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STEPS TOWARDS "PATIENT-TAILORED THERAPY" IN INTENSIVE CARE: THE ROLE OF MICROVASCULAR MONITORING.

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❖ INTRODUCTION

➢ Research Proposal:

The patient-tailored approach is a relatively new concept in Intensive Care, although the art of tailoring treatments to the patient dates back at least to the time of Hippocrates. The patient-tailored approach combines the EBM (Evidence Base Medicine) provided by international guidelines with a more single-patient-focused attitude, and it takes advantage from the knowledge of the complex and interactions among patient's characteristics, disease physiopathology and response to treatments. Guidelines and protocols remain undeniably essential but benefit from further weapons; and microvascular assessment could be one of them.

First "personalised medicine" was based on developments in cancer research, where it was mainly centred on genomics, biomarkers, and information obtained from large data sets. However, in the setting of critical illness, and particularly in sepsis, this approach may not perfectly suit, because of the heterogeneity of the pathophysiology of critical illness that makes it difficult to recognise precise subtypes. [1]

How could microvascular monitoring add further information to this concept? We will try to answer this question through the current scientific knowledge and the results of the studies presented.

➢ The microcirculation:

The microcirculation is defined as the smallest vessels where gas and nutrient exchanges with tissues take place. It is composed of a network of capillaries, venules and arterioles that connects the arterial and venous systems, and it is characterized by a diameter of vessels smaller than 100 μm. Below the threshold of 100 μm, different diameters correspond to different structures and to different functions. [2-3] The morphology of the microvascular network is, also, highly heterogeneous toward organs and

this anatomical heterogeneity reflects the peculiar differences in the regional metabolical demand of organs.

One of the primary functions of microcirculation is to ensure adequate oxygen delivery to meet the oxygen demand of tissue cells and thereby to sustain organ function.

Microcirculation also plays a key role in the homeostasis of the immune system and of coagulation and is implied in specialized functions as thermoregulation and hormone release to the parenchymal cells. It conveys nutrients and drugs to target-cells and it removes metabolites and toxins from tissues. In order to achieve the intents, the healthy microcirculation auto-regulates via myogenic, metabolic and neurohumoral mechanisms, and it responds accordingly with the changes in metabolic demand or of functional status of organs.

Because of critical illness, or as source of critical illness, microcirculation can be severely impaired, and if this derangement is not corrected, microcirculatory dysfunction can lead to respiratory distress in tissue cells and to subsequent organ injury. [4] This mechanism finds high relevance in the pathophysiology of sepsis, where endothelial cell dysfunction is associated with glycocalyx degradation, vascular leakage, altered local perfusion pressures, and functional shunting of oxygen transport. [5]

Anatomical descriptions of microcirculation are well-known since olden times, but the functional monitoring is relatively recent, particularly in critical care.

First direct evaluation of the microcirculation was performed with Orthogonal Polarization Spectral (OPS) imaging by Slaaf et al; SDF imaging (Sidestream Dark Field- introduced by Goedhart and colleagues) first, and IDF (Incident Dark Field) technology then, made further improvements of the direct monitoring, with a frank increase of optical resolution of the technique, easiness in daily use at bedside and standardization of capture. [6-9]

The sublingual mucosa is the main site for evaluating microcirculation and the most standardized one, and Boerma's study demonstrated that it is an excellent mirror of splanchnic vessels. [10] Further

and more specific description of the technique of microvascular monitoring, and of the main parameters of microcirculation will be more extensively descripted in Chapter 1.

- **Role of microcirculation in critical care:**

Critical illness includes a wide range of different diseases, as sepsis, cardiac, renal and respiratory failure, major trauma and high-risk surgery. All these conditions are characterized by impaired tissue oxygenation and are related to different patterns of compromise in the cardiovascular and immunological systems.

Hemodynamic optimization is one of the most important cornerstones and challenges in the management of critically ill patients. Its main objective is to assure correct perfusion to organs and tissues and to prevent and treat organ failure. However, the normalization of "macrohemodynamic" variables (as cardiac output, stroke volume or mean arterial pressure) is not a guarantee to adequate organ supply and to reversal of shock.

Why? Because shock is a cellular status! It is defined as the condition whereby the circulation is unable to deliver adequate blood flow to meet the metabolic demand of the parenchymal cells, and/or where cellular metabolism dysfunction occurs. [11] Therefore, macrohemodynamics is just one of the determinants of shock, and just one of the parameters the makes resuscitation successful.

The physiology of systemic circulation, of microcirculation, and of cellular function are equally relevant to understand how organs will response to treatments. My previous experience as honorary fellow in the laboratories of Prof M Singer (University College London) allowed me to increase my knowledge about cellular compromise and mitochondrial dysfunction. This thesis will be focused instead on the relevance of microcirculatory disfunctions.

The healthy coupling between macro- and micro-circulation is named "hemodynamic coherence" and it can be considered part of a wider concept, called "Personalized physiological medicine".

The concept of “hemodynamic coherence” between the macrocirculation and the microcirculation was introduced by Ince C in 2015. [12] This is “the condition in which resuscitation procedures aimed at the correction of systemic hemodynamic variables are effective in parallel improvement in the perfusion of microcirculation and in the regional oxygen delivery to parenchymal cells, so that the cells are able to perform their functional activities in support of organ function”. [4, 12]

In critical care patients, with different categories of diseases, and first of all in sepsis, the homeostatic coupling between macro- and micro-circulation is lost (“Loss of hemodynamic coherence”). [4, 12--14] In addition, medications themselves and resuscitation fluids can furtherly affect this coupling. [ex.15-17]

Four subtypes of microcirculatory alterations have been classified, and they are associated with different states of cardiovascular compromise. [12] They can occur individually or in combination (also as result of our treatments) and they are all characterized by reduction in functional capillaries and thereby loss of the capacity of the microcirculation to transport oxygen to the tissues.

Type 1, in particular, is evident in septic patients and it is characterized by heterogeneity in microcirculatory perfusion, with obstructed capillaries next to capillaries with flowing blood; several studies identified the persistence of this state as independent determinant of adverse outcome. [16-17] Type 2 dysfunction is related to hemodilution, in which dilution of blood causes loss of red blood cell (RBC)-filled capillaries and it results in increased diffusion disturbances to tissue cells. It can be frequently recognized in patients undergoing cardiac surgery and it is particularly associated to renal failure [18-19]; type 3 pattern can be found during excessive vasoconstriction (associated to vasoactive or inotropic treatment or to hyperoxia) and/or tamponade, and it results in microcirculatory ischemia and raised venous pressures. [21] Type 4 is consistent with increased diffusion distances between the RBCs and tissue cells: this pattern of loss in coherence is due to tissue edema and caused by capillary leak, endothelial cell damage, loss of glycocalyx barriers, and/or compromise of adherent and tight cell junctions. It can be iatrogenic (quality and quantity of fluid therapy) or disease-related

(hyperinflammatory, malarian) and it complicates sepsis and hyperinflammatory states (for example it can be found in acute hematological conditions characterized by endotheliitis). [ex. 22-23]

Loss of coupling cannot be detected, unless explicitly monitored. Several non-specific indicators of perfusion have been studied (lactate, capillary refilling time, peripheral-to-central temperature difference, central venous saturation of oxygen, arterial-to-venous gap of CO2), but none of them resulted to be sufficient, alone, to assess the coupling. [24-26]

Direct observation of the nature of microcirculatory alterations using hand-held video microscopes (HVM) should be considered when the loss of hemodynamic coherence may be of clinical relevance.

Unfortunately, microvascular monitoring is not widely available, and it presents important limitations at bedside approach that will be discussed later in this manuscript.

Identification of the presence or absence of hemodynamic coherence and of the response of microcirculation to therapies form the third of four pillars of "Personalized Physiological Medicine". [27] The concept of Personalized Physiological Medicine comprehends also the assessment of fitness and frailty of the patient (to determine physiological reserve), the evaluation of organ function (focused on the regulatory capacity of the organ systems to stress factors) and the integration of the three pillars with information from humoral biomarkers and genetic screening of unfavorable polymorphisms.

- **Introduction to adjunctive therapies in critical care septic patients:**

In the first urgent salvage phase, the clinical practice on sepsis and septic shock is standardized by the international guidelines, that guarantee that the patient receives early broad-spectrum antibiotics, source control, aggressive fluid resuscitation and cardiovascular support with vasopressor/s and inotropes. [28] Then, the guidelines consider other therapeutic options for advanced treatment of sepsis and they describe adjunctive therapies without any final recommendation, because the results

have so far resulted disappointing, and the Randomized Controlled clinical Trials (RCTs) mostly failed to prove efficacy.

A large part of the adjunctive treatments aims to control both the overwhelming systemic inflammatory response of the host and the immunosuppressive component, through immunomodulation. [29] The aim immunomodulation is to recover the homeostasis of the immune system. Immunological adjunctive therapies comprehend a number of pharmaceutical agents targeting different pathways of immune response and extracorporeal therapies that act through removal of excessive inflammation. Some of them will be discussed later. [30]

So, several decades of clinical research on sepsis and septic shock provided us with new adjunctive therapies for advanced management of critical care patients, but none of them have been successfully introduced into current practice. Are we doing it the right way? It can be supposed that it is not a complete failure of the weapon, but of the way we use it.

The causes of these failures are likely several-fold. The heterogeneity of the human response to infections makes a single-drug therapy unlikely to be successful for all the individuals. Use of individualized patient therapy may direct the future use of specific targeted therapy.

In our studies we focused on the effect of new treatment options to the sublingual and peripheral microcirculation.

- **Contents of the manuscript:**

Chapter 1 is a research article published in 2016, that reports the state of art of microvascular monitoring in critical care, through 6 traditional questions (5 W and 1 H). It focuses on the technique of bedside monitoring and describes the main parameters of sublingual microcirculation and their meanings. Furthermore, the article highlights the open issues and the current restrictions for the use of the device for routine clinical practice.

Chapter 2 is a retrospective subgroup analysis on traumatic patients enrolled for the Microcirculation DAIly MONitoring in critically ill patients study (MicroDAIMON - NCT 02649088). MicroDAIMON is a single-center prospective observational study conducted in our Intensive Care Unit (ICU), where sublingual microcirculation and peripheral oxygenation measurements were performed in 97 adult critically ill patients (with respiratory, post-traumatic and medical/surgical acute problems) on a daily basis from admission to ICU-discharge/death. The subgroup analysis specifically investigated the association between organ dysfunction and microvascular alterations in critically ill patients admitted in ICU with major trauma, on the light of previous (limited) literature about microvascular behavior during traumatic shock.

Chapter 3 transports microvascular monitoring in the operating room, and it explores changes in microvascular perfusion in patients undergoing elective open abdominal aortic aneurysm repair (and managed with a goal-directed hemodynamic approach) confronting balanced anesthesia and totally intravenous one with TIVA-TCI (target-controlled infusion) technique (NCT03510793).

Chapter 4 reports a very recent observational study, the manuscript is under preparation. It researched a correlation between MR-proADM (midregional pro-Adrenomedullin), as marker of organ dysfunction, and MFI (Microvascular Flow Index) at admission in ICU in 20 adult patients diagnosed with infection, sepsis or septic shock. It assessed the prognostic value of MR-proADM in term of ICU-mortality and SOFA (Sequential Organ Failure Assessment) score.

Chapter 5 and 6 explore the response of the microvasculature to two different immunomodulatory (adjunctive) therapies in sepsis and septic shock.

Chapter 5 reports the results of a single-centre, randomized, double-blind, placebo-controlled phase II trial, performed in our ICU from January 2016 to December 2017 (NCT 02655133). It included 20 adult patients with sepsis, severe sepsis or septic shock (the original study protocol was approved before the publication of the Sepsis-3 definitions). [ ] Patients were randomized to receive IgM-enriched immunoglobulins (Pentaglobin, Biotest Pharma GmbH, Dreieich, Germany) or Placebo

(same volume of normal saline solution NaCl 0.9%) as a continuous intravenous infusion for 72 hours. We aimed to test whether the infusion of IgM-enriched immunoglobulins improved microvascular perfusion, peripheral (skeletal muscle) tissue oxygenation and/or microvascular reactivity.

In chapter 6 we evaluated the response of sublingual microcirculation, cytokines and haemodynamics during blood purification with Cytosorb haemoadsorber (CytoSorbents Corporation, Monmouth Junction, NJ, USA), in septic patients that underwent continuous renal replacement therapy (RRT) for acute renal failure (NCT03456180).

