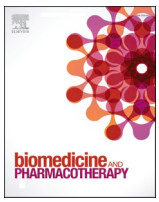
Biomedicine & Pharmacotherapy 171 (2024) 116147

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



Unlocking endothelial barrier restoration: FX06 in systemic capillary leak syndrome and beyond

Maddalena Alessandra Wu [a,*], Laura Locatelli [b], Chiara Cogliati [a,b], Riccardo Colombo [c,1], Jeanette A. Maier [b,1]

[a] Division of Internal Medicine, ASST Fatebenefratelli Sacco, Luigi Sacco Hospital, University of Milan, Milan, Italy
[b] Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy
[c] Division of Anesthesiology and Intensive Care, ASST Fatebenefratelli Sacco, Luigi Sacco Hospital, University of Milan, Milan, Italy

ARTICLE INFO

Keywords:
Idiopathic systemic capillary leak syndrome
Endothelial permeability
Paroxysmal permeability disorders
Treatment
VE-Cadherin
Cytoskeleton
Nitric oxide
FX06

ABSTRACT

Increased vascular permeability is a prevalent feature in a wide spectrum of clinical conditions, but no effective treatments to restore the endothelial barrier are available. Idiopathic systemic capillary leak syndrome (ISCLS) is a life-threatening Paroxysmal Permeability Disorder characterized by abrupt, massive plasma extravasation. This condition serves as a robust model for investigating therapeutic approaches targeting interendothelial junctions. We conducted a single-center, interventional in vitro study at the Referral Center for ISCLS in Italy, involving four diagnosed ISCLS patients, aiming at investigating the effects of FX06, a Bβ15–42 fibrin-derived peptide binding to VE-Cadherin, on endothelial barrier exposed to intercritical and acute ISCLS sera. The Transwell Permeability Assay was used to assess the permeability of human umbilical vein endothelial cells (HUVECs) exposed to ISCLS sera with or without FX06 (50 μg/ml). Acute ISCLS serum was also tested in a three-dimensional microfluidic device. Nitric oxide (NO), VE-Cadherin localization, and cytoskeletal organization were also assessed. In two and three-dimensional systems, ISCLS sera increased endothelial permeability, with a more pronounced effect for acute sera. Furthermore, acute sera altered VE-Cadherin localization and cytoskeletal organization. NO levels remained unchanged. FX06 restored the endothelial barrier function by influencing cellular localization rather than VE-Cadherin levels. In conclusion, FX06 prevents and reverts the hyper-permeability induced by ISCLS sera. These preliminary yet promising results provide initial evidence of the in vitro efficacy of a drug targeting the underlying pathophysiological mechanisms of ISCLS. Moreover, this approach may hold potential for addressing hyperpermeability in a spectrum of clinical conditions beyond ISCLS.

1. Introduction

Idiopathic systemic capillary leak syndrome (ISCLS), also known as Clarkson's disease [1], is a rare disease which belongs to the nosological category of Paroxysmal Permeability Disorders (PPDs) [2]. It represents a potentially rapidly progressing clinical condition due to extensive alteration of the endothelial barrier function. Patients affected by ISCLS may experience non-specific prodromal symptoms and signs (e.g., profound fatigue, oligo-anuria, peripheral edema, presyncope or syncope, and abdominal pain), which may suddenly evolve into a dramatic distributive shock, frequently misdiagnosed. Myocardial edema with increased left ventricular wall thickness and impaired diastolic and systolic function may further complicate the clinical picture, with cardiogenic shock overlapping hypovolemic shock [3–5].

Complications, such as renal failure, rhabdomyolysis, compartment syndrome, venous and arterial thromboses may develop during ISCLS flares and often persist in the post-acute phase, with possible long-term consequences (e.g., chronic renal failure and peripheral neuropathy) [6]. The recurrent nature and unpredictability of attacks make this condition particularly challenging, leading to a remarkable physical, psychological, and social burden to patients.

Regarding the underlying pathophysiological mechanisms, ISCLS shares with other PPDs a transient microvascular barrier dysfunction, in the absence of detectable inflammatory, degenerative, or ischemic injury of the endothelial cells.

The maintenance of the endothelial barrier is fundamental for tissue

* Correspondence to: Division of Internal Medicine, ASST Fatebenefratelli Sacco, Luigi Sacco Hospital, University of Milan, Via G.B. Grassi 74, 20157 Milan, Italy.
E-mail addresses: maddalena.ale.wu@gmail.com, wu.maddalena@asst-fbf-sacco.it, maddalena.wu@unimi.it (M.A. Wu).
[1] Joint last authors

https://doi.org/10.1016/j.biopha.2024.116147
Received 9 November 2023; Received in revised form 7 January 2024; Accepted 10 January 2024
Available online 18 January 2024

homeostasis and relies on the intercellular junctions between endothelial cells [7]. In particular, VE-Cadherin, the principal component of adherens junctions, is identified as the master regulator of the endothelial barrier [8,9]. It is a very dynamic adhesion molecule that responds to mechanical and chemical signals in both physiological and pathological contexts [9]. When VE-Cadherin is internalized through clathrin-coated vesicles, cell-cell junctions loosen and endothelial permeability increases, thus causing edema [7]. In vitro studies have shown that episodic sera from ISCLS patients induce the internalization of VE-Cadherin [10,11]. Also endothelial apoptosis has been advocated as a possible mechanism of increased permeability during ISCLS attacks [12–14]. In addition, it has been suggested that increased amounts of humoral factors such as Vascular Endothelial Growth Factor (VEGF) and Angiopoietin-2 (Ang2) may shape actin stress fiber formation and endothelial cytoskeletal rearrangement and contraction, which result in paracellular hyperpermeability without cell apoptosis [10].

Furthermore, since nitric oxide (NO) cascade and the pathway of NO synthase are among the main factors increasing vascular permeability, a role for NO in the pathogenesis of ISCLS flares has been hypothesized [15].

Over the decades, various prophylactic and acute treatment regimens have been investigated with alternate efficacy.

Sadly, there are no proven therapies for ISCLS flares. The use of intravenous immunoglobulins (IV Ig) has been advocated as the most useful prophylactic treatment, and IV Ig withdrawal is associated with increased mortality and higher rates of ISCLS recurrence [16]. Administration of IV Ig close to the onset of ISCLS-related symptoms might be associated with a favorable outcome, preventing further circulatory derangement at a very early phase of the ISCLS attack [17]. However, this precise timing of IV Ig treatment right after the onset of ISCLS flares remains anecdotal. IV Ig are thought to act as immunomodulators, not merely as a means to restore plasma volume. Specific additional mechanisms still need to be elucidated.

