronchial and gastrointestinal (GI) tract during endoscopy. This technique delivers high-quality images that are magnified up to 1000-fold, enabling the visualization of the capillary architecture in the mucosal layer after injection of a contrast agent (fluorescein).

The aim of the current pilot study was to evaluate the feasibility of probe-based confocal laser endomicroscopy (pCLE) for in vivo imaging of microcirculatory alterations in various GI mucosal beds and to quantify the influence of volume therapy in the early phase of shock in a porcine model of septic shock. Moreover, we investigated microvascular architecture in the early phase of severe sepsis in patients to assess the feasibility of studying microcirculatory failure in humans.

**Methods**

**Animals**

Seven female, white cross pigs (source: GbR Fraatz, Pölzig, Germany) with a mean [standard deviation (SD)] weight of 29.8 (3.5) kg were investigated. Animals were adapted to climate- and light-cycle-controlled environment for at least 7 days before experiments. All animals were allowed standard laboratory food and water ad libitum.

Surgical procedure and sepsis induction

The right jugular vein and the right femoral artery were surgically exposed. For drug and fluid administration, a central venous catheter was inserted into the superior vena cava and a balloon-tipped thermodilution pulmonary artery catheter (139HF75, 7.5-Fr, Edwards Lifesciences, Irvine, CA, USA) was inserted via the right jugular vein. A 4-Fr catheter with an integrated thermistor and fiberoptic module (Pulsiocath 4F PV 2024L, Pulsion Medical Systems, Munich, Germany) was inserted into right femoral artery. Catheters and infusion systems were not heparinized. The abdominal cavity was opened via median laparotomy. Ileostomy was constructed using an opened loop of the ileum. A caecal incision of 2 cm was made and faecal material was aspirated. Then, the caecotomy was closed and a catheter was positioned intra-abdominally before the abdomen was closed with a suture. After the surgical preparation, towels were placed to avoid heat loss and animals were allowed to recover for 120 min before baseline measurements were performed. Continuous infusion of Ringer’s solution (10 ml kg<sup>-1</sup> h<sup>-1</sup> intravenously) was given during surgery and the post-surgical period until the induction of sepsis. To induce sepsis, autologous faeces was performed under a protocol approved by the local committee of animal use and care.

**Anaesthesia and monitoring**

Animals received i.m. premedication with 5 mg kg<sup>-1</sup> ketamine (Ratiopharm, Ulm, Germany). After placement of a peripheral venous cannula into an ear, vein anaesthesia was induced by i.v. propofol (AstraZeneca, Wedel, Germany). Pigs were then orally intubated and placed in the supine position. Anaesthesia and analgesia were maintained with an infusion of 10 mg kg<sup>-1</sup> h<sup>-1</sup> propofol and 0.02 mg kg<sup>-1</sup> h<sup>-1</sup> fentanyl (Actavis Deutschland, Langenfeld, Germany). Animals were mechanically ventilated with an inspiratory oxygen fraction (F<sub>1</sub> <sub>1</sub>) of 0.3, positive end-expiratory pressure of 5 cm H<sub>2</sub>O, and a tidal volume of 8 ml kg<sup>-1</sup>. The respiratory rate was adjusted between 12 and 15 min<sup>-1</sup> to maintain arterial CO<sub>2</sub> partial pressure between 4.6 and 5.9 kPa. Body core temperature was kept at > 37°C by using an infrared lamp and warmed solutions.

**Fig 1** Time course of the animal model of septic shock.
(0.75 g kg⁻¹) were suspended in 150 ml of isotonic NaCl (37 °C) and injected through the abdominal catheter. After induction of peritonitis, the catheter was used to drain ascites. The time course of the experiment is shown in Figure 1. I.V. volume therapy was performed by infusion of 30 ml kg⁻¹ gelatine (MW 30 kDa; Serumwerk Bernburg, Germany) within 90 min after establishment of a septic shock in order to achieve a mean arterial pressure (MAP) of > 65 mm Hg.