Chapter 7 describes the study protocol and preliminary results of a prospective observational study performed in our ICU in the last 2 years, where we evaluate the effect of paracetamol (acetaminophen) on free circulating Haemoglobin (freeHb) and on the sublingual microcirculation of 50 pyrexical septic critical care patients (NCT02750163). The primary objective of this study is to research an improvement, if exists, of Perfused Vessel Density (PVD) 30 minutes after the infusion of paracetamol. Other aim of the study is, also, to examine the response of freeHb (measured on plasma), of oxidative stress markers, and of PD1/Pd-L1 to administration of paracetamol.

This study also evaluates the prevalence of two pharmacogenetic polymorphisms (*rs776746 e rs8330*) in the sample population and the association of the two polymorphisms with the pharmacokinetic of paracetamol and with the response of the sublingual microcirculation to it.

It is great pleasure to report this interdisciplinarity project in my thesis. The protocol required an inter-departmental collaboration among intensivists, pharmacologists, respiratory physicians and biologists, and it was awarded of honor and grant as one of the best scientific research projects 2016-2017 from Università Politecnica della Marche.

I participated to it from the design stage to the analysis of the results, and it allowed me to improve my skill in lab research and to contribute to most of the analysis performed,

We performed a half-sample preliminary analysis (on n=25) patients on the main parameters of microcirculation and of pharmacogenomics. These data were reported as poster presentation and oral presentation during the last edition of SMART congress (Smart Meeting Anaesthesia Resuscitation and inTensive care) in Milan (May 2019) and will be summarized in the chapter.

Chapter 8 examines tachycardia as marker of sympathetic overstimulation in septic shock patients treated with norepinephrine. This is a multicentre international retrospective observational study, that included more than 700 patients throughout Europe, and it is now submitted to *Journal of Critical Care*. Differently from the previous studies, it does not involve microvascular monitoring but it earns a position within the others because it evaluates the response of the organism to "stress", and researches a marker of excessive adrenergic stimulation.

In conclusion chapter 9 will discuss the results of the previous chapters from my personal point of view and it will conclude the manuscript. The intention of the discussion will be merely to draw some possible food for thought from the studies reported, and to focalize to limits and future perspectives of bed-side microvascular monitoring in the patient-tailored therapy approach to critically ill patients.

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Chapter 1

The 5 W of microcirculatory assessment in the critically ill patients.

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Abstract.

Microcirculatory monitoring revealed great impact in predicting the severity of organ dysfunction and the outcome of critically ill patients.

Clinicians are trying to introduce this technology in the clinical practice, as a supplement to routine surveillance; unfortunately, despite decades of researches, there are still unsolved questions and practical limitations to this.

The purpose of this article is to summarize the state of the art regarding microcirculation through 6 traditional questions (5 W and 1 H), but also to highlight the open issues and the current restrictions for the use of the device as routine.

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Introduction.

The microcirculation is a complex network of capillaries, venules and arterioles. All the vessels characterized by a diameter smaller than 100 μm are part of the microcirculation. Several functions have been attributed to it, included oxygen delivery, removal of metabolites and nutrient's supply to tissues.

Microcirculatory perfusion is controlled by myogenic, metabolic and neurohumoral mechanisms and several studies have shown that the endothelial cell works as active determinant of this autoregulation, through cell-to-cell interactions and autocrine and paracrine signaling. [1-4]

The regulatory dysfunction of microcirculation is specifically involved in the systemic response to inflammation and infection, and it can lead to organ dysfunction and multiorgan failure. Microcirculatory monitoring revealed to be a strong predictor of the outcome of critical care patients with severe sepsis and septic shock: presence of microvascular alterations and heterogeneity, failure in improving microcirculatory perfusion with fluid resuscitation and vasoactive drugs have been all related to lower survival and higher severity of organ dysfunction. [1, 5-7]

Despite years of clinical and experimental studies, there are still unsolved questions about the pathophysiology underlying this deregulation and there are also technical limitations to the bedside use of the technique as complement to macrohemodynamic monitoring. However microcirculatory assessment could be strongly instructive in all those situations where the optimal tissue's perfusion represents the cornerstone of the treatment.

We believe that 5 Ws and 1 H is a perfect formula for getting the complete state of art and to understand the progresses of microcirculatory investigation.

"*How": Technology underlying microvascular monitoring.*

For a long time, microvascular monitoring has been limited to semi-transparent tissues allowing only experimental or ex vivo studies, preventing it to the clinical setting.

Slaaf and colleagues introduced the first real bedside monitoring with the technology of the Orthogonal Polarization Spectral imaging (OPS).[8] Therefore, OPS has been experimented in different settings of severe illness and emergency medicine. [9-13]

SDF imaging (Sidestream Dark Field- introduced by Goedhart and colleagues) first, and IDF (Incident Dark Field) Illumination then, made further improvements as they obtained higher optical resolution and they increased the percentage of success in acquisition and therefore the possibility to analyze the records collected. [14-15]

Cytocam® (Braedius Medical, Huizen, The Netherlands) is the most recent technology introduced in commerce and it is based on IDF illumination; if compared to Microscan ® (MicroVision Medical, Amsterdam, The Netherlands) that is based on SDF imaging, IDF illumination seems to offer advantages for the operator, derived from the introduction of digital signal, the reduction in weight of the device (better handling of the probe and lower pressure artifacts) and the higher optical resolution. Some validation studies are already available in literature and they report superiority of this novel video-microscope on the previous devices. [16-18]

Core of the technology for vessel's detection is an incoming ring light (LEDs light) characterized by a specific wavelength of 530 nm. 530 nm represents the isosbestic point of absorption spectra of the hemoglobin contained in red blood cells (RBC) so that RBC impressed by the light are visualized as flowing granules that indirectly highlight just those vessels that are perfused, hiding vessels that are not perfused at all. [11-14]

"How" to capture good quality videos?

As nicely described by De Backer and colleagues, saliva, fresh blood and contusions are often the main responsibles for an inadequate visualization of vessels; for a good quality of records, all secretions should be carefully removed using tissues or gauzes and high attention should be taken to optimize focus, illumination and contrast (Figure 1).[19] Other disturbances can derive from pressure's and motion's artifacts: pressure artifacts can be focal or global, and they are determined by excessive pressure applied on the mucosa that occludes RBC flow; they can be detected by the observation of large venules, where impaired flow of venules is suggestive of excessive pressure; motion's artifacts are more frequent in awake patients even more in confused and agitated ones, because they are unable to remain steady for long and to cooperate with the observer. A firm but gentle grip of the probe on patient's chin usually facilitates stabilization of the image. [19-20]

"How" to analyze videos?

As Bezemer R at al reported, the off-line analysis is the gold standard for this technology. [21] All capillaries have to be manually detected and drew, then blood flow is individually characterized. Semi-automated softwares are available in commerce. AVA (Automated Vascular Analysis) is the most used software package worldwide, it facilitates the operator in some of the main steps of off-line analysis, but nevertheless it remains time-consuming and partially operator-dependent, so we are far from a real resolution of the limits of this methodology. We should aim to reach a complete automatization of the procedure both to reduce inter-operator dependency and to introduce microcirculatory monitoring in the daily clinical practice in the setting of critical care patients where an evaluation of tissue's perfusion is undoubtedly important. [21]

"Who": importance of a well-trained observer.

As with many other devices introduced into clinical practice, such as Echocardiography and Transcranial Doppler, both video capture and off-line analysis of microcirculation require expertise

and skills, and a well-trained observer is fundamental to obtain good-quality images, to avoid artifacts and to give the right meaning to the results.

A well-trained observer can easily recognize and avoid pressure and motion artifacts and he can therefore optimize the acquisition. The operator must also be skilled in the off-line evaluation of the principal parameters. As previously mentioned, inter-operator deviation of the method could be completely deleted just when a totally automated software will be available. [20, 22-23]

Massey MJ, Shapiro NI and colleagues have recently created a score of quality called "Microcirculation Image Quality Score" with the aim to rate image's quality before off-line analysis and they have introduced 6 different features to control(illumination, duration, focus, content, stability and pressure), to assign the score of optimal, sub-optimal, or unacceptable quality of the record. [22] Further assessment of this score is mandatory, but it is unquestionable that we need a quality control to ensure high relevance studies. [23]

"What": guide to microcirculatory parameters.

De Backer D, Ince C, et al. published the new roundtable Guidelines in 2007, in order to formulate a consensus statement on microvascular evaluation. These guidelines are still the main guidance for both acquisition and analysis of images. [19]

Participants of the round table were some of the principal experts in "microhemodynamic". All of the experts agreed that a measure of vascular density, of perfusion of the vessels and of heterogeneity of flow should be always performed and registered. They decided that 20 μm in diameter was the right cut-off to divide small vessels (mostly capillaries) from medium and large vessels (20-100 μm). Vessels with a diameter smaller than 20 μm are the most physiologically active vessels for oxygen exchange. Medium and large vessels predominantly represent small venules and also rare arterioles. [19-20]

Measures of vessel density are TVD (Total Vessel Density) and De Backer Score; PVD (Perfused Vessel Density) instead is an estimate of functional capillary density (FCD). PVD is calculated by multiplying vessel density by the Proportion of Perfused Vessels (PPV). It is one of the most relevant variables, because it identifies and differentiates patterns of bad and good perfusion of small vessels: "no flow" and "intermittent flow" are identified as determinants of a poor oxygenation. Most of these indices are derived from the number of intersections of capillaries with the horizontal and the vertical arbitrary grid-lines and from the measurement of the total capillary length of the surface. [20-21]

The Microcirculatory Flow Index (MFI) is a parameter of perfusion. In this case just one horizontal and one vertical line divide the image in 4 equal quadrants, in which the observer reports the predominant type of flow using an ordinal scale (0 for absent flow, 1 as intermittent flow, 2 for sluggish flow and 3 for normal). The average of the 4 quadrants is the final MFI. [24-25] Boerma and colleagues in their trials found a high intra- and inter-observer reproducibility of MFI. [19,26]

Heterogeneity of perfusion is another determinant of oxygen extraction efficacy. It is characterized by alternation of normally perfused areas and poorly perfused ones. When the distance among capillaries increases, those cells that are distant from capillaries find it difficult to exchange gas and metabolites, and a certain degree of cellular distress can appear as result of hypoxia. Therefore, high heterogeneity of vascular distribution is generally less tolerated than a homogeneous but sluggish flow. [19-20] 5 spots of sublingual surface should be captured to assess vascular heterogeneity, and at least 3 of the 5 records should be analyzed.

"Where" and _"when"_: targets for microvascular examination.

Sublingual mucosa is the most common and standardized site for evaluation of microcirculation and Boerma's study demonstrated that it is an excellent mirror of splanchnic vessels.[26] Other sites of interest are small intestine (villi), colon (crypts), rectum (crypts), liver (sinuses) and gingival tissue:

several studies have been performed, but they mostly involve ex vivo evaluation or experimental trials.

A recent multicentre trial called “MicroSOAP study” showed a high prevalence of microvascular alteration in the critically ill patients. The study consisted in a one-shot evaluation of microcirculation of ICU patients. It involved 36 ICUs worldwide and it tried to correlate specific patterns of microcirculation with specific pathologies; an on-going monocentric study of Donati’s group is trying to characterize patterns of alterations starting from patient’s diagnosis, and also to follow the course of the pathology from the admission throughout all the ICU's stay. [27]

Sepsis and septic shock are the main field of application for microcirculatory assessment. [1, 28-30]

The new definition of sepsis proposed by Singer M, Angus DC and colleagues describes sepsis as “a life-threatening organ dysfunction caused by a dysregulated host response to infection”. [31]

Decades of studies demonstrated that in the pattern of distributive shocks like septic one, there is an activation of the cytokine cascade that causes derangement of the auto-regulatory mechanisms and of endothelial cell's function, anomalous production of nitric oxide and altered response of the circulation to vasoactive drugs. When this infection-induced derangement persists, it causes qualitative and quantitative alterations of microcirculation (reduction in total and perfused vessel density and increased heterogeneity of flow) that affect tissue perfusion and organ function. [1, 4, 30]

Further promising fields of application for microvascular monitoring have been studied: hypovolemic and hemorrhagic shock, cardiac failure and diabetes, organ perfusion in anemia and hemodilution, multiorgan failure and organ failure related to polytrauma. [32-35]

The effects on microcirculation have also been reported for several drugs (catecholamine, nitroglycerin, recombinant activated protein C, beta-blockade), fluids (albumin, crystalloids, colloids and blood transfusion), and procedures (normobaric hyperoxia, ischemia-reperfusion syndrome,

cardiac resynchronization and cardiac surgery) in the pattern of septic and non-septic patients. [36-44]

"Why": The rationale behind microcirculatory monitoring

Shoemaker's "supranormal values" theory, River's Early Goal Directed therapy and all subsequent studies have shown the importance of hemodynamic optimization as treatment's target for septic and critically ill patients. [45-49]

However, the normalization of macrohemodynamic parameters, such as Cardiac Output (CO) and arterial pressure, is not a guarantee for good oxygen delivery and organ perfusion: there is often a mismatch between macro and micro-hemodynamic.[50-51] Even if there are some surrogates to assess the adequacy of the organ perfusion, as lactate, peripheral temperature and capillary refill, all of them are characterized by well-known limitations: blood lactates are the result of a balance among production, metabolism and excretion, and they do not represent a direct marker of anaerobic metabolism and poor tissue perfusion; the capillary refill time and peripheral temperature suffer from environmental factors, altered thermoregulatory mechanisms and vascular disorders. None of them gives us a true picture of organ perfusion, and moreover none of them is a precocious marker of inadequate treatment: in these cases, the direct visualization of microcirculatory alterations, through direct monitoring at the patient's bedside, may be an additional resource for clinicians to guide their treatment and to formulate a right prognosis for their patients.