During the shock phase, the management is mainly supportive. To date, no specific treatment is available for acute crises, and the most rational strategy often implies attempting to avoid harmful interventions rather than targeting the underlying pathophysiological mechanisms. It should be noted that therapies usually applied in shock from other etiologies are often ineffective and may cause further severe complications that ultimately lead to death by multiple organ failure. Thus, a “less is more” approach is recommended: judicious fluid replacement, cautious use of low dose vasopressors and rigorous monitoring to identify the indicators of the transition from the acute to the post-acute phase are the cornerstones of appropriate management [6].

FX06 is a naturally occurring peptide, Bβ15–42, derived from the E1 fragment of fibrin, which has considerable therapeutic potential for all diseases and pathological conditions associated with increased vascular permeability. FX06 binds to VE-Cadherin in its native intercellular configuration, stabilizing the endothelial barrier [18–20].

Based on its characteristics, we considered FX06 suitable for the treatment in diseases primarily characterized by capillary leakage and specifically for Clarkson’s disease.

We conducted a study to assess the effects of FX06 on the endothelial barrier function by culturing human endothelial cells in two-dimensional (2D) and three-dimensional (3D) systems and exposing them to sera of ISCLS patients.

2. Materials and methods

2.1. Patients and setting

Patients with a diagnosis of ISCLS in follow-up at the Luigi Sacco Hospital in Milan, Italy, were considered eligible for enrollment in the study. ISCLS was identified according to the established criteria of one or more episodes of distributive shock characterized by marked hemoconcentration and hypoalbuminemia, generalized or segmental non-pitting edema, in the absence of secondary causes of capillary leakage [1,6].

Sera from four patients with a diagnosis of ISCLS were collected. Table 1 shows patients’ characteristics and disease features.

Remarkably, one patient (patient 1) underwent blood withdrawals during the acute phase of ISCLS triggered by SARS-CoV-2 infection and blood samples were collected at the zenith of clinical-biochemical compromise. The other three patients were sampled during the intercritical phase.

The study was approved by the local Ethical Committee (Comitato Etico Area 1, Milan, reg. number 0002793 approved on 22nd January 2021). Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 2013 Declaration of Helsinki.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Basel, Switzerland) were cultured on 2% fibronectin-coated dishes using EBM-2 culture medium and used up to passage 6 according to manufacturer’s instructions. In 3D experiments, HUVECs were cultured in microfluidic devices fabricated using a standard soft-lithography technique, as previously described [21–23], with a shear stress of 0.4 Pa, for four days before beginning the treatments, and then they were fixed for immunofluorescence. Fetal Bovine Serum (FBS) of the culture media was replaced with 10% of serum from CTR or ISCLS patients (collected during the acute crisis or in an intercritical phase).

Before testing FX06 (F4 Pharma GmbH, Wien, Austria) on HUVECs exposed to the patients’ sera, the effect of FX06 on cell viability was preliminarily assessed at increasing drug concentrations (Supplement Methods, Viability assay and Supplement Fig. 1).

We selected 50 μg/ml of FX06 for all the following experiments, and the timeline of each experiment’s steps is graphically depicted in Fig. 1.

2.3. Permeability assays

The Transwell Permeability Assay was performed in a 24-well receiver plate with individual hanging cell culture inserts (Transwell Permeable Supports, 0.4 μm micropores, Euroclone). HUVECs were seeded into the inserts and, when confluent, treated with bradykinin (10 μM), inflammatory cytokines (simultaneously added interleukin (IL)− 1β and Tumor Necrosis Factor (TNF) α, each at 10 ng/ml) or different ISLCS sera with and without FX06. Sera from healthy volunteers were used as negative controls (CTR). At the end of the treatment, 1 mg/ml Fluorescein isothiocyanate labelled–albumin (FITC–BSA) (Sigma–Aldrich, St. Louis, Missouri, USA) was added in the upper part of the transwell and the degree of permeability was determined by measuring the fluorescence of the FITC–BSA passed in the lower compartment of the transwell. Fluorescence was detected using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA) ($\lambda exc = 495$ nm, $\lambda emm = 519$ nm) [24].

The X-PerT assay was used to measure permeability in the 3D microfluidic devices [2]. Microfluidic devices were coated with biotin-fibronectin (20 μg/ml, Cytoskeleton Inc, Denver, CO, USA). Briefly, at the end of the experiment, Avidin-555 (Thermo Fisher Scientific) was added to the medium (final concentration 2.5 μg/ml) for two minutes, followed by two washes with Phosphate Buffered Saline (PBS). Avidin-555 specifically binds to the biotin-fibronectin coating, generating fluorescence in areas where spaces exist between cells. Subsequently, the microfluidic devices were fixed and processed for immunofluorescence, as described below. In the 3D analysis, permeability was measured by quantifying the intensity of the Avidin-555 staining using ImageJ. The experiments were performed in triplicate three times, and data are expressed as percentages compared to the untreated control (CTR) ± standard deviation (SD).

Table 1
Characteristics of the patients.

	Age, years	Gender	Disease phase	Prophylactic regimen	Concomitant condition	ISCLS crises, n
Patient 1	67	Female	Acute	IVIG (1 g/kg of body weight)	COVID-19, MGUS (IgG k, 2.8 g/l)	Three
Patient 2	30	Female	Intercritical	none	Absolute IgA deficiency; no monoclonal component at serum electrophoresis	Two
Patient 3	42	Male	Intercritical	IVIG (1 g/kg of body weight), inadequate compliance to therapy	MGUS (IgG k, 1.7 g/l)	Ten
Patient 4	59	Female	Intercritical	IVIG (1 g/kg of body weight), started after blood withdrawal	None	Two

IVIG, intravenous immunoglobulin; MGUS, monoclonal gammopathy of undetermined significance.

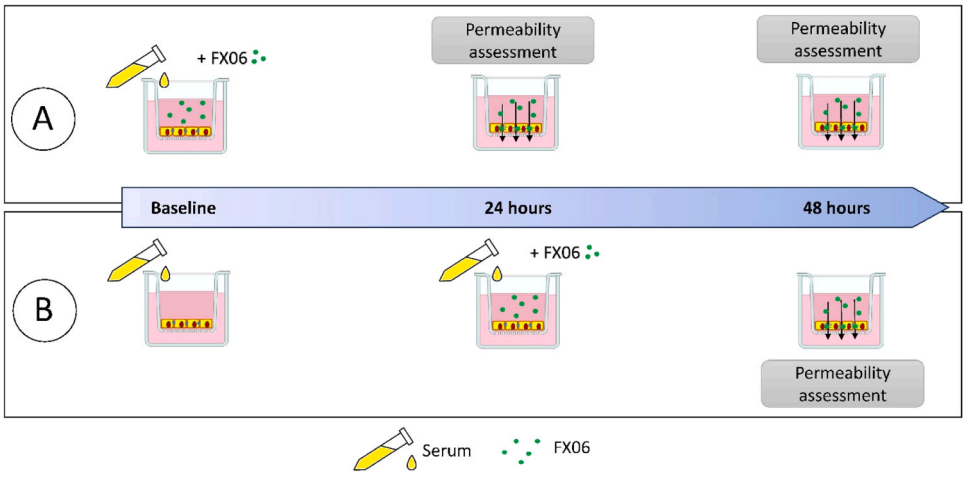


Fig. 1. Timeline of the Experiments. (A) At time 0, HUVECs were treated simultaneously with FX06 and sera from ISCLS patients or healthy controls (CTR). Permeability was measured 24 and 48 h later. (B) At time 0, HUVECs were treated with the sera either from ISCLS patients or healthy controls (CTR). After 24 h, media were replaced with freshly added sera and FX06 for additional 24 h. Then, permeability was measured, as described.