Haemodynamics

MAP, central venous pressure, and pulmonary artery occlusion pressure were recorded from calibrated pressure transducers (Edwards Lifesciences). Values were obtained at the end of expiration and referred to mid-chest level. Cardiac output was determined by thermodilution using a cardiac output monitor (Vigilance; Edwards Lifesciences, Newbury, UK) and data represent the mean of three injections of 5 ml of ice-cold 5% glucose randomly spread over the respiratory cycle. Arterial and mixed venous blood was sampled in heparinized syringes and immediately analysed (System 860 including co-oximetry system 835, Bayer Diagnostics, Newbury, UK) to determine blood gases, pH, \( P_{aO_2} \), \( P_{aCO_2} \), and oxygen saturation. Systemic oxygen delivery (DO₂) and consumption (VO₂) were calculated according to standard formulae.

Patients with severe sepsis and healthy controls

The study was approved by the ethics committee of the Friedrich-Schiller-University (2024-05/07) and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from the nearest relatives of all patients before study entry. The study was registered at the Federal Ministry of Education and Research (BMBF), Germany, FKZ: 01EO1002. Ten patients with severe sepsis according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference were included in the study. Exclusion criteria were renal failure (serum creatinine > 1.5 ULN), international normalized ratio > 1.5 and platelet count < 50,000 µl⁻¹. The Acute Physiology and Chronic Health Evaluation (APACHE) II score was obtained on admission. Treatment for septic shock was standardized, including vasopressors (norepinephrine) to maintain an MAP of > 65 mm Hg in addition to repeated fluid challenges with crystalloids to allow the lowest dose of vasopressors. If needed, dobutamine was added up to a dose of 20 µg kg⁻¹ min⁻¹. All patients were initially mechanically ventilated. Sedation with midazolam up to 4 mg h⁻¹ and analgesia with sufentanil 0.5–2 µg kg⁻¹ h⁻¹ was provided according to individual needs.

Temperature, heart rate, arterial pressure, and central venous pressure were measured at study inclusion. Arterial and mixed venous blood samples were withdrawn simultaneously, and blood gases, haemoglobin, and lactate concentrations were measured using an ABL700 Radiometer (Copenhagen, Denmark).

Septic patients were compared with eight patients who underwent a gastroscopy to rule out diseases of the upper GI tract in the presence of unspcific abdominal symptoms.

Confocal laser endomicroscopy

The Cellvizio-GI cLE system (Mauna Kea Technologies, Paris, France) comprises three parts: a laser scanning unit, an acquisition and image analysis device, and an imaging pCLE probe. The pCLE probe (GastroFlex, Mauna Kea Technologies) has a 2.5 mm outer diameter and can be introduced through the working channel of any standard endoscope. The laser excitation wavelength is 488 nm. After conventional endoscopic examination, fluorescein isothiocyanate (FITC)-labelled dextran (70 kDa; TdB Consultancy AB, Upplands, Sweden; 5 ml of a sterile solution) was injected i.v. into septic animals and endomicroscopy was performed. Five to seven areas of gastric and duodenal (access via the oral route), and also ileal (via an ileostomy) and rectal mucosa (via rectal insertion of the scope) were examined in septic animals. The distal tip of the probe was placed in gentle contact with the mucosa to avoid possible influence of pressure on microvascular perfusion. Two experienced endoscopists (C.S. and A.S.) trained in confocal endomicroscopy performed the examinations simultaneously in stomach and duodenum and also in ileum and rectum in the animal study.

Patients with severe sepsis and healthy controls were investigated evaluating only the duodenal mucosa. Patients were investigated within 24 h after the onset of sepsis using 1 ml fluorescein 10% (Alcon Pharma GmbH, Freiburg, Germany) i.v. as a contrast agent. Confocal imaging was performed within 10 min after the injection of fluorescein. Healthy control patients were investigated during their hospital stay. Examinations in septic patients and healthy controls were performed by one single endoscopist (C.S.).