Conclusion:

Several years of researches demonstrated that microcirculation is important in the physiopathology of sepsis, septic shock and many other pathologies.

Real time monitoring of microcirculation may represent an additional weapon for the diagnosis and a guide to treatment. Unfortunately, the technology is not yet ready to ensure that the instrument is versatile and quick enough to be routinely used in clinical practice. The current limitations to a broad spectrum use of the device are: the necessity of specific expertise of the observer, the time-consuming procedure of analysis and moreover the lack of agreement upon the range of normal values for the main parameters in healthy people. Despite the difficulties, the increasing space given from literature is a clear sign of recognition and legitimation of this technique and a stimulation to find answers to the open questions through high-quality clinical and experimental trials.

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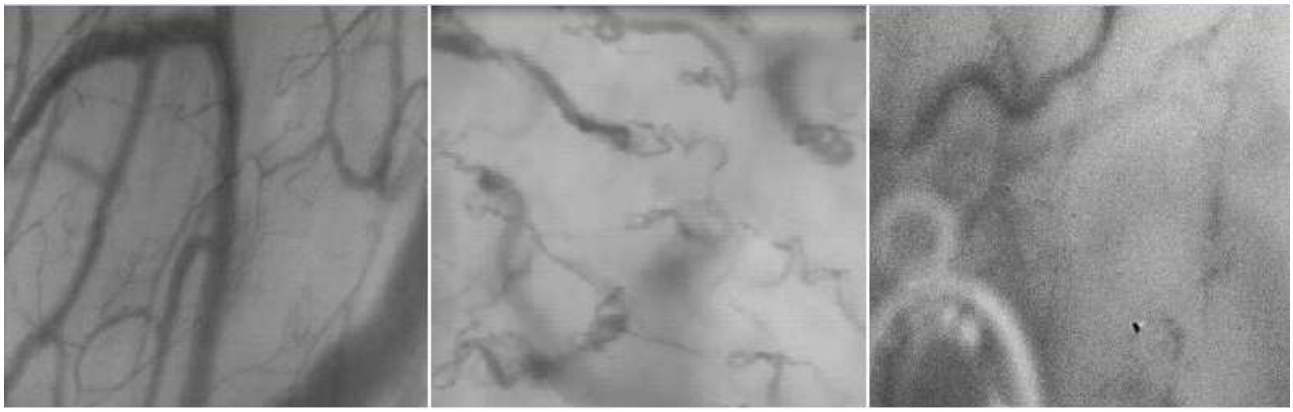

Figure 1: Examples of quality of capture. The image on the left represents a good quality video, while the middle and right ones respectively represent examples of incorrect area of evaluation and of artifacts derived from saliva bubbles and pressure artifacts.

Chapter 2

Association between sublingual microcirculation, tissue perfusion and organ failure in major trauma: a subgroup analysis of a prospective observational study.

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Abstract

Introduction:

Previous studies described impaired microvascular perfusion and tissue oxygenation as reliable predictors of Multiple Organ Failure in major trauma. However, this relationship has been incompletely investigated. The objective of this analysis is to further evaluate the association between organ dysfunction and microcirculation after trauma.

Materials and methods:

This is a retrospective subgroup analysis on 28 trauma patients enrolled for the Microcirculation DAIly MONitoring in critically ill patients study (NCT 02649088). Patients were divided in two groups according with their Sequential Organ Failure Assessment (SOFA) score at day 4. At admission and every 24 hours, the sublingual microcirculation was evaluated with Sidestream Darkfield Imaging (SDF) and peripheral tissue perfusion was assessed with Near Infrared Spectroscopy (NIRS) and Vascular Occlusion Test (VOT). Simultaneously, hemodynamic, clinical/laboratory parameters and main organ supports were collected.

Results:

Median SOFA score at Day 4 was 6.5. Accordingly, patients were divided in two groups: D4-SOFA ≤6.5 and D4-SOFA >6.5. The Length of Stay in Intensive Care was significantly higher in patients with D4-SOFA>6.5 compared to D4-SOFA≤6.5 (p=0.013). Total Vessel Density of small vessels was significantly lower in patients with high D4-SOFA score at Day 1 (p=0.002) and Day 2 (p=0.006) after admission; the Perfused Vessel Density was lower in patients with high D4-SOFA score at Day 1 (p=0.007) and Day 2 (p=0.033). At Day 1, NIRS monitoring with VOT showed significantly faster tissue oxygen saturation downslope (p=0.018) and slower upslope (p=0.04) in patients with high D4-SOFA.

Discussion:

In our cohort of major traumas, sublingual microcirculation and peripheral microvascular reactivity were significantly more impaired early after trauma in those patients who developed more severe organ dysfunctions. Our data would support the hypothesis that restoration of macrocirculation can be dissociated from restoration of peripheral and tissue perfusion, and that microvascular alterations can be associated with organ failure.

Introduction:

In the last decades, advancements in prehospital and emergency hospital care led to a reduction in early mortality after multiple trauma. However, the occurrence of multi-organ failure (MOF) remains a major problem in severely injured patients and is responsible for delayed mortality, morbidity and prolonged stay in intensive care units [1,2].

Tissue damage and blood loss following trauma induce a systemic inflammatory response (SIRS) [3-5], with activation of complex haemostatic, inflammatory, endocrine and neurological processes that aggravate the initial damage caused (mainly) by hypoperfusion and reperfusion [6]. Cytokines and pro-inflammatory mediators are responsible for endothelial and glycocalyx dysfunction, increased capillary leakage, leukocyte activation and tissue oedema, which may lead to microcirculatory alterations similar to those observed during sepsis [2, 7-9]. An impairment in microvascular perfusion and tissue oxygenation has been described after traumatic haemorrhagic shock and was associated with higher risk of MOF and death [10-15]. Nonetheless, only a few studies investigated the pathophysiological and prognostic significance of microcirculatory dysfunction in patients with multiple trauma, both in the experimental and human settings [11-14].

Herein, we report a retrospective subgroup analysis of the single-centre prospective observational Microcirculation DAIly MONitoring in critically ill patients study (MicroDAIMON– NCT 02649088), in which we aimed to test the hypothesis that early alterations in microcirculation and tissue oxygenation are associated with the occurrence of organ failure in a heterogeneous cohort of adult patients admitted to the ICU for major trauma (major trauma defined as an Injury Severity Score being greater than 15) [16].

Materials and methods:

This is a retrospective subgroup analysis of the Microcirculation DAIly MONitoring in critically ill patients study (MicroDAIMON– NCT 02649088). The MicroDAIMON was a single-center prospective observational study where sublingual microcirculation and peripheral oxygenation measurements were performed in 97 adult critically ill patients (with respiratory, post-traumatic and general medical/surgical acute problems) on a daily basis from admission to ICU-discharge/death.

The study protocol was approved by the Local Ethics Committee (Comitato Etico Azienda Ospedaliero Universitaria Ospedali Riuniti Umberto I – G.M. Lancisi – G. Salesi of Ancona, currently named Comitato Etico Regione Marche – CERM; protocol number 212639, approval in date 14/02/2013) and it conformed to the principles of Helsinki declaration (last revision, Edinburgh 2000). A written informed consent was obtained, by signing the appropriate informed consent paperwork, from all the subjects or from their next of kin, in compliance with national applicable laws.

The aim of the study was to evaluate the association between microcirculatory alterations and outcome in a general ICU population.

Lack of written informed consent, age <18 years, pathophysiological conditions interfering with the acquisition of sublingual microcirculation videos (e.g. major maxillo-facial trauma) were exclusion criteria.

This retrospective analysis is focused on the subgroup of patients admitted in the ICU with a diagnosis of major trauma. The main objective of this analysis was to evaluate sublingual microcirculation and tissue perfusion after trauma and to assess if a correlation existed with the occurrence/persistence of organ failure, as identified by an elevated Sequential Organ Failure Assessment (SOFA) score. In a previous study by Tachon et al in patients with traumatic haemorrhagic shock, the SOFA score at day 4 after ICU admission was chosen to split the patients in high/low SOFA score groups, as ICU length of stay (LOS) in the two groups was significantly different [10]. Similarly, we divided the patients in

two groups based on their SOFA score at day 4 (higher and lower or equal than the median value of SOFA score in the whole sample).

All patients were included in the study within the first 12 hours from ICU admission. At the moment of inclusion and every 24 hours thereafter, the sublingual microcirculation and peripheral tissue perfusion were evaluated. Simultaneously, arterial and central venous blood samples were withdrawn in order to assess blood gases, lactate levels and base excess. Baseline demographic data (age, sex, weight and height), hemodynamic parameters (Heart Rate, Mean Arterial Pressure, and Cardiac Output monitoring whenever available), clinical/laboratory data, and main organ supports were collected in digital excel spreadsheet.

Sublingual microcirculation was monitored with Sidestream DarkField (SDF) imaging technique (Microscan; Microvision Medical BV, Amsterdam, The Netherland).

Extensive details on the SDF imaging technique have been described in previous papers, however the below gives a brief summary of the technique. Microscan is equipped with a ring of green light-emitting diodes (LEDs) located at the end of a probe. The LEDs light of Microscan is characterized by a specific wavelength of 530 nm that is absorbed by the haemoglobin contained in red blood cells (RBC) so that RBC are visualized as flowing granules that indirectly highlight just those vessels that are perfused, hiding vessels that are not perfused [17,18]. We tried to obtain the highest quality of picture by avoiding pressure and movement artefacts, improving focus and illumination and cleaning the sublingual mucosa from saliva and blood. Videos from at least 5 different sites of sublingual microcirculation were recorded for each session, and the best 3 of them were analysed offline using a dedicated software package (Automated Vascular Analysis Software; Microvision Medical BV).

Microcirculatory parameters were calculated offline with the Automated Vascular Analysis software (AVA v3, Microvision Medical, Amsterdam, NL), according to the 2007 round table conference and “the microcirculation image quality score”: Massey et al [17,19].

The analysis of microvascular flow focused on vessels with a diameter of 0–20 μm (small-size vessels). Medium-size vessels (diameter 20-50 μm), that consist in precapillary arterioles and postcapillary venules, were used as quality index and as tool to identify pressure artefacts and mechanical occlusion to flow (small vessels with disturbed microcirculatory flow along with flow disruptions in medium vessels) [17-19]

Total vessel density (TVD) and perfused vessel density (PVD), De Backer score, the proportion

of perfused vessels (PPV) and the microvascular flow index (MFI), were calculated for small-size vessels (diameter ≤ 20 μm) in all the videos analysed.

Peripheral skeletal muscle tissue oxygenation was evaluated using Near-InfraRed Spectroscopy (InSpectra Model 650; Hutchinson Technology Inc., Hutchinson, MN, USA) with a 15-mm probe on the thenar eminence. A vascular occlusion test (VOT) was performed in order to assess the variations in tissue oxygen saturation (StO2) during a transient ischemic challenge.