2.4. Immunofluorescence and confocal imaging

The cells were fixed in PBS containing 4% paraformaldehyde and 2% sucrose (pH 7.6), permeabilized with Triton 0.3% and incubated with anti-VE-Cadherin antibodies (Thermo Fisher Scientific) overnight at 4 °C, and then stained with an Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific). To stain the actin cytoskeleton, the cells were incubated with TRITC-phalloidin (for 2D experiments) or Alexa Fluor 647-phalloidin (for 3D experiments) (Thermo Fisher Scientific). 4′,6-Diamidine-2′-phenylindole dihydro-chloride (DAPI, Sigma-Aldrich) was used to stain the nuclei. Finally, the cells were mounted with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific), and images were acquired using a 40x objective in oil by a SP8 Leica confocal microscope. The image stacks were processed to analyze fluorescence intensity using the ImageJ.

2.5. Measurement of nitric oxide (NO)

Since the half-life of NO in the circulation is shorter than 0.1 s, we used the Griess assay to measure circulating NO metabolites ($NaNO_2$) directly on the collected sera. Briefly, a freshly prepared Griess reagent, made with sulfanilamide and N-(1-naphthyl)ethylenediamine (1:1), was added to the collected sera. The absorbance was measured at 550 nm using Varioskan Lux (Thermo Fisher Scientific), and the amounts of nitrites in the samples were determined using a standard curve generated with known concentrations of sodium nitrite ($NaNO_2$). All these experiments were performed three times in triplicate, and data are expressed as μM of $NaNO_2 \pm$ SD.

2.6. Western blot

HUVECs were lysed in 10 mM Tris-HCl (pH 7.4) containing 3 mM $MgCl_2$, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protein inhibitors, separated on SDS-PAGE and transferred to nitrocellulose sheets at 400 mA for two hours at 4 °C. Western blot analysis was performed using antibodies against VE-Cadherin (Thermo Fisher Scientific) and actin (Tebu Bio-Santa Cruz, Magenta, Italy), which was used as a loading control. After extensive washing, secondary antibodies labelled with horseradish peroxidase (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) were used. Immunoreactive proteins were detected by the Super-Signal chemiluminescence kit (Thermo Fisher Scientific). All the experiments were performed at least three times, and a representative blot is shown. Densitometry of the bands was performed with the software ImageLab (Bio-Rad, Hercules, CA, USA) on three blots.

2.7. Statistical analysis

Results of the experiments were analyzed using one-way repeated measures ANOVA followed by post hoc Bonferroni test for multiple comparisons. Statistical significance was defined as a p-value ≤ 0.05 for a two-tail test.

3. Results

3.1. FX06 prevents bradykinin and cytokine-induced endothelial hyperpermeability

After assessing that FX06 had no toxicity on HUVECs (Supplement

Fig. 1), we investigated whether 24 h exposure to FX06 (50 μg/ml) affects the total amounts and the localization of VE-Cadherin under normal culture conditions. Western blot shows that FX06 does not affect the levels of the protein (Fig. 2A). Confocal microscopy reveals no differences in VE-Cadherin localization (green) and in actin fiber organization (red) in HUVECs treated or not with FX06 (Fig. 2B).

Then, we evaluated FX06 effect in HUVECs treated with the permeabilizing agent bradykinin (BK) or with the pro-inflammatory cytokines IL-1β and TNFα added together (10 ng/ml each) for 24 h. The simultaneous addition of FX06 hampers the increase of permeability induced by BK and cytokines (Fig. 2C).

3.2. FX06 prevents or restores endothelial hyperpermeability in response to sera from patients suffering from ISCLS

Sera collected from ISCLS patients were added to the culture medium and endothelial permeability was measured using the transwell method. We found that both at 24 and 48 h the episodic and the intercritical sera increased the permeability of HUVECs, as compared to the healthy control sera (CTR) (Fig. 3). Afterwards, we utilized FX06 at a 50 μg/ml concentration to investigate its activity on sera-induced endothelial hyperpermeability. We treated HUVECs simultaneously with FX06 and sera (named "+FX06″) and measured permeability both at 24 (A) and 48 h (B) from the beginning of the experiments. We demonstrate that FX06 prevents the permeabilizing effects of the sera. Moreover, we