All images obtained by pCLE were recorded as digital sequences of at least 60 s duration per localization (12 frames per second) and analysed thereafter.

Vessel detection software algorithm

Five images per site were analysed to determine FCD and mean capillary diameter using a specially designed detection software algorithm (provided by Mauna Kea Technologies, Paris, France). The size of each individual round image was 600 µm in diameter. The vessel detection algorithm detects the medial axis and the border of tubular structures represented by fluorescein-induced contrast in a specified region of interest (ROI). The length, area, and diameter of the capillaries were calculated as a mean of these tubular structures. For quantification of FCD, the quotient of the capillary area divided by the total area of a given ROI was calculated.

Statistical analysis

Data were analysed using SPSS for Windows, version 18.0 (Somers, NY, USA). After verification of normal distribution of data, univariate one-way analysis of variance was used to detect statistical differences in FCD, capillary length, and mean vessel diameter. Statistical differences were then calculated using Student’s t-test and results are presented mean (±). \( P \leq 0.05 \) were considered to be significant. Bonferroni
Results

Porcine model of sepsis

After induction of sepsis, the animals developed a hyperdynamic state associated with high cardiac output and low systemic vascular resistance. Table 1 represents haemodynamic parameters at baseline, after induction of septic shock and after volume therapy.

Microvascular parameters

The microvascular architecture of the normal gastric mucosa varied with respect to the investigation part of the stomach. The gastric body showed a honeycomb-like subepithelial capillary network pattern surrounding gastric pits (Fig. 2A), whereas the gastric antrum revealed a coil-shaped subepithelial capillary network pattern.

In the duodenum, villi showed a hairpin-like capillary network within each single villus. Capillaries were displayed along the longitudinal villus axis in the loose connective tissue of the lamina propria just below the epithelial lining (Fig. 2B). In the ileum, the capillary structures were similar to that of the duodenum. The normal mucosal surface of the rectum was characterized by hexagonal, honeycomb-like microvascular architecture (Fig. 2C) with luminal crypt openings appearing as black holes projected onto the surface of the mucosa. In general, the capillaries were brightly high-lighted and blood cells could be observed as dark spots within lumen.

To quantitatively analyse the microcirculatory network, functional capillary density (FCD) and also capillary diameter and total capillary length were measured on a total of 420 images. FCD of the area showed a significant reduction under the conditions of sepsis ($P<0.01$) in all intestinal segments indicated (Fig. 3C). After administration of 30 ml kg$^{-1}$ of gelatine, FCD improved significantly in all segments examined ($P<0.05$). FCD partially improved in the stomach (95.4% of pre-sepsis values), ileum (94.4%), and rectum (97%), but remained significantly lower after volume therapy in the duodenum compared with baseline (90.0%) (Fig. 3C). The mean capillary diameter decreased significantly in the upper GI tract (stomach and duodenum) and in the ileum ($P<0.01$), whereas it remained constant in the rectum (Fig. 3A). Moreover, in the upper GI tract, the mean capillary diameter was significantly larger in comparison with baseline values after volume therapy whereas it returned to a pre-septic diameter in the ileum. The total capillary length showed an inhomogeneous alteration under the conditions of sepsis and after treatment (Fig. 3A).

During sepsis, the microcirculatory dysfunction in the mucosa was characterized by an alteration in the distribution of blood flow. In contrast to unaltered flow in the larger microcirculatory vessels, blood flow was stagnant in the smallest capillaries in all segments of the GI tract. Representative video sequences of pCLE from stomach, duodenum, ileum, and rectum are shown in Supplementary videos 1–4, respectively (supplemental digital content).

Correlation of haemodynamic parameters with FCD

Haemodynamic and vital parameters (Table 1) at baseline, after induction of sepsis, and after volume therapy were correlated with FCD at the respective points of time after Bonferroni correction. In summary, none of the parameters tested was significantly correlated with FCD ($P>0.04$). In particular, MAP and FCD were uncoupled after volume therapy of septic shock (Fig. 4).