A sphygmomanometer cuff was placed around the forearm and kept inflated to 50 mm Hg above the systolic arterial pressure until the StO2 reached 40%. The StO2 downslope (%/minute) was calculated from the regression line of the StO2 decay during vascular occlusion and provides an index of O2 extraction and consumption rate. The StO2 upslope (%/minute) was calculated from the regression line of the reperfusion phase and reflects microvascular reactivity, capillary recruitment and post-ischemic vasodilatation. The area under the curve (AUC) of StO2 represents the hyperaemic response [20]. (S1 Fig)

The Kolmogorov-Smirnov test was check for normality of distribution of continuous variables. Since most of the parameters showed a non-normal distribution, data are presented as median (and InterQuartile range, IQR) or as N° (and %) for nominal variables. Non-parametric tests (Mann–Whitney and $\chi 2$ with Fisher's exact tests) were used as appropriate for comparison between

independent samples. Differences were considered significant at P values of less than 0.05 (two-sided).

The area under the Receiver Operating Characteristic (ROC) curve was calculated to evaluate the discriminative ability of microvascular parameters and NIRS variables towards SOFA score at day 4 (D4-SOFA).

Statistical analysis was performed using the Statistical Package for Social Science software, version 17.0 (SPSS Inc).

The study protocol was approved by the Local Ethics Committee and it conformed to the principles

of Helsinki declaration (last revision, Edinburgh 2000). A written informed consent was obtained, by signing the appropriate informed consent paperwork, from all the subjects or from their next of kin, in compliance with national applicable laws.

Results:

Of the cohort of 97 patients enrolled in the MicroDAIMON study, 39 were admitted with a diagnosis of multiple trauma.

Median age was 55 (35-74) years, 30 patients (77% of total) were male, most of them were previously healthy (71% with less than two comorbidities at admission in ICU) and the leading causes of trauma were road collisions. Head-and-neck and chest were injured in most of the patients. 18 patients (46%) were transfused in the Emergency Room and 18 (46%) received surgery before admission in ICU (S1 Table). Median values for Apache score at admission in ICU was 14 (7-18) and for SOFA score was 7 (4-9); the 87% of the patients were haemodynamically stable at admission in ICU.

Median ICU length of stay (ICU-LOS) was 7 days (4-15), with a hospital mortality of 20.5% (8 patients).

Three of the 39 patients deceased in ICU (7.7%) within 72 hours from admission, 5 patients were discharged from ICU before Day 4, three further patients missed data for SOFA score calculation at day 4 and they were excluded a priori. Therefore, this analysis includes 28 patients in total.

Median SOFA score at Day 4 was 6.5 (4-9). Accordingly, patients were divided in two groups: D4-SOFA ≤6.5 and D4-SOFA >6.5.

ICU-LOS was significantly higher in patients with D4-SOFA>6.5 with a median of 15 (9-25) versus 7 (4-13) days for patients with D4-SOFA≤6.5 (p=0.013). Patients with high SOFA score at Day 4 were younger than patients with lower SOFA score: 44 (29-76) versus 69 years (41-74) years, but the difference wasn't statistically significant (p=0.458). The admission SOFA score was higher in those patients who had D4-SOFA score> 6.5 (8 [7-9.5] versus 5 [3-9]; p=0.037).

In the first 4 days, the two groups did not differ for Mean Arterial Pressure (MAP), Heart Rate (HR), Lactate, Central Venous Saturation (ScVO2), haemoglobin and RBC transfusion requirements (Table 1).

Norepinephrine infusion was significantly higher in high SOFA group at Day 1, Day 3 and Day 4. Total Vessel Density of small vessels (TVDs) was significantly lower in patients with D4-SOFA >6.5 both at Day 1 (17.33 [16.58-21.63] versus 23.24 [18.51-23.72] mm/mm2; p=0.002) and at Day 2 (17.45 [15.82-22.51] versus 22.92 [20.64-25.58] mm/mm2, p=0.006); Perfused Vessel Density of small Vessels (PVDs) showed similar results at Day 1 and Day 2 with lower PVDs in patients with D4-SOFA score>6.5 (16.55 [17.94-23.93] versus 20.91 [17.57-23.45] mm/mm2 at Day 1, p=0.007; 16.52 [14.24-19.92] versus 21.32 [17.94-23.93] mm/mm2 at Day 2, p=0.033 (Fig 1).

MFI and PPV did not show any difference between the groups, De Backer score tended to be lower in the high SOFA score group at Day 1 and Day 2 although the difference was not significant (Fig 1).

Changes in NIRS-derived parameters in the two groups are shown in Fig 2.

StO2 was similar in the first 4 days between patients with lower and higher D4-SOFA. At Day 1 desaturation was faster for patients with D4-SOFA> 6.5 with a median StO2 downslope of -9.3 (-11.6, --7.5) versus -7.12 [IQR (-8.5,-6.2] %/minute for patients with D4-SOFA≤6.5 (p=0.018). The StO2 upslope was lower in high-SOFA patients at D1 (141 [75-190] versus 170 [150-193] %/min in low-SOFA patients (p=0.04). Similar results were evident at D2, however the difference was not statistically significant (Fig 2).

ROC curve analysis showed that low PVDs and TVDs at admission in ICU were significant predictors of D4-SOFA score >6.5 with an Area Under the curve of 0.805 for PVDs (95% confidence interval [CI] 0.627-0.983, p=0.08) and AUC of 0.85 for TVDs (95% CI= 0.699-0.993; p=0.03) (Fig 3).

The StO2 Downslope and StO2 Upslope at D1 were also able to predict a higher D4-SOFA (AUC 0.773 [95%CI 0.57-0.97] p=0.02 and 0.74 [95%CI 0.52-0.96] p=0.04, respectively).

Discussion:

In this retrospective analysis of prospectively collected data on 28 patients with major trauma, patients with higher SOFA score at day 4 (D4-SOFA>6.5) after admission in ICU showed significantly lower sublingual microvascular density in the first two days in the ICU as compared to those with a SOFA score ≤6.5 at day 4, while no differences were observed in parameters of microcirculatory blood flow quality. In addition, a higher peripheral tissue oxygen extraction rate and impaired microvascular reactivity at admission were associated with a SOFA score >6.5 at day 4. These microcirculatory disturbances occurred despite early haemodynamic stabilization, as suggested by similar values of MAP, HR, lactate and ScvO2 in the two groups, although patients with a D4-SOFA>6.5 required higher doses of norepinephrine in the first 4 days.

Our results are consistent with data from previous literature. The microvascular response to major trauma was studied from Tachon et al that demonstrated that disturbances in the microcirculation in

the first 72 hours after ICU admission were associated with organ failure and with longer ICU stay in their cohort of traumatic haemorrhagic shock. However, differently from our results, MFI and PPV were significantly different between the groups and Functional Capillary Density was assessed instead of PVD and TVD [10]. The two studies can be easily compared as Perfused Vessel Density (PVD) is an estimate of Functional Capillary Density (FCD), calculated by multiplying vessel density by the proportion of perfused vessels.

Gòmez et al performed NIRS measurements and evaluated the response to a VOT in ICU trauma patients and healthy controls. They showed slower StO2 recovery upslopes in trauma patients than in controls, with similar downslope curves, suggesting a local reduction in microcirculatory reserve, however microcirculatory flow distribution was not assessed in this study [21].

Duret et al showed significantly altered StO2 and desaturation slopes at admission were associated with no improvement or worsening organ failure in the first 72 hours in patients with traumatic haemorrhagic shock [22]. In the present study, StO2 was similar at all timepoints between the two groups, suggesting that StO2 alone may not be an adequate predictor of tissue hypoperfusion at least in some patient categories, and that the ischaemic challenge derived from VOT may be more sensitive to highlight signs of altered tissue perfusion and oxygenation [23].

Although the retrospective design of our analysis does not allow to speculate on potential confounding factors, our data suggest that a reduction in microvascular perfusion and reactivity in the first hours after a multiple trauma may predispose patients to the development of tissue oxygen deficit and organ failure.

Impaired tissue perfusion and reduced microvascular density may be considered markers of insufficient fluid resuscitation and persistent low-grade hypovolaemia: microvascular disturbances have been previously showed in hypovolaemic shock and are characterized by deterioration of Functional Capillary Density and reduction of TVD [24-25]. Fluid resuscitation can cause an apparent improvement in systemic macrocirculation, while leaving inadequate regional oxygenation and

microvascular perfusion. This pattern was demonstrated both in hypovolaemic and haemorrhagic shock that are the main features of early traumatic shock [24-28]

Afterwards, trauma may induce further mechanisms of complex microvascular impairment: the extensive tissue damage and tissue hypoperfusion may induce release of inflammatory mediators and reactive oxygen species and it can produce Systemic Inflammatory Response Syndrome with deterioration of the endothelial function and of glycocalyx, alterations in red blood cell deformability and increase in leukocyte adhesion. Any one of these alterations either alone or acting together can lead to a more distributive shock characterized by a loss of microvascular integrity, reduction of capillary density, increase in vascular permeability and interstitial oedema. The enhanced oxygen diffusion distance can result in tissue hypoxemia and organ dysfunction.

We can suggest that in severe trauma patients, as in sepsis and other patterns of shock a loss of coherence between macro and microhemodynamic exists, and medical interventions aimed at the correction of systemic hemodynamic variables may fail to be effective in correcting regional and microcirculatory perfusion and oxygen delivery to the parenchymal cell. [27]

Finally, the patients with D4-SOFA>6.5 received higher norepinephrine doses than the group with a SOFA score ≤6.5 at day 4. We cannot exclude a direct relationship between vasopressor use and the observed microcirculatory alterations, because high dose norepinephrine, together with inadequate fluid resuscitation, can induce excessive vasoconstriction and capillary de-recruitment and increasing MAP with norepinephrine showed no impact on improvement of microcirculatory perfusion in different patterns of shock. [28-320] However, while norepinephrine dose was consistently different in the two groups even at Day 3 and Day 4 post-admission in ICU, microcirculatory alterations were not evident after Day 2, supporting the hypothesis that vasopressor use alone cannot explain the microvascular pattern demonstrated early after ICU admission. Unfortunately, our study is not powered to answer these questions.

Nevertheless, as in our patients a persistent microcirculatory under-resuscitation in the presence of normalized systemic hemodynamic was associated with adverse clinical outcome, this study suggests that early identification of microvascular and tissue hypoperfusion in trauma patients may be relevant in the clinical practice to detect patients at higher risk to develop multiple organ failure and to guide the clinician to a tailored intensive care treatment in term of fluids and/or vasoactive drugs. Moreover, further studies on this specific field will help us to understand if a more accurate resuscitation, titrated on bedside assessment of microcirculation instead of hemodynamic alone would be important in the intensive care treatment of major trauma.

Our study has several limitations. First, the retrospective design did not allow to control for potential confounders. Second, this was a subgroup analysis of a prospective observational study with a different primary goal: some of the analyses may thus be underpowered to detect statistically significant differences. In addition, the small sample size prevented evaluate of the relationship between microvascular alterations and other outcomes, such as mortality. Third, the observational design of our study does not allow us to clarify whether the implementation of microcirculation-targeted therapies may be able to modify the outcome and prevent the development of organ dysfunctions. Fourth, the lack of cardiac output monitoring impeded a more comprehensive overview of the haemodynamic state and of its relationship with microvascular perfusion.

Conclusion:

In our cohort of patients with major trauma, sublingual microcirculation and peripheral microvascular reactivity were significantly impaired among those patients who then showed more severe organ dysfunction. Our data suggest that early impairment in microvascular perfusion after severe trauma may be associated with development of organ failure. Our study would support the hypothesis that restoration of macrocirculation can be dissociated from restoration of peripheral and tissue perfusion.

Therefore, evaluating the microcirculation together with the macrohemodynamic variables in this patient category may represent a tool to identify those patients with higher risk of MOF, who could benefit from closer monitoring and additional therapeutic efforts. Further studies are needed to clarify the role of microvascular dysfunction in the pathophysiology of MOF after multiple trauma.

Short title:

Sublingual microcirculation and tissue perfusion in traumatic organ failure

Acknowledgements:

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Supporting information:

S1 Fig: Near-InfraRed Spectroscopy (NIRS), StO2 response to vascular occlusion test (VOT).

S1 Table: Baseline characteristics of the 39 trauma patients included in MicroDAIMON study; n (%). aER=Emergency Room

S2 Table: Supporting information; anonymized datasheet with main data collected. SOFA= Sequential Organ failure Score; PVDs= perfused small vessel density; TVDs= total small vessel density; PPV= percentage of perfused vessels; MFI= microvascular flow index; StO2= tissue oxygen saturation; AUC StO2= area under the curve of tissue oxygen saturation; LOS= Length of Stay; ICU= Intensive Care Unit; APACHE= Acute Physiology, Age, Chronic Health Evaluation; NE=Norepinephrine; HR= Heart Rate; MAP= Mean Arterial Pressure; Hb= Haemoglobin.