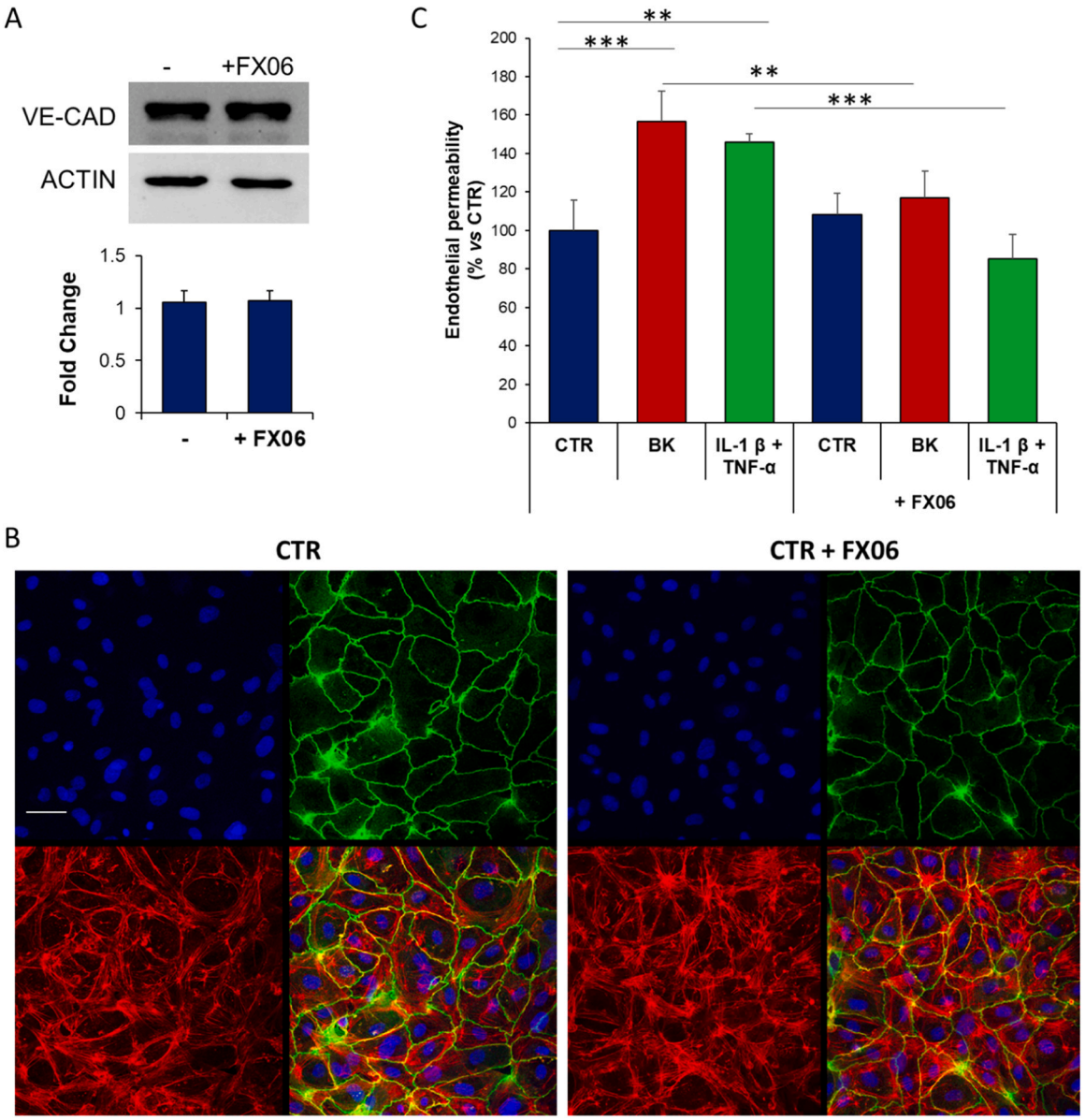


Fig. 2. The effect of FX06 on VE-Cadherin levels and endothelial permeability. (A) Western blots were performed on protein lysates derived from HUVECs treated for 24 h with control medium (-) or medium containing FX06 (+FX06) using antibodies against VE-Cadherin (VE-CAD). Anti-β-actin antibodies were used as control of equal loading. Densitometry of the bands was performed with the software ImageLab and expressed as the fold change compared to the untreated control ± standard deviation (SD). A representative blot of three independent experiments is shown. (B) HUVECs cultured on a glass coverslip were treated for 24 h with control medium (CTR) or with control medium containing 50 μg/ml of FX06 (CTR + FX06). The cells were incubated with anti-VE-Cadherin antibodies and then stained with an Alexa Fluor 488 secondary antibody (green) or TRITC-phalloidin (red). DAPI (blue) was used to stain the nuclei. In CTR and CTR+FX06, the lower right panel shows the merged image. Images were acquired using a 40 × objective in oil by an SP8 Leica confocal microscope. Scale bar: 100 μm. (C) HUVECs were seeded on transwell inserts and then treated for 24 h with some permeability-inducing treatments with or without FX06 and the permeability was measured. Data are expressed as the percentage compared to the untreated control (CTR) ± standard deviation (SD). * * $p \leq 0.01$; * ** $p \leq 0.001$.

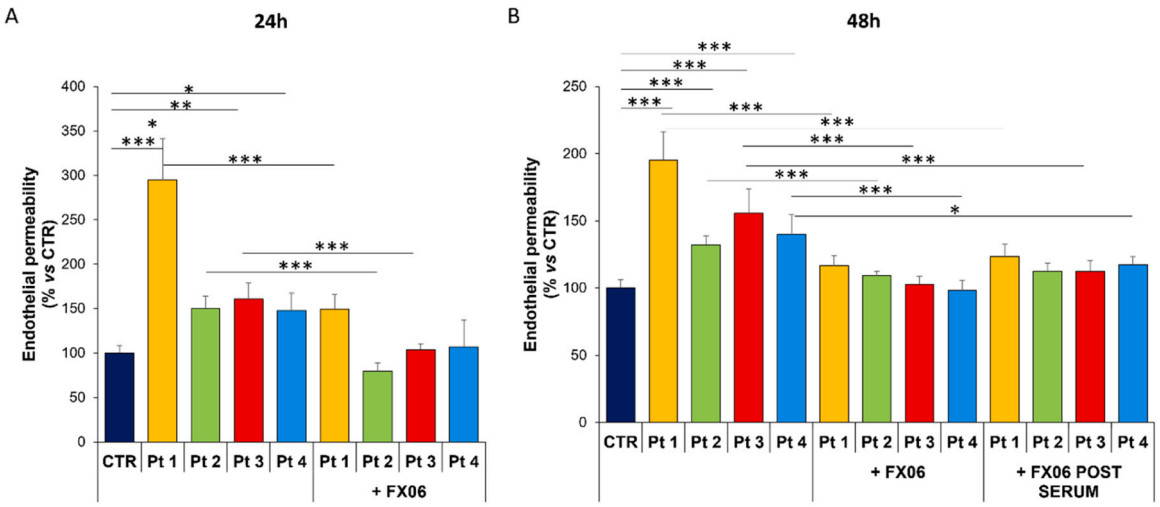


Fig. 3. Endothelial permeability after the treatment with sera from patients suffering of ISCLS with or without FX06. HUVECs were seeded on transwell inserts and then treated for 24 (A) or 48 h (B) with serum with or without FX06 and the permeability was measured. Data are expressed as the percentage of the untreated control (CTR) ± standard deviation (SD). * $p \leq 0.05$; * * $p \leq 0.01$; * ** $p \leq 0.001$.

added FX06 after 24 h of serum treatment (named "+FX06 post serum") and measured permeability after additional 24 h (B) of culture. We show that FX06 restores endothelial barrier function.

3.3. Nitric oxide (NO) does not change in the sera from patients suffering of ISCLS

Measurement of circulating NO metabolites ($NaNO_2$) revealed no modulation of NO levels in ISCLS sera compared to control, suggesting that NO is not the mediator of the increased permeability in these patients (Fig. 4).