Human study

We examined 10 patients admitted to the intensive care unit (two females/eight males) median (range) age 61 (48–81) yr. The APACHE II was 21 (14–32). Sources of infection were abdominal in four patients (2× peritonitis, splenic abscess, and pancreatitis), endocarditis in two patients, pneumonia in two patients and catheter-associated bloodstream infection and a knee empyema in one patient each. At the time of pCLE imaging, patients were treated with norepinephrine at a median dose of 0.12 μg kg$^{-1}$ min$^{-1}$ (range 0–0.83 μg kg$^{-1}$ min$^{-1}$), while four patients still had a MAP of < 65 mm Hg. Two healthy control patients were male, six were female, age 64 (48–78) yr (Table 2). None of these patients suffered from an infectious condition.

Microvascular parameters

Vascular architecture in the duodenum of patients with severe sepsis and healthy controls was similar to that of animals, showing a typical villous appearance. Because of the use of fluorescein instead of FITC-labelled dextran leakage of the contrast agent into the surrounding tissue vessel was

| Table 1 Haemodynamic and vital parameters at baseline, 4 h after induction of septic shock, and after volume therapy in the porcine model. Data are presented as mean values (1 sd). |
|-----------------|-----------------|-----------------|
|                  | Baseline        | Septic shock    | After therapy   |
| Temperature (°C) | 36.8 (0.6)      | 39.7 (1.4)      | 39.3 (1.7)     |
| MAP (mm Hg)      | 78 (13.6)       | 59 (17.3)       | 91 (12.4)      |
| Heart rate (bpm) | 88 (12.6)       | 160 (39.8)      | 138 (59.9)     |
| Central venous pressure (mm Hg) | 8 (2.2) | 8 (1.3) | 11 (1.9) |
| Mean pulmonary artery pressure (mm Hg) | 19 (0.0) | 23 (0.0) | 28 (3.5) |
| Pulmonary artery occlusive pressure (mm Hg) | 10 (0.7) | 11 (0.7) | 14 (0.7) |
| Systolic volume variation (%) | 10 (1.3) | 27 (4.4) | 17 (12.5) |
| Oxygen delivery (ml min$^{-1}$) | 282 (59.4) | 328 (17.7) | 304 (38.9) |
| Oxygen consumption (ml min$^{-1}$) | 144 (12.0) | 191 (31.1) | 221 (56.6) |
observed. Despite lower contrast in patients compared with animals reproducible measurements were possible. In accordance with the animal data, we found no significant difference in the total vessel length between septic patients and healthy controls (5.0 vs 5.4 mm; not significant). However, the mean vessel diameter was reduced significantly in septic patients (13.4 vs 14.0 μm; P<0.01) and FCD was reduced accordingly (0.27 vs 0.30; P<0.05).

**Discussion**

In severe sepsis, major alterations in microvascular perfusion have been described in several clinical studies in children and adults and are of pathophysiological and clinical relevance. Disturbed microcirculation of the GI tract is of major importance because it not only represents a complication of sepsis but also facilitates infection and inflammation by...
Promoting translocation of bacteria from the gut into the bloodstream.\textsuperscript{21–26} We showed a significant decrease in the FCD and mean vessel diameter throughout duodenal, ileal, gastric, and rectal mucosal beds in a porcine model of septic shock and validate these data by \textit{in vivo} assessment of the duodenal microcirculation in patients with severe sepsis.

This is the first study reporting the assessment of microvascular alterations during sepsis in different sections of the GI tract. Although other imaging techniques, such as intravital microscopy or laser Doppler flowmetry, are available for the assessment of microcirculation, they require specific surgical preparation or exteriorized mucosa to gain access to the vascular bed.\textsuperscript{12} Imaging techniques such as OPS and SDF have been incorporated into hand-held devices that can easily be used in the assessment of the microcirculation in clinical settings but access to intestinal mucosal areas, however, is limited.