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Table 1: Hemodynamic variables in the first four days of ICU admission for the two groups of patients (SOFA[a] score at D4≤6.5 and SOFA score at D4>6.5).

		SOFA≤6.5 at D4	*SOFA>6.5 at D4*	*p value*
		15 (53.6%)	13 (46.4%)	
MAP[b], mmHg				
	D1	86 [75-99]	85 [77-95]	0.964
	D2	82 [71-99]	89 [71-95]	0.648
	D3	89 [84-102]	88 [79-97]	0.413
	D4	91 [74-106]	88 [84-89]	0.932
HR[c], bpm				
	D1	63 [57-83]	83 [74-102]	0.142
	D2	77 [64-100]	84 [60-101]	0.717
	D3	71 [62-88]	78 [63-92]	0,964
	D4	75 [64-87]	74 [70-85]	0.843
Norepinephrine, mcg/kg/min				
	D1	0.00 [0.00-0.22]	0.23 [0.026-0.48]	***0.033***
	D2	0.00 [0.00-0.182]	0.22 [0.02-0.28]	0.085
	D3	0.00 [0.00-0.00]	0.09 [0.04-0.33]	***0.001***
	D4	0.00 [0.00-0.00]	0.1 [0.04-0.17]	***0.001***
Arterial pH, AU				
	D1	7.43 [7.4-7.58]	7.45 [7.38-7.49]	0.329
	D2	7.48 [7.44-7.49]	7.45 [7.42-7.49]	0.892

	D3	7.47 [7.43-7.52]	7.48 [7.43-7.53]	0.440
	D4	7.48 [7.44-7.49]	7.47 [7.45-7.51]	0.936
BE[d], mmol/L				
	D1	0.6 [(-)0.9,-2.3]	0.6 [(-)2.7-3.8]	0.880
	D2	4.5 [2.2-8.5]	4.4 [1.7-6.2]	0.751
	D3	5.5 [3.7-9.4]	6.4 [2.1-7.6]	0.892
	D4	5.2 [3.7-9.7]	7.7 [4.2-11.8]	0.538
Lactate, mmol/L				
	D1	1.3 [0.9-2.0]	1.7 [1.1-2.2]	0.235
	D2	1.0 [0.7-2.0]	1.1 [0.7-2.0]	0.217
	D3	0.9 [0.8-1.1]	0.9 [0.7-1.2]	0.413
	D4	0.7 [0.7-1.1]	0.9 [0.6-1.4]	0.590
ScvO2[e], %				
	D1	79.4 [75.6-81.7]	74.3 [59.2-84.4]	0.161
	D2	74.8 [67.1-80.8]	79.3 [70.7-85.2]	0.347
	D3	75 [67.5-78.6]	72 [71.6-90.6]	0.219
	D4	72.1 [68.6-74.4]	78.6 [73.3-81.0]	0.148
Hb[f], g/dl				
	D1	10.8 [9.0-12.4]	10.4 [10.2-11.2]	0.751
	D2	10.3 [9.2-11.5]	9.3 [8.4-9.9]	0.170
	D3	9.3 [8.9-11.4]	9.8 [9.5-10.9]	0.316
	D4	9.4 [8.2-11.3]	9.8 [9.3-10.8]	0.630

Hct[g], %				
	D1	30.2 [27.1-3.8]	30.7 [29.2-32.]	0.856
	D2	30.9 [26.0-34.3]	27.4 [24.4-29.7]	0.170
	D3	27.7 [27.0-34.8]	29.5 [28.6-31.4]	0.440
	D4	29.0 [25.8-33.9]	29.6 [27.8-31.9]	0.79
Patients transfused, n°(%)				
	D1	7 (47)	3 (23)	0.254
	D2	0 (0)	5 (39)	***0.013***
	D3	2 (13)	3 (23)	0.639
	D4	3 (25)	2 (17)	0.99

Hemodynamic data were collected daily, at the same time of microcirculatory assessment. Data presented as median [IQR] or number [%].

aSOFA = Sequential Organ Failure Assessment;

bMAP= Mean Arterial Pressure;

cHR= Heart Rate;

dBE= Base Excess;

eScVO2= Central venous Saturation

fHb= Haemoglobin;

gHct= Haematocrit.

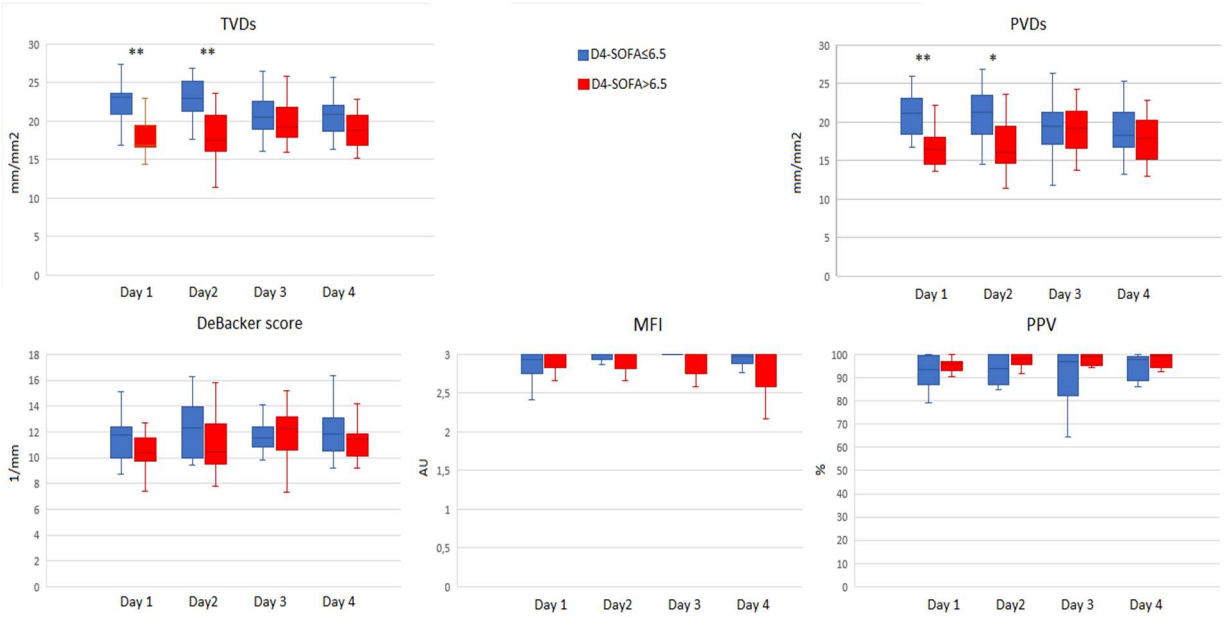


Fig 1: Changes in the sublingual microvascular parameters in the first 4 days of admission in ICU for the two groups of patients (SOFA score at D4≤6.5 and SOFA score at D4>6.5). * p<0.05; ** p<0.01. TVDs: total small vessel density; PVDs: perfused small vessel density; MFI: microvascular flow index; PPV: percentage of perfused vessels.

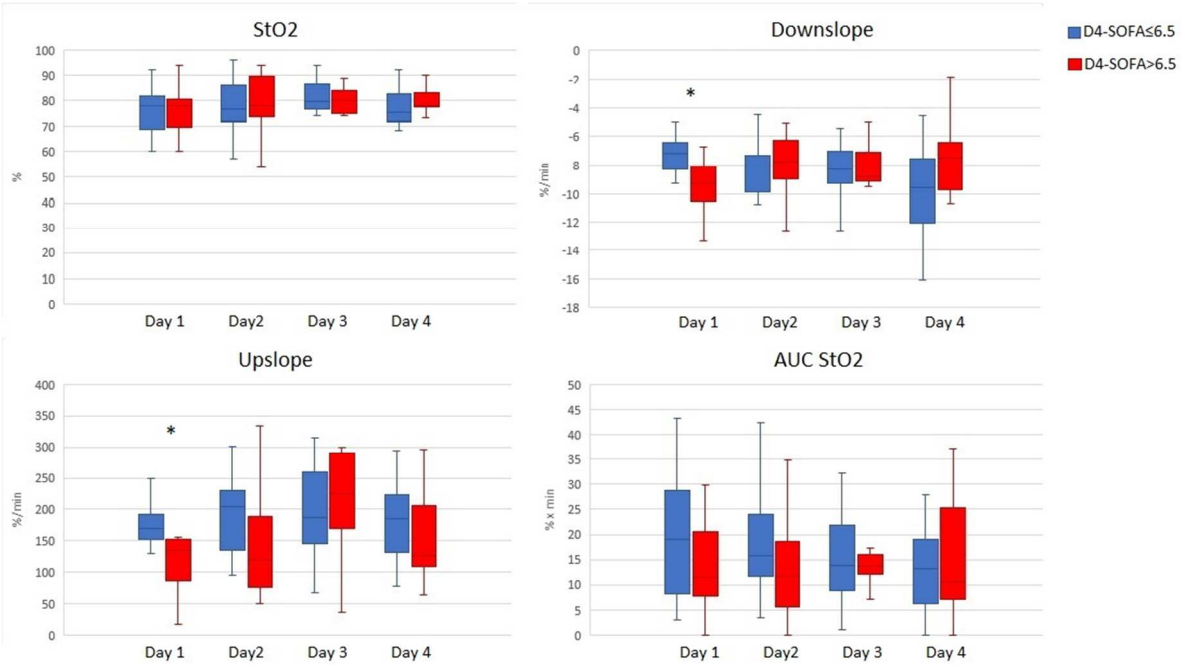


Fig 2: Changes in NIRS-derived parameters in the first 4 days of admission in ICU for the two groups of patients (SOFA score at D4≤6.5 and SOFA score at D4>6.5). * p<0.05. StO2: tissue oxygen saturation; AUC StO2: area under the curve of tissue oxygen saturation.

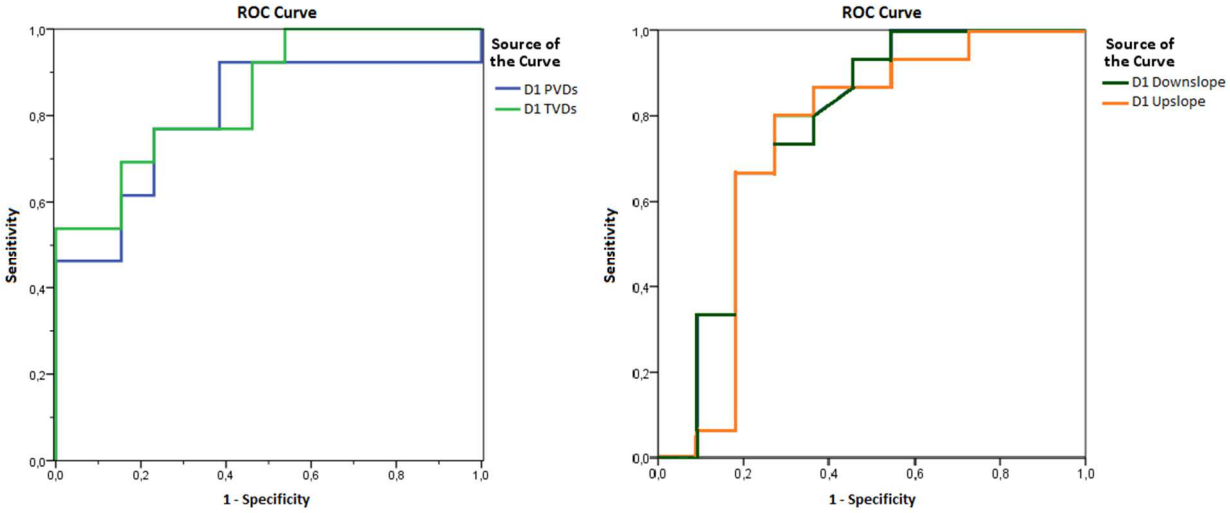
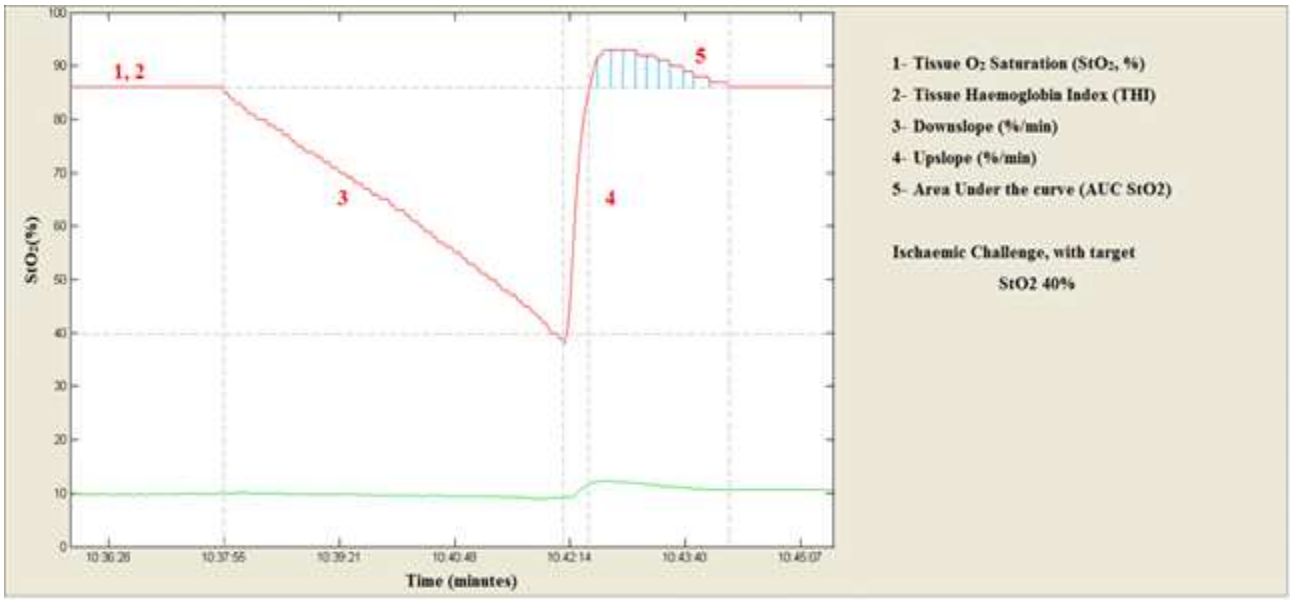