3.4. FX06 prevents or restores VE-Cadherin maldistribution in response to the serum collected during an acute ISCLS phase: experiments in 2D

To get insights into the mechanisms involved in the modulation of endothelial permeability, we analyzed VE-Cadherin localization by confocal microscopy in HUVECs treated for 24 h with the serum from patient 1, which was collected during the acute phase, in the presence or absence of FX06. While in intact, healthy endothelial cells VE-Cadherin is continuously distributed along cells borders (Fig. 5A, left panel), the addition of the serum alone induces the formation of gaps between the cells (Fig. 5A, white arrows). Simultaneously added FX06 prevents this effect (Fig. 5A, right panel). We also looked at the cytoarchitecture of the cells [23] and found that the treatment with the serum does not impact the cytoskeleton visualized by actin staining (Fig. 5A in red).

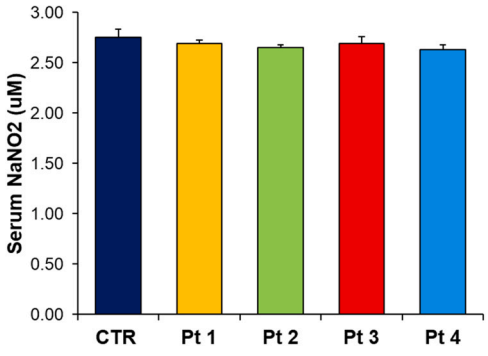


Fig. 4. Nitric oxide in the sera from patients suffering from ISCLS. The levels of $NaNO_2$ were measured in the sera from healthy donors (CTR) and in the sera from ISLCS patients using the Griess method. Data are expressed as μM of $NaNO_2$ ± standard deviation (SD).

We also added FX06 after 24 h of exposure to the serum (Pt 1 "+FX06 post") for an additional 24 h. As expected, gaps between cells persist after 48 h of treatment with the serum (Fig. 4B, white arrows in central panel). The addition of FX06 in the last 24 h restores the normal VE-Cadherin staining (Fig. 5B, right panel). Moreover, we found that the treatment with the serum does not alter the cytoskeleton visualized through actin staining at 48 h of treatment (Fig. 5B in red).

Similar results were obtained when the sera from intercritical patients were utilized (Supplement Fig. 2-4).

3.5. FX06 prevents or restores VE-Cadherin disorganization in response to the serum collected during an acute ISCLS phase: experiments in a 3D microfluidic chip

To better mimic the geometric features of a vessel exposed to a constant flow, we cultured HUVECs in a 3D microfluidic chip to study the effects of the serum from patient 1 with or without FX06 (Fig. 6). In the 3D device, the exposure for 24 h to the ISCLS episodic serum induces the formation of gaps in the VE-Cadherin staining, which appears uniformly distributed in the controls (Fig. 6A). This relocalization parallels a remarkable increase in endothelial permeability, as detected by the yellow staining nearby the gaps between cells and its quantification (Figs. 6B, D). On the contrary, the sample treated for 24 h with the drug together with the serum (Pt 1 + FX06) shows the proper localization of VE-Cadherin on the membrane and no increase of permeability, confirming that in 3D in the presence of shear stress, the drug restores endothelial barrier function (Fig. 6C). Intriguingly, in the 3D microfluidic chip the episodic ISCLS serum (patient 1) disorganizes the cytoskeleton. The cells have fewer and thinner stress fibers than the CTR, associated to an increase of cortical actin (Fig. 6, red staining). It is noteworthy that these features are detectable in 3D but not in 2D [23]. Even in this case, the simultaneous treatment with FX06 (Pt 1 + FX06) restores cellular cytoarchitecture, which returns comparable to the untreated control.

4. Discussion

Our results highlight that the Bβ15–42 fibrin-derived peptide FX06 can prevent and revert endothelial hyperpermeability induced by the sera of ISCLS patients, with a net impact on the localization of VE-Cadherin, the key player of endothelial permeability. These effects were also confirmed in endothelial cells challenged with serum