Data concerning a possible correlation of microperfusion in sublingual and intestinal areas are conflicting. In a porcine model of septic shock, a strong correlation between severity and time course of microcirculatory changes in the sublingual and the gut mucosa was described.\textsuperscript{23} However, there is evidence that sublingual perfusion cannot be expected to fully reflect microvascular perfusion all over the GI tract: first, sublingual and ileal microcirculatory alterations did not correlate during the early phase of sepsis in patients with an ileostomy.\textsuperscript{12} Secondly, despite restoring microvascular flow at the sublingual and intestinal serosal level, fluid resuscitation therapy may not restore altered flow in intestinal mucosal capillaries.\textsuperscript{26} Using direct assessment of mucosal perfusion by pCLE, we were able to overcome this problem.

Diagnostic tools to assess intestinal microcirculation should be able to detect the heterogeneity of microvascular perfusion in sepsis\textsuperscript{24} to monitor qualitative derangements of perfusion with non-perfused in close vicinity to well-perfused capillaries. By the use of pCLE, we were able to validate the heterogeneity of microvascular perfusion in various parts of the GI mucosa. During septic shock, some villi in the small intestine and in the honeycomb-like capillary network of the stomach and rectum showed preserved perfusion, whereas perfusion in other areas was sluggish or absent.

In our study on early sepsis, volume therapy with gelatine for septic shock improved microcirculatory perfusion in the upper and lower GI tract. Ospina-Tascon and colleagues\textsuperscript{28} showed improved sublingual microcirculation in septic patients after ‘early’ (within 24 h) but not ‘late’ (after 48 h) volume substitution despite similar increases in global haemodynamic parameters. In accordance with these observations, volume therapy may be effective to improve intestinal microvascularization only in the early phase of septic shock. We also found that global haemodynamic parameters did not correlate with mucosal intestinal microcirculatory alterations and FCD in our study. Gelatine restored mucosal microcirculatory alterations only partially, and microvascular flow was uncoupled from macrovascular flow especially in the duodenum. Indeed, the critical aspect of uncoupled micro- and macrocirculation has been recently demonstrated in a rodent sepsis model\textsuperscript{29} and has been observed in patients with abdominal sepsis using OPS imaging.\textsuperscript{12} In patients with septic shock, both survivors and non-survivors had similar haemodynamic profiles during the course of sepsis.\textsuperscript{11} However, microcirculatory alterations improved rapidly in survivors after septic shock, but not in non-survivors with multiple organ failure, regardless of whether shock had been resolved.\textsuperscript{11} These data further support the importance of direct assessment of mucosal microcirculation in sepsis to identify patients with a poor prognosis and also treatment responders.

However, our study has some technical limitations. The quantification of vessel parameters with the software used is not yet optimal. According to the algorithm-based selection process of vessels, capillaries may either be not identified or overestimated in extension. However, this may be a systematic
error, and for our interpretations the perceptions remained constant. Furthermore, because of repeated measurements, the decrease in FCD could be underestimated in septic animals as FITC–dextran may still stick in the capillaries after the first application of the substance even during the second and third examination. Because of the limitations and technical difficulties of this technique, the use of pCLE may not be suitable for routine application in clinical settings in the current form. The time-consuming analysis should be replaced by fast, automated analysis for pCLE to become a suitable approach in daily clinical practice.

In conclusion, pCLE is a promising tool to evaluate GI microcirculation and to assess influences of therapeutic approaches in sepsis treatment, such as volume therapy or vasopressors. In addition to further evaluation of this promising methodology in animal studies, subsequent larger studies in humans are necessary to define the role of pCLE to assess microcirculation in septic patients.

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

Declaration of interest
None declared.
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Authors’ contributions
C.S. study design, data collection, data analysis and writing up of the first draft of the paper. C.L. data analysis, B.P. data collection. Y.S. patient recruitment. G.M. study design. A.S. study design, data collection and data analysis. All authors read and approved the final version of the manuscript.

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