Fig 3: Receiver Operating Characteristic (ROC) curves. Discriminative ability of PVDs and TVDs (on the left) and StO2 Downslope and Upslope (on the right) at admission in ICU towards D4 SOFA score. TVDs: total small vessel density; PVDs: perfused small vessel density. StO2: tissue oxygen saturation.



S1 Fig: Near-InfraRed Spectroscopy (NIRS), StO2 response to vascular occlusion test (VOT).

S1 Table: Baseline characteristics of the 39 trauma patients included in MicroDAIMON study; n (%).

aER=Emergency Room

Mechanism of trauma, n(%)		**n (%)**
	Traffic	20 (51.3%)
	Fall	9 (23.1%)
	Knife wound	3 (7.7%)
	Other	7 (17.9%)
Injured area, n(%)		
	Head and neck	19 (48,7%)
	Face	8 (20,1%)
	Chest	19 (48,7%)
	Abdomen	15 (38,5%)
	Extremity	12 (30,1%)
	Other	7 (17,9%)
Crush Syndrome, n(%)		
		5 (12.8%)
Patients transfused in the ER[a]		
		18 (46%)
Surgery after ER		
		18 (46%)

Chapter 3

Changes in the sublingual microcirculation following aortic surgery under balanced or total intravenous anaesthesia: a prospective observational study.

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Abstract

Background: In vascular surgery with aortic cross-clamping, ischemia/reperfusion injury induces systemic haemodynamic and microcirculatory disturbances. Different anaesthetic regimens may have a varying impact on tissue perfusion. The aim of this study was to explore changes in microvascular perfusion in patients undergoing elective open abdominal aortic aneurysm repair under balanced or total intravenous anaesthesia.

Methods: Prospective observational study. Patients undergoing elective open infrarenal abdominal aortic aneurysm repair received balanced (desflurane + remifentanil, n=20) or total intravenous anaesthesia (TIVA, propofol + remifentanil using target-controlled infusion, n=20) according to the clinician's decision. A goal-directed haemodynamic management was applied in all patients. Measurements were obtained before anaesthesia induction (baseline) and at end-surgery and included haemodynamics, arterial/venous blood gases, sublingual microvascular flow and density (incident dark field illumination imaging), peripheral muscle tissue oxygenation and microcirculatory reactivity (thenar near infrared spectroscopy with a vascular occlusion test).

Results: The two groups did not differ for baseline characteristics, mean aortic-clamping time and requirement of vasoactive agents during surgery. Changes in mean arterial pressure, systemic vascular resistance index, haemoglobin and blood lactate levels were similar between the two groups, while the cardiac index increased at end-surgery in patients undergoing balanced anaesthesia. The sublingual microcirculation was globally unaltered in the TIVA group at end-surgery, while patients undergoing balanced anaesthesia showed an increase in the total and perfused small vessel densities (from 16.6±4.2 to 19.1±5.4 mm/mm2, $p<0.05$). Changes in microvascular density were negatively correlated with changes in the systemic vascular resistance index. The area of reactive hyperaemia during the VOT increased in the balanced anaesthesia group (from 14.8±8.1 to 25.6±14.8 %*min, $p<0.05$). At end-surgery, the tissue haemoglobin index in the TIVA group was lower than that in the balanced anaesthesia group.

Conclusions: In patients undergoing elective open abdominal aortic aneurysm repair with a goal-directed hemodynamic management, indices of sublingual or peripheral microvascular perfusion/oxygenation were globally preserved with both balanced anaesthesia and TIVA. Patients undergoing balanced anaesthesia showed microvascular recruitment at end-surgery.

Trial registration: NCT03510793, https://www.clinicaltrials.gov, date of registration April 27th 2018, retrospectively registered.

Keywords

Aortic surgery, ischemia/reperfusion injury, anaesthesia, haemodynamics, microcirculation

Background

Ischemia/reperfusion (I/R) injury is common in patients undergoing aortic clamping for vascular surgery and leads to systemic inflammation and organ dysfunction [1-3]. The production of pro-inflammatory molecules and oxidative stress increase especially in the reperfusion phase and are responsible for microvascular alterations similar to those observed during sepsis [4-6]. These include an impairment in blood flow, capillary shunting with reduction in microcirculatory density and increased perfusion heterogeneity, resulting in a mismatch between oxygen delivery and consumption [4, 5]. The severity of microcirculatory dysfunction increases along with the duration of the ischemic phase [2, 3, 7].

A number of studies explored how drugs can modulate microvascular perfusion in different disease states. In septic patients with impaired blood flow auto-regulation, vasoactive agents may have limited potential for microvascular recruitment [8]: the use of norepinephrine titrated to reach a mean arterial pressure (MAP) >65 mmHg was unable to improve microcirculatory perfusion during septic shock [9, 10]. Dobutamine increased intestinal microvascular blood flow in animal models of sepsis [11] but failed to restore capillary perfusion in patients with septic shock [12]. Conversely, after cardiac surgery a rise in MAP by norepinephrine infusion induced an increase in splanchnic oxygen extraction without altering the intestinal mucosal perfusion, possibly because of autoregulation phenomenon [13-15].

Anaesthetics can affect microvascular perfusion as well. Propofol causes vasodilation by stimulating nitric oxide production: this may result in microvascular shunting with reduction in capillary density, increased blood flow heterogeneity and reduced tissue oxygen delivery [16-18]. Volatile anaesthetics also cause dose-dependent vasodilation [19, 20]. In anesthetised dogs, desflurane, unlike isoflurane and halothane, was able to maintain myocardial, hepatic, intestinal and skeletal muscle blood flow [21]. In patients undergoing thoracoscopic surgery, desflurane-remifentanil anaesthesia was associated with better microvascular perfusion as compared to propofol-remifentanil anaesthesia

[22]. In female patients undergoing breast surgery, the use of sevoflurane, unlike propofol, was associated with a significant decrease in the capillary filtration coefficient, suggesting a lower risk of interstitial oedema and tissue perfusion alteration [23]. Microcirculatory dysfunction was found in different surgical settings despite the optimization of systemic hemodynamic parameters [24] and was associated with the development of post-operative complications [25].

We aimed to verify whether desflurane might have a better impact on the microcirculation as compared to propofol in patients at risk for I/R injury due to aortic surgery. The goal of this study was to explore changes in sublingual and peripheral muscle microcirculation in patients undergoing elective open abdominal aortic aneurysm repair under balanced or total intravenous anaesthesia (TIVA).

Methods

The study was approved by our local ethic committee of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona in Italy (ClinicalTrials.gov identifier: NCT03510793, www.clinicaltrials.gov, principal investigator: Prof. Abele Donati, date of registration: April 27th 2018, retrospectively registered). A written informed consent was obtained from all patients before enrolment. This was designed as a prospective observational study on 40 patients undergoing elective open infrarenal abdominal aortic aneurysm repair with prosthetic aorto-aortic or aorto-bisiliac bypass under general anaesthesia. The study was performed at Vascular Surgery of the Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona in Italy between September 2013 and November 2017.

Inclusion criteria were: elective infrarenal abdominal aortic open repair; use of a protocol of intraoperative hemodynamic goal-directed therapy; American Society of Anaesthesiology (ASA) class $\leq$ III. Exclusion criteria were: age <18 years, pregnancy, endovascular aneurysm repair, presence of infections, trauma, emergency surgery.

Patients received balanced anaesthesia or TIVA based on the attending physician's decision, resulting in two study groups of 20 patients each. The choice of the anaesthetic regimen was based exclusively on the physician preference and was taken before and independently of the patient's enrolment, the anaesthesiologist was unaware of baseline microcirculatory measurements. The patients' distribution between the two groups was initially due to chance; the enrolment was then stopped in the balanced anaesthesia group once the required sample size (n=20) had been reached, in order to achieve the same number of patients in the TIVA group. Patients undergoing balanced anaesthesia received desflurane (with an inspiratory oxygen fraction of 0.4-0.5) and remifentanil, administered as a target-controlled infusion (Minto model). TIVA was performed with a target-controlled infusion of propofol (Schnider model) and remifentanil (Minto model). In all patients, anaesthetic depth was monitored with spectral entropy and the anaesthetic dose was titrated in order to maintain a state entropy value of 35 to 45%, resulting in an age-adjusted minimum alveolar concentration of 0.8 for desflurane and an estimated effect-site target concentration (Ce) of 2-4 mcg/ml and 2.5-5 ng/ml for propofol and remifentanil, respectively. Neuromuscular blockade was achieved with a bolus of 0.6 mg/kg rocuronium at anaesthesia induction; additional boluses (10 mg) were provided based on train-of-four neuromuscular monitoring. Haemodynamic parameters were monitored in all patients with Flotrac/Vigileo (Edwards Lifesciences); arterial and central venous blood gases were monitored according to routine clinical practice. A protocol of intraoperative goal-directed therapy was applied in all patients for haemodynamic optimization, according to standard care in our unit [26]. This includes: first, stroke volume optimization by fluid therapy (targeting an increase in stroke volume <10% after a 200 ml fluid bolus over 5 minutes); second maintenance of a MAP >70 mmHg by titrated vasopressor administration; third, maintenance of a cardiac index (CI) ≥2.5 ml/kg per m^2 by titrated inotropic therapy [26].

Measurements were collected at baseline (before induction of anaesthesia) and at end-surgery (before anaesthesia discontinuation) and included haemodynamic parameters, arterial and central venous

blood gases and assessment of the sublingual microcirculation and peripheral muscle tissue oxygenation and microvascular reactivity.

Evaluation of the sublingual microcirculation

The sublingual microcirculation was assessed with incident dark field (IDF) imaging that is incorporated into a handheld video microscope (CytoCam, Braedius, Amsterdam, The Netherlands) and enables a real time visualisation of microvascular blood flow. Details on this technique have been described elsewhere [27]. After gentle removal of saliva and other secretions with a gauze, the probe was applied on the sublingual mucosa. Three videos of 10 seconds' duration were recorded on three different areas with adequate focus and contrast. All efforts were made to avoid pressure artifacts. Videos were analysed offline with a dedicated software (Automated Vascular Analysis v3.0, Microvision Medical, Amsterdam, The Netherlands). For small (diameter <20 micron) and medium (diameter 20-50 micron) vessels, we calculated the Total Vessel Density (TVD), Perfused Vessel Density (PVD), De Backer score, Percentage of Perfused Vessels (PPV), Microvascular Flow Index (MFI) and Flow Heterogeneity Index (FHI), as previously described [28].