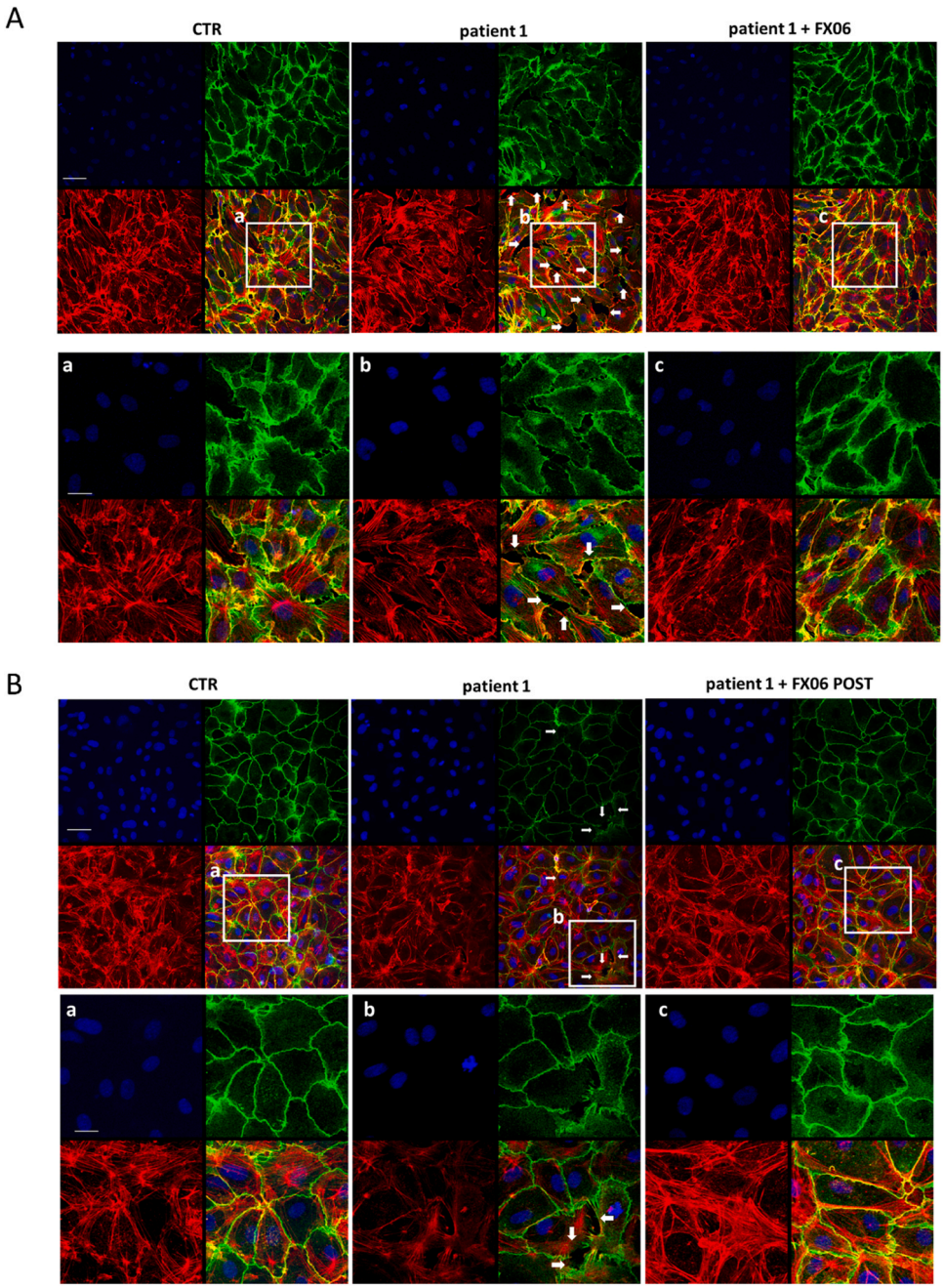


Fig. 5. The effect on VE-Cadherin localization and cytoskeletal organization of 24 h and 48 h exposure of HUVECs to the serum from patient 1 with or without FX06. (A) HUVECs were cultured on glass coverslips and exposed to serum with or without FX06 for 24 h. (B) HUVECs cultured on glass coverslips were initially exposed to serum from patient 1. After 24 h FX06 was added to the medium (Pt 1 "+FX06 (POST)") for an additional 24 h. The cells were incubated with anti-VE-Cadherin antibodies and stained with an Alexa Fluor 488 secondary antibody (green) or TRITC-phalloidin (red). DAPI (blue) was used to stain the nuclei. Images were acquired using a 40 × objective in oil by an SP8 Leica confocal microscope. Scale bar upper panels: 100 μm. White arrows highlight gaps between cells (black spots) or VE-Cadherin staining interruptions. White boxes highlight the zoommed areas in a, b, c. Scale bar zoommed panels: 30 μm.

withdrawn from a critically ill patient with complex features because of ISCLS flare and overt COVID-19 pneumonia, thus with an extreme derangement of all the pathways that affect the endothelial barrier function. Furthermore, in vitro FX06 restored both the barrier function and the cytoarchitecture.

Of interest, we also employed an innovative approach utilizing three-dimensional microfluidic systems, where HUVECs uniformly coat the surfaces of artificial vessels under dynamic flow conditions. This cutting-edge methodology allows the observation of cellular elongation and alignment in the direction of the flow, closely mimicking physiological conditions. By utilizing these advanced experimental conditions, we were able to capture early alterations in endothelial function that may elude traditional two-dimensional (2D) cultures [2]. In ISCLS, the role of shear stress becomes crucial, as it is likely to increase during acute flares due to hemoconcentration [25]. Our investigation using the 3D microfluidic models revealed novel insights. ISCLS episodic serum not only increased endothelial permeability by altering VE-Cadherin localization but also induced significant cytoskeletal alterations. This phenomenon remained undetected in 2D cultures. These findings highlight the potential of this innovative method in unravelling the intricate mechanisms underlying ISCLS pathophysiology. Our findings may pave the way for further investigation into the molecular mechanisms mediating

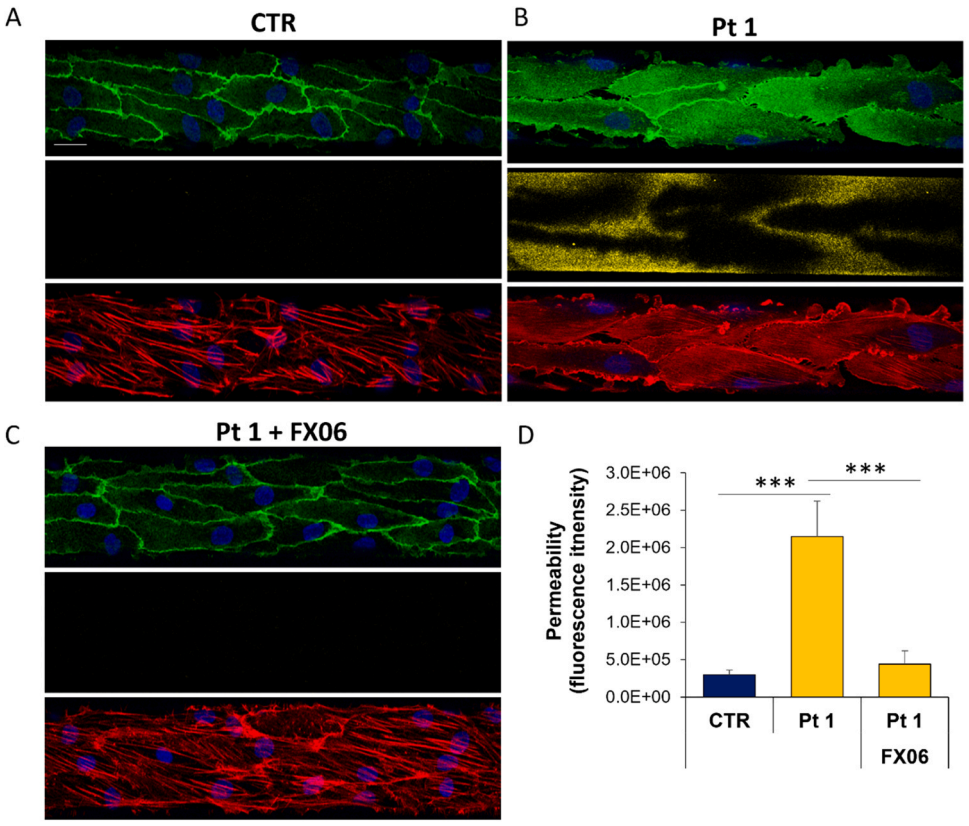


Fig. 6. The effect on VE-Cadherin localization, endothelial permeability, and cytoskeletal organization of HUVECs cultured in a 3D chip and exposed to the serum from patient 1 with or without FX06. HUVECs cultured in 3D microfluidic devices were treated for 24 h with serum alone or in combination with FX06. Before fixation, avidin-555 was added to the medium to detect gaps between cells (yellow), and permeability was measured. The cells were then fixed and incubated with anti-VE-Cadherin antibodies and stained with an Alexa Fluor 488 secondary antibody (green) or with Alexa Fluor 647-phalloidin (red). DAPI (blue) was used to stain the nuclei. Images were acquired using a 40 × objective in oil by an SP8 Leica confocal microscope. Scale bar: 20 μm. (A) HUVECs exposed to serum from healthy controls show uniformly distributed VE-Cadherin in all cells, no increase of endothelial permeability, and no alterations of the cytoskeleton. (B) HUVECs exposed to serum from an ISCLS patient during an acute flare exhibit the development of gaps in the VE-Cadherin staining, as well as disorganization of the cytoskeleton. This relocalization of VE-Cadherin corresponds to a remarkable increase in endothelial permeability, as detected by the yellow staining near the gaps between cells. (C) HUVECs treated for 24 h with the drug together with the serum (Pt 1 + FX06) show the proper localization of VE-Cadherin on the membrane and no increase of permeability, as well as preserved cellular cytoarchitecture. (D) Permeability of HUVECs exposed to serum from an ISCLS patient during an acute flare, with or without the drug, depicted as fluorescence intensity.