Evaluation of tissue oxygenation and microvascular reactivity

Near-infrared spectroscopy (NIRS) (InSpectra^{TM} Model 650, Hutchinson Technology Inc, Hutchinson, MN, USA) was applied on the thenar eminence with a 15-mm sized probe to measure peripheral muscle tissue oxygen saturation (StO_2) and tissue haemoglobin index (THI) [29] before and during a vascular occlusion test (VOT), as described previously [30]. This was performed by inflating a sphygmomanometer cuff placed on the forearm to 50 mmHg above the systolic blood pressure. Arterial inflow was arrested until the StO_2 decreased to 40%. StO_2 was recorded during the ischemic and the reperfusion phases until stabilization [31]. NIRS-derived parameters were calculated

by using a software package (version 3.02 InSpectra Analysis Program; Hutchinson Technology Inc.). The StO_2 downslope was calculated from the regression line of the StO_2 decay after occlusion, providing an index of tissue oxygen extraction rate [31]. The desaturation slope may vary during the ischaemic phase of the VOT, becoming more or less steep before reaching the 40% StO_2 threshold: the inflection point was identified and the downslope was calculated separately for the first and the last part of the desaturation curve (downslope 1 and downslope 2, respectively), as previously described [32]. Whenever a change in the slope was not observed, the desaturation curve was divided into two halves for the calculation of the two downslope values. The delta-downslope was calculated as the difference between the last and the first part of the desaturation slope (downslope 2 - downslope1), so that a positive value indicated a flattening in the second part of the slope (slower StO_2 decay) [32]. The StO_2 upslope during the reperfusion phase and the area under the curve of the StO_2 (AUC StO_2) during the hyperaemic response were calculated as indices of microcirculatory reactivity [31].

Statistical analysis

The analysis was performed using Graphpad Prism 6 (GraphPad Software, La Jolla, CA, USA). The Kolmogorov-Smirnov test was used to check the normality of distribution. Data were expressed as mean ± standard deviation or median [1st-3rd quartile], as appropriate. A chi-square test or Student's t test (or Mann Whitney U test) were applied for between-group comparisons of nominal or continuous variables, as appropriate. A two-way analysis of variance (ANOVA) with the Sidack's multiple comparison test was used whenever possible. Alternatively, the Wilcoxon and Mann-Whitney U test were used for non-normally distributed variables for comparisons between the two time points in the same group and between the two groups at the same time point. The Spearman rho was calculated to evaluate the correlation between variables. A (two-sided) p value <0.05 was used to indicate statistical significance. For sample size calculation, we considered a difference in MFI at

end-surgery of 0.4 (standard deviation 0.4) as clinically relevant [28]. A sample size of 16 patients per group was sufficient to detect such a difference with a power of 80% and an alpha error of 0.05. We included 40 patients (20 per group) to allow for missing data or dropouts.

Results

Figure 1 shows the study flow diagram, with details on the number of patients evaluated for eligibility and reasons for exclusion. The two groups did not differ for age, gender distribution, comorbidities, ASA score, and intraoperative data including clamping time, fluid balance at end-surgery and vasoactive agent requirements (Table 1). Changes in haemodynamics and blood gases from baseline to end-surgery are reported in Table 2. MAP and systemic vascular resistance index (SVRI) decreased in both groups. The CI increased in patients undergoing balanced anaesthesia, while it remained substantially stable in the TIVA group. The two groups showed similar changes in haemoglobin (Hb), central venous oxygen saturation ($ScvO_2$), arterial lactate levels and arterial base excess.

Changes in microcirculatory and NIRS-derived parameters are shown in Table 3. The TVD and PVD for small vessels increased at end-surgery in the balanced anaesthesia group, while the small vessel MFI and PPV remained unaltered in both groups (Figure 2). At both time points, the TVD and PVD for medium vessels were lower in the TIVA group as compared to those in the balanced anaesthesia group (Table 3). Changes in microvascular density were negatively correlated with changes in SVRI (TVD for small vessels: r=-0.479 p=0.002; PVD for small vessels: r=-0.451 p=0.003). These correlations remained if considering the two groups separately, although appearing weaker in the TIVA group (TVD for small vessels: r=-0.453 p=0.045, PVD for small vessels: r=-0.362 p=0.116) as compared to those observed the balanced anaesthesia group (TVD for small vessels: r=-0.526 p=0.017, PVD for small vessels: r=-0.515 p=0.020). No correlation was found between microvascular parameters and CI, MAP or mean norepinephrine dose during surgery. In all patients, a negative correlation was found between changes in SVRI and mean norepinephrine dose (r=-0.387, p=0.022).

The StO_2 did not vary from baseline to end-surgery in either group (Figure 3). The StO_2 downslope 1 increased in the TIVA group at end-surgery (indicating a slower desaturation rate in the initial part of the ischemic phase), while it remained stable in the balanced anaesthesia group (Figure 3). The StO_2 downslope 2, delta-downslope and StO_2 upslope did not vary in either group (Table 3). The AUC StO_2 increased at end-surgery in the balanced anaesthesia group (Figure 3). The THI tended to decrease and was significantly lower at end-surgery in the TIVA group, while it remained stable in the balanced anaesthesia group (Figure 4).

Discussion

In this prospective observational study on 40 patients undergoing elective open abdominal aortic aneurysm repair with an intraoperative goal-directed haemodynamic optimization, we aimed to explore whether different anaesthetic regimens could have a varying impact on microvascular perfusion in a condition of potential I/R injury. Our first finding is that both balanced anaesthesia and TIVA were associated with overall maintenance of microvascular perfusion and tissue oxygenation. Patients receiving balanced anaesthesia showed an increase in microvascular density, which was inversely related to a reduction in systemic vascular resistance. In patients undergoing TIVA we observed a minor reduction in the skeletal muscle tissue oxygen extraction rate during a VOT and lower THI at end-surgery as compared to that observed in the balanced anaesthesia group. Balanced anaesthesia was associated with an increase in microvascular reactivity, as reflected by a greater area of hyperaemia after the VOT as compared to that seen at baseline.

The activation of the inflammatory response may occur early during aortic surgery [33] with maximal production of oxygen free radicals within 5 minutes of lower-limb reperfusion [34]. Previous studies showed that oxidative stress during supraceliac or infrarenal aortic cross clamping and reperfusion leads to distributive alterations in microvascular oxygenation and perfusion of splanchnic organs [35]. Heterogeneous flow distribution is characterized by capillaries with stagnant blood cells next to

capillaries with normal flow [36] and can be found in microvascular beds far from the site of direct ischemic damage [37]. In rats subjected to I/R injury of the small bowel, the intestinal microvascular blood flow was reduced early in the first 10 minutes of reperfusion and failed to normalize in the first 2 hours [38]. Microcirculatory disturbances during I/R injury may occur independently of changes in macro-haemodynamics and tissue hypo-perfusion may persist despite a normalisation of systemic cardiocirculatory parameters due to a loss of haemodynamic coherence [39]. In our study, we did not find an impairment in microvascular perfusion at end-surgery. We hypothesize that intraoperative hemodynamic optimization with the routine use of a protocol of goal-directed therapy prevented the development of microvascular derangements. In pigs subjected to colon anastomosis, a goal-directed colloid administration was able to improve microvascular perfusion and oxygenation of healthy and perianastomotic colon [40]. Although we could not find any correlation with MAP or CI, changes in sublingual capillary density were inversely related with changes in systemic vascular resistance, reflecting a certain degree of coherence between the macro- and micro-haemodynamic responses in our population [41].

Sublingual microvascular density increased at end-surgery in patients receiving balanced anaesthesia, while it remained unaltered in the TIVA group. It cannot be excluded that the microvascular changes observed were the effect of vasodilating metabolites (e.g. adenosine monophosphate, hypoxanthine) released during reperfusion, however we would expect this reactive hyperaemia to disappear in a late reperfusion phase at end-surgery [3]. Even if the observational design of our study prevents to define cause-effect relationships, the between-group homogeneity in baseline and intraoperative characteristics (including clamping time, fluid balance, blood losses, type and dose of vasoactive agents) would corroborate the hypothesis of direct and separate actions of desflurane and propofol on the microcirculation.

Previous studies showed that both propofol and sevoflurane can exert a protective effect towards I/R injury by modulating the inflammatory response, reducing oxidative stress and tissue apoptosis [42].

Halogenated anaesthetics induce peripheral vasodilation [43, 44] and reduce vascular permeability [23], thus favouring microvascular recruitment and tissue oxygen diffusion. On the other hand, propofol can modulate the inflammatory response and oxidative stress [45], thus increasing tolerance to hypoxia also in tissues far from those directly exposed to ischemia [46]. In this study, desflurane may have been responsible for vasodilation and vascular recruitment in the balanced anaesthesia group due to dilation of resistive arterioles. The increased area of hyperaemia after the VOT would also be consistent with a greater microvascular recruitment following the ischemic challenge in the balanced anaesthesia group.

The rationale of evaluating the microcirculatory response to therapies is that improving microvascular perfusion will guarantee tissue oxygenation and prevent the development of organ dysfunction. In a previous study in patients undergoing abdominal aortic aneurysm surgery, we showed that portal lactate, intramucosal sigmoid pH and arterial-sigmoid delta pCO_2 (as indices of splanchnic hypo-perfusion) were able to predict the occurrence of post-operative complications [47]. In the present study, we could not show a better impact on anaerobic metabolism of desflurane or propofol, as a clinically relevant hyper-lactataemia was not found in either group at end-surgery. This was consistent with the absence of major microcirculatory alterations in both the balanced anaesthesia and the TIVA groups.

While the StO_2 remained stable in both groups, the desaturation rate in the first part of the ischemic challenge decreased at end-surgery in patients undergoing TIVA. This may result from a reduction in peripheral microvascular density and would be consistent with the lower THI at end-surgery as compared to that seen in the balanced anaesthesia group, which seems to be related to a lower tissue perfusion rather than to a reduction in Hb levels as these remained similar in the two groups. Baseline differences in StO_2 downslope between the groups, although not significant, could also explain our results: the observed variation may only depend upon a rebalance in the metabolic pattern in the TIVA group without a dysfunctional meaning. In critically ill patients, we showed that a decrease in the

desaturation rate in the last part of the ischaemic phase of the VOT is associated with worse outcome and could suggest impaired microcirculatory auto-regulation [32]. In this study, the StO_2 downslope was again similar between the groups in the final part of vascular occlusion and the delta-downslope did not differ between the two groups. Therefore, the type of anaesthesia may not significantly affect peripheral microcirculatory auto-regulation.

Limitations of the study

First, the observational design does not allow drawing any cause-effect relationship between the type of anaesthesia and the microvascular changes observed. Moreover, lack of randomization and the relatively small sample size prevented to control for possible confounding factors. This study was designed as a preliminary exploratory analysis with the aim to detect any possible influence of different anaesthetic regimens on microvascular perfusion alterations during aortic surgery. Future randomized trials are needed to confirm our findings. Second, we cannot exclude that baseline differences in some parameters influenced our results. Even if the main haemodynamic parameters were similar between the groups at baseline, the lower $ScvO_2$ in the balanced anaesthesia group, as well as differences in baseline microvascular medium vessel density, may indicate a higher initial tissue O_2 demand/consumption. However, baseline microvascular small vessel density and NIRS-derived parameters were similar between the two groups, suggesting a similar pattern of tissue perfusion. Third, a relevant number of patients among those assessed for eligibility was excluded, therefore we cannot exclude the presence of a selection bias. Lastly, the microcirculation was globally preserved in both groups, suggesting an overall low prevalence of I/R injury syndrome in our sample. Our study may have been underpowered to detect significant microvascular derangements following aortic surgery. Moreover, monitoring of the sublingual microcirculation may not be sensitive enough to detect tissue perfusion alterations in splanchnic organs [48]. Unfortunately, we did not evaluate the incidence of organ dysfunction or post-operative complications.

Conclusions

In patients undergoing open abdominal aortic aneurysm repair under general anaesthesia with a protocol of intraoperative hemodynamic goal-directed therapy, microvascular perfusion and peripheral tissue oxygenation were generally preserved, however the use of balanced anaesthesia was associated with increased microvascular density and reactivity, while these remained unaltered with TIVA. Further studies are needed to clarify whether the choice of different anaesthetic regimens may contribute to prevent I/R injury in aortic surgery.