endothelial barrier dysfunction in ISCLS, as well as acquired systemic capillary leak. It is noteworthy that actomyosin-dependent contraction leading to disruption of endothelial integrity has been reported to be associated with RhoB-controlled inhibition of Rac1 activity and trafficking in response to exposure to inflammatory cytokines such as TNF. This occurs through the regulation of downstream signaling pathways, such as the NF-κB–dependent pathway [26]. The report of a fatal case of pediatric ISCLS linked to a point mutation in p190BRhoGAP and prolonged RhoB activation resulting in the inhibition of endothelial cell barrier recovery appears to be consistent with the involvement of this pathophysiological mechanism [27].

Furthermore, our results suggest that hyperpermeability due to ISCLS sera was independent of the nitric oxide pathway. This observation is particularly significant considering that NO, known for its potent vasodilator activity and close association with inflammation, coagulation and oxidative stress [28], has often been envisaged to be involved in ISCLS and even some treatments targeting this specific pathway have been advocated for the acute crisis, such as methylene blue [15].

We found that both acute and intercritical ISCLS sera increased the endothelial permeability as compared to the effect of sera from healthy controls. Although intercritical sera have not been shown to affect endothelial permeability by other authors in vitro [10] and induced less marked effects than episodic sera in our study, we speculate that the difference found between the results with intercritical and control sera should be considered meaningful from a clinical point of view. Hence, our results may be consistent with the "threshold hypothesis", suggesting that ISCLS patients may exhibit a chronic predisposition to endothelial hyperpermeability. This susceptibility may be easily overcome when the patients encounter other potentially exacerbating factors. This hypothesis is consistent with results by other teams showing ongoing endothelial dysfunction induced by remission ISCLS sera, as demonstrated, for instance, by the significant increase of several endothelial-glycocalyx-related components in both acute and remission ISCLS sera (in the absence of overt clinical symptoms) compared to controls [29].

Currently, there are no effective treatments for the shock phases of the ISCLS crises. IV Ig represent the standard of care as prophylactic treatment and have been advocated for potentially preventing further clinical worsening when given at the very early stages of ISCLS flares. Both in vitro and in vivo studies have highlighted the good safety profile of FX06, which has been previously used with limited experience in non-PPDs and in a case of vascular leakage due to Ebola virus disease with promising results [30,31]. FX06 has no nephrotoxicity, typically complicating high-dose IV Ig use, especially in the hypovolemic state characterizing the ISCLS flares.

A crucial point deserving specific mention is that FX06 was added to the culture media with varying time schedules to investigate whether its efficacy was confirmed at variable time points after exposure of

endothelial cells to ISCLS sera. The encouraging result is that this drug has beneficial effects on the endothelial cells when co-administered with ISCLS sera, as well as when given after a relatively prolonged exposure of the cells to ISCLS sera alone. Therefore, we hypothesize that FX06 might be effective in vivo even when administered after the onset of an acute crisis. On the other hand, its effect also on endothelial cell cultures exposed to intercritical sera may suggest the potential usefulness of a very early administration when the patient is still experiencing prodromal symptoms and the "permeability threshold" is going to be reached.

5. Conclusions

Our study presents the first report of preliminary yet highly promising findings regarding the effectiveness of a drug targeting the underlying pathophysiological mechanisms of ISCLS. FX06 shows potential in restoring impaired endothelial barrier function during acute ISCLS crises, marking a significant breakthrough in managing this life-threatening condition, for which no proven effective therapy currently exists.

While the rarity of ISCLS poses challenges in conducting large interventional randomized controlled trials, further studies are needed to confirm the efficacy of FX06 in vivo during ISCLS crises and, ideally, in the prodromal phase to prevent clinical worsening.

Considering that endothelial hyperpermeability is a shared pathophysiological mechanism observed in various clinical conditions, we hypothesize that ISCLS could serve as a paradigmatic model for permeability disorders. This unique model may be less susceptible to the influence of complex confounding factors. Future perspectives should explore the application of FX06 in a broader spectrum of clinical scenarios characterized by increased endothelial permeability. This investigation could encompass conditions such as Acute Respiratory Distress Syndrome, sepsis of varying origins, extensive burns and trauma, ischemia-reperfusion injury, hemorrhagic fever, carbon monoxide poisoning, and organ transplantation.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Wu Maddalena Alessandra: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Maier Jeanette:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Colombo Riccardo:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. **Cogliati Chiara Beatrice:** Investigation, Resources, Supervision, Visualization, Writing – review & editing. **Locatelli Laura:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing.

Declaration of Competing Interest

None.

Acknowledgements

We thank our colleagues Chaouqi Misbah and Lionel Bureau (LIPhy, Université Grenoble Alpes) for providing the 3D chips.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116147.

References

[1] B. Clarkson, D. Thompson, M. Horwith, E.H. Luckey, Cyclical edema and shock due to increased capillary permeability, Am. J. Med. 29 (1960) 193–216.
[2] M.A. Wu, D. Tsvirkun, L. Bureau, I. Boccon-Gibod, M. Inglebert, A. Duperray, et al., Paroxysmal permeability disorders: development of a microfluidic device to assess endothelial barrier function, Front. Med. 6 (2019) 89.
[3] M.A. Wu, E. Catena, C. Cogliati, D. Ottolina, A. Castelli, R. Rech, et al., Myocardial edema in paroxysmal permeability disorders: the paradigm of Clarkson's disease, J. Crit. Care 57 (2020) 13–18.
[4] A. Ertel, D. Pratt, P. Kellman, S. Leung, P. Bandettini, L.M. Long, et al., Increased myocardial extracellular volume in active idiopathic systemic capillary leak syndrome, J. Cardiovasc Magn. Reson 17 (2015) 76.
[5] M. Pineton de Chambrun, A. Mathian, C.E. Luyt, A. Combes, Z. Amoura, G. EureClark Study, Myocardial dysfunction is frequent in systemic capillary-leak syndrome (Clarkson disease) severe episodes, J. Allergy Clin. Immunol. 141 (2018) 1539–1540.
[6] M.A. Wu, R. Colombo, G.M. Podda, M. Cicardi, Handling shock in idiopathic systemic capillary leak syndrome (Clarkson's disease): less is more, Intern. Emerg. Med. 14 (2019) 723–730.
[7] L. Claesson-Welsh, E. Dejana, D.M. McDonald, Permeability of the endothelial barrier: identifying and reconciling controversies, Trends Mol. Med. 27 (2021) 314–331.
[8] Y.A. Komarova, K. Kruse, D. Mehta, A.B. Malik, Protein interactions at endothelial junctions and signaling mechanisms regulating endothelial permeability, Circ. Res. 120 (2017) 179–206.
[9] M.G. Lampugnani, E. Dejana, C. Giampietro, Vascular endothelial (VE)-Cadherin, endothelial adherens junctions, and vascular disease, Cold Spring Harb. Perspect. Biol. 10 (2018).
[10] Z. Xie, C.C. Ghosh, R. Patel, S. Iwaki, D. Gaskins, C. Nelson, et al., Vascular endothelial hyperpermeability induces the clinical symptoms of Clarkson disease (the systemic capillary leak syndrome), Blood 119 (2012) 4321–4332.
[11] W.J. Lesterhuis, A.J. Rennings, W.P. Leenders, A. Nooteboom, C.J. Punt, F. C. Sweep, et al., Vascular endothelial growth factor in systemic capillary leak syndrome, Am. J. Med. 122 (2009) e5–e7.
[12] R. Assaly, D. Olson, J. Hammersley, P.S. Fan, J. Liu, J.I. Shapiro, et al., Initial evidence of endothelial cell apoptosis as a mechanism of systemic capillary leak syndrome, Chest 120 (2001) 1301–1308.
[13] B.R. Johansson, C.G. Lofdahl, Ultrastructure of the microvessels in skeletal muscle in a case of systemic capillary leak syndrome, Acta Med. Scand. 206 (1979) 413–416.
[14] P. Kapoor, P.T. Greipp, E.W. Schaefer, S.J. Mandrekar, A.H. Kamal, N.C. Gonzalez-Paz, et al., Idiopathic systemic capillary leak syndrome (Clarkson's disease): the Mayo clinic experience, Mayo Clin. Proc. 85 (2010) 905–912.
[15] M. Umbrello, M. Gardinali, D. Ottolina, G. Zanforlin, G. Iapichino, Systemic capillary leak syndrome: is methylene blue the silver bullet? Case Rep. Crit. Care 2014 (2014) 141670.
[16] Q. Moyon, M. Pineton de Chambrun, M. Gousseff, M. Hie, G. Urbanski, F. Verlicchi, et al., Intravenous immunoglobulins tapering and withdrawal in systemic capillary-leak syndrome (Clarkson Disease), J. Allergy Clin. Immunol. Pr. (2022).
[17] M.S. Pecker, M. Hammudi, R. Melchio, A.R. Eisch, F. Verlicchi, K.M. Druey, Management of acute episodes of clarkson disease (Monoclonal Gammopathy-Associated Systemic Capillary Leak Syndrome) with intravenous immunoglobulins, Ann. Intern. Med.: Clin. Cases 1 (2022).
[18] M. Groger, W. Pasteiner, G. Ignatyev, U. Matt, S. Knapp, A. Atrasheuskaya, et al., Peptide Bbeta(15-42) preserves endothelial barrier function in shock, PLoS One 4 (2009) e5391.
[19] S. Bergt, M. Gruenewald, C. Beltschany, A. Grub, T. Neumann, M. Albrecht, et al., The fibrin-derived peptide bbeta15-42 (FX06) ameliorates vascular leakage and improves survival and neurocognitive recovery: implications from two animal models of cardiopulmonary resuscitation, Crit. Care Med. 44 (2016) e988–e995.
[20] E.H. Adam, B. Schmid, M. Sonntagbauer, P. Kranke, K. Zacharowski, P. Meybohm, Fibrin-derived peptide Bbeta15-s salvage treatment in critically ill patients with COVID-19-associated acute respiratory distress syn42 (FX06) adrome, Crit. Care 24 (2020) 574.
[21] D. Tsvirkun, A. Grichine, A. Duperray, C. Misbah, L. Bureau, Microvasculature on a chip: study of the endothelial surface layer and the flow structure of red blood cells, Sci. Rep. 7 (2017) 45036.
[22] M. Inglebert, L. Locatelli, D. Tsvirkun, P. Sinha, J.A. Maier, C. Misbah, et al., The effect of shear stress reduction on endothelial cells: a microfluidic study of the actin cytoskeleton, Biomicrofluidics 14 (2020) 024115.
[23] L. Locatelli, M. Inglebert, R. Scrimieri, P.K. Sinha, G.V. Zuccotti, P. Milani, et al., Human endothelial cells in high glucose: new clues from culture in 3D microfluidic chips, FASEB J. 36 (2022) e22137.
[24] L. Locatelli, G. Fedele, J.A. Maier, The role of txnip in mediating low-magnesium-driven endothelial dysfunction, Int. J. Mol. Sci. 24 (2023).
[25] R. Colombo, M.A. Wu, A. Castelli, T. Fossali, R. Rech, D. Ottolina, et al., The effects of severe hemoconcentration on acid-base equilibrium in critically ill patients: the forgotten role of buffers in whole blood, J. Crit. Care 57 (2020) 177–184.

[26] B. Marcos-Ramiro, D. Garcia-Weber, S. Barroso, J. Feito, M.C. Ortega, E. Cernuda-Morollon, et al., RhoB controls endothelial barrier recovery by inhibiting Rac1 trafficking to the cell border, J. Cell Biol. 213 (2016) 385–402.
[27] R.W. Pierce, J. Merola, J.P. Lavik, M.S. Kluger, A. Huttner, M.K. Khokha, et al., A p190BRhoGAP mutation and prolonged RhoB activation in fatal systemic capillary leak syndrome, J. Exp. Med. 214 (2017) 3497–3505.
[28] Y. Zhao, P.M. Vanhoutte, S.W. Leung, Vascular nitric oxide: Beyond eNOS, J. Pharm. Sci. 129 (2015) 83–94.
[29] Z. Xie, M. Borset, K. Sveen, O.W. Boe, E.C. Chan, J.B. Lack, et al., Markers of endothelial glycocalyx dysfunction in Clarkson disease, J. Transl. Med. 20 (2022) 380.
[30] D. Atar, P. Petzelbauer, J. Schwitter, K. Huber, B. Rensing, J.D. Kasprzak, et al., Effect of intravenous FX06 as an adjunct to primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction results of the F.I.R.E. (Efficacy of FX06 in the Prevention of Myocardial Reperfusion Injury) trial, J. Am. Coll. Cardiol. 53 (2009) 720–729.
[31] T. Wolf, G. Kann, S. Becker, C. Stephan, H.R. Brodt, P. de Leuw, et al., Severe Ebola virus disease with vascular leakage and multiorgan failure: treatment of a patient in intensive care, Lancet 385 (2015) 1428–1435.