List of abbreviations

I/R ischemia/reperfusion, *MAP* mean arterial pressure, *TIVA* total intravenous anaesthesia, *ASA* American Society of Anaesthesiology, *Cp* plasma target concentration, *CI* cardiac index, *IDF* Incident Dark Field, *TVD* total vessel density, *PVD* perfused vessel density, *PPV* percentage of perfused vessels, *MFI* microvascular flow index, *FHI* flow heterogeneity index, *NIRS* near infrared spectroscopy, StO_2 tissue oxygen saturation, *THI* tissue haemoglobin index, *VOT* vascular occlusion test, *AUC* StO_2 area under the hyperaemic phase, *SVRI* systemic vascular resistance index, *Hb* haemoglobin, $ScvO_2$ central venous oxygen saturation.

Declarations

Ethics approval and consent to participate

The study was approved by our local ethic committee of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona in Italy (ClinicalTrials.gov identifier: NCT03510793, www.clinicaltrials.gov, principal investigator: Prof. Abele Donati, date of registration: April 27th 2018, retrospectively registered). A written informed consent was obtained from all patients before enrolment.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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None.

Authors' contributions

SL contributed to the study design, acquisition of the data, interpretation of the results and drafted the manuscript. NM, ED collected the data and drafted the manuscript. BM, EA, CS, RD, AC, SP and GP participated in the acquisition and analysis of the data and revised the manuscript. LC and AD contributed to the study design and interpretation of the results and revised the manuscript critically for important intellectual content. All authors read the manuscript and gave final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Table 1 – General and intraoperative data for the two groups of patients receiving balanced anaesthesia or total intravenous anaesthesia.

	Balanced anaesthesia	**TIVA**	**p**
Age (years)	75±7	74±6	0.464
Gender (nr, % of males)	16, 80%	18, 90%	0.376
ASA score (nr, %)			0.127
2	13, 65%	18, 90%	
3	7, 35%	2, 10%	
Comorbidities (nr, %)			
Arterial hypertension	20, 100%	18, 90%	0.149
Cardiopathy	6, 30%	10, 50%	0.197
Renal Failure	3, 15%	5, 25%	0.429
COPD	12, 60%	8, 40%	0.206
Dyslipidaemia	10, 50%	10, 50%	0.999
Vasculopathy (lower limbs or carotid)	10, 50%	8, 40%	0.525
Clamping time (min)	65±29	69±31	0.691
Fluid balance (ml)	-200 [-1100-+150]	-195 [-670-+300]	0.657
Norepinephrine			
number, %	20, 100%	20, 100%	0.999
mean dosage (mcg/kg/min)	0.15 [0.08-0.20]	0.12 [0.09-0.15]	0.466
Dobutamine			
number, %	13, 65%	16, 80%	0.288
mean dosage (mcg/kg/min)	2.00 [1.23-3.00]	2.39 [1.67-3.92]	0.416
Esmolol (nr, %)	14, 70%	10, 50%	0.197
Levosimendan (nr, %)	3, 15%	5, 25%	0.429
Blood transfusions			
number, %	10, 50%	10, 50%	0.999
average amount (ml)	540 [450-1060]	580 [450-780]	0.837

Data are expressed as mean ± standard deviation or median [1st-3rd quartile], as appropriate.

Cardiopathy was defined by the presence of echocardiographic alterations including left ventricular hypertrophy and valvulopathy, or a history of coronary artery disease and/or heart failure and reduced left ventricular ejection fraction. Renal failure was defined by the presence of an estimated glomerular filtration rate lower than 60 ml/min/1.73mq.

TIVA total intravenous anaesthesia, *ASA* American Society of Anesthesiology, *COPD* chronic obstructive pulmonary disease.

Table 2 – Changes in haemodynamics and blood gases from baseline to end-surgery in the two groups of patients receiving balanced anaesthesia or total intravenous anaesthesia.

	Balanced anaesthesia		TIVA		p for interaction (2-way ANOVA)
	baseline	end-surgery	baseline	end-surgery	
MAP (mmHg)	94±18	86±13*	92±14	81±12**	0.642
HR (bpm)	67±13	71±11	70±13	71±14	0.521
CI (ml/m^2/min)	2.4±0.7	3.2±0.6***	2.6±0.5	2.8±0.7	0.005
SVRI (dyn*s*cm^{-5})	2800 [2352-3224]	1839 [1691-2085]***	2488 [2203-2952]	2119 [1754-2350]***	-
pH	7.42 [7.39-7.47]	7.40 [7.38-7.43]	7.43 [7.42-7.44]	7.41 [7.37-7.45]	-
PaO_2 (mmHg)	70 [62-88]	159 [145-207]**	72 [63-166]	194 [134-235]***	-
$PaCO_2$ (mmHg)	38±4	38±4	37±3	39±5*	0.027
Base excess (mEq/l)	0.5 [-0.3, +2.5]	-0.4 [-3.2, +1.2]**	0.5 [-1.4, +1.2]	-0.4 [-1.9, +2.4]	-
$ScvO_2$ (%)	72 [69-78]	90 [82-92]***	79 [70-90]#	91 [86-94]**	-
Lactate (mmol/l)	0.6 [0.6-0.8]	1.3 [1.1-1.9]***	0.7 [0.6-1.0]	1.3 [1.0-1.5]***	-
Hb (g/dl)	13.3±2.0	11.8±0.8***	13.4±1.6	11.8±1.3***	0.793
Glucose (mg/dl)	96 [86-107]	121 [105-131]***	102 [95-112]	125 [106-141]***	-

*p<0.05 **p<0.01 ***p<0.001 versus baseline, #p<0.05 versus time-matched balanced anaesthesia. Two-way Analysis of Variance (ANOVA) with Sidack's multiple comparisons test or Wilcoxon and Mann-Whitney U test, as appropriate. Data are expressed as mean ± standard deviation or median [1st-3rd quartile], as appropriate. *TIVA* total intravenous anaesthesia, *MAP* mean arterial pressure, *HR* heart rate, *CI* cardiac index, *SVRI* systemic vascular resistance index, *PaO_2* arterial oxygen tension, *$PaCO_2$* arterial carbon dioxide tension, *$ScvO_2$* central venous oxygen saturation, *Hb* haemoglobin.

Table 3 – Changes in sublingual microcirculation and NIRS-derived parameters from baseline to end-surgery in the two groups of patients receiving balanced anaesthesia or total intravenous anaesthesia.

	Balanced anaesthesia		TIVA		p for interaction (2-way ANOVA)
	Baseline	**end-surgery**	**baseline**	**end-surgery**	
MFI (small vessels) [AU]	2.67 [2.50-2.75]	2.83 [2.37-2.98]	2.75 [2.58-2.90]	2.67 [2.42-2.81]	-
Abnormal MFI[a] (n, %)	9, 45%	6, 30%	7, 35%	9, 45%	-
FHI [AU]	0.15 [0.00-0.29]	0.18 [0.02-0.36]	0.23 [0.09-0.28]	0.19 [0.10-0.38]	-
TVD (small) (mm/mm2)	18.4±3.8	21.0±4.7*	19.8±4.0	20.7±4.5	0.216
TVD (medium) (mm/mm2)	1.5 [0.9-2.2]	1.2 [1.1-1.5]	0.8 [0.6-1.1]##	0.5 [0.1-0.8]###	-
PVD (small) (mm/mm2)	16.6±4.2	19.1±5.4*	18.1±4.2	19.2±4.9	0.302
PVD (medium) (mm/mm2)	1.5 [0.9-2.2]	1.2 [1.1-1.5]	0.8 [0.6-1.1]##	0.5 [0.1-0.8]###	-
PPV (small, %)	91 [83-97]	91 [82-97]	96 [83-98]	95 [90-97]	-
StO_2 (%)	83±5	86±7	82±6	85±9	0.975
StO_2 Downslope 1 (%/min)	-8.0 [-10.8, -6.8]	-7.8 [-10.4, -6.9]	-8.8 [-12.0, -7.8]	-8.1 [-11.1, -5.9]**	-
StO_2 Downslope 2 (%/min)	-8.2±3.3	-8.0±4.8	-8.2±2.8	-8.0±3.4	0.962
Delta Downslope (2-1) (%/min)	1.1 [-0.5, 2.4]	-0.1 [-1.6, 1.8]	0.9 [-0.8, 3.9]	-0.7 [-1.4, 1.0]	-
StO_2 Upslope (%/min)	226±64	206±103	264±84	247±77	0.900
AUC StO_2 (%*min)	14.8±8.1	25.6±14.8*	20.4±6.0	26.1±19.1	0.359
THI [AU]	15.2±2.0	15.3±3.1	14.6±3.3	12.8±3.6#	0.140

*p<0.05 **p<0.01 versus baseline, #p<0.05 ##p<0.01 ###p<0.001 versus time-matched balanced anesthesia. Two-way Analysis of Variance (ANOVA) with Sidack's multiple comparisons test or Wilcoxon and Mann-Whitney U test, as appropriate. Data are expressed as mean ± standard deviation or median [1^{st}-3^{rd} quartile], as appropriate.

[a] An abnormal MFI was defined as an MFI lower than 2.6 [25].

NIRS near infrared spectroscopy, *TIVA* total intravenous anaesthesia, *MFI* microvascular flow index, *AU* arbitrary unit, *FHI* flow heterogeneity index, *TVD* total vessel density, *PVD* perfused vessel density, *PPV* percentage of perfused vessels, *StO_2* tissue oxygen saturation, *AUC StO_2* area under the curve of StO_2, *THI* tissue haemoglobin index.

Figure 1 – Study flow diagram.

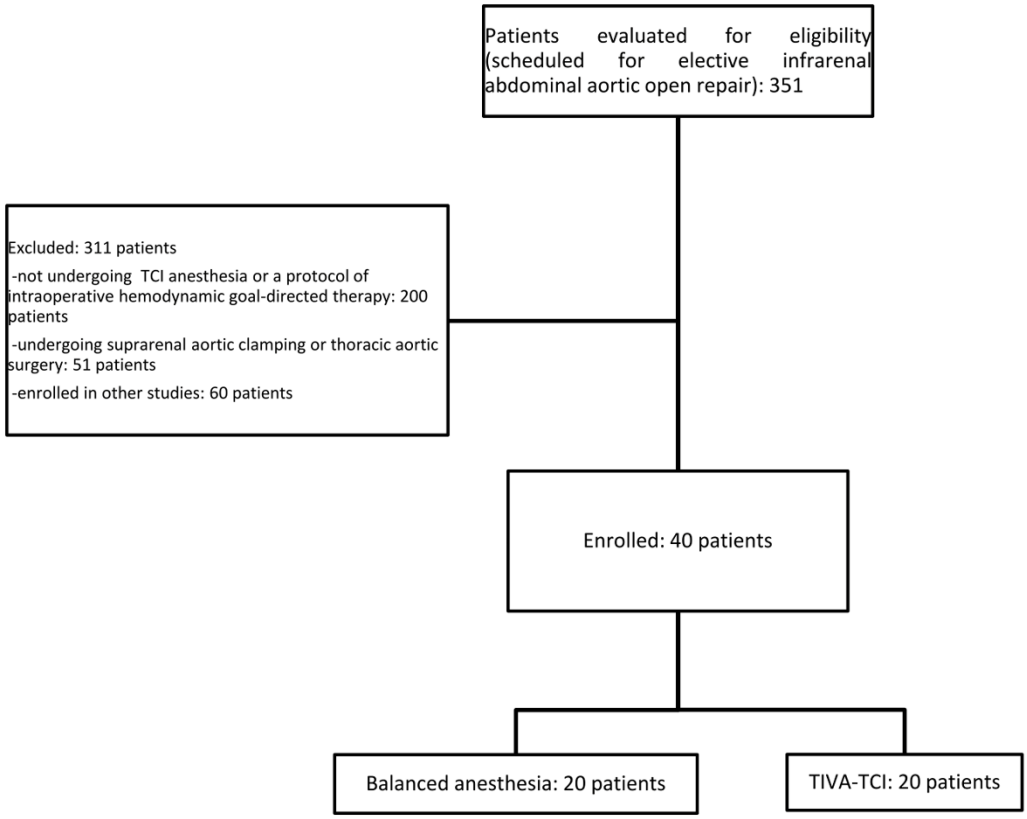


Figure 2 – Changes in sublingual microvascular parameters from baseline to end-surgery in the two groups of patients receiving balanced anaesthesia or total intravenous anaesthesia (TIVA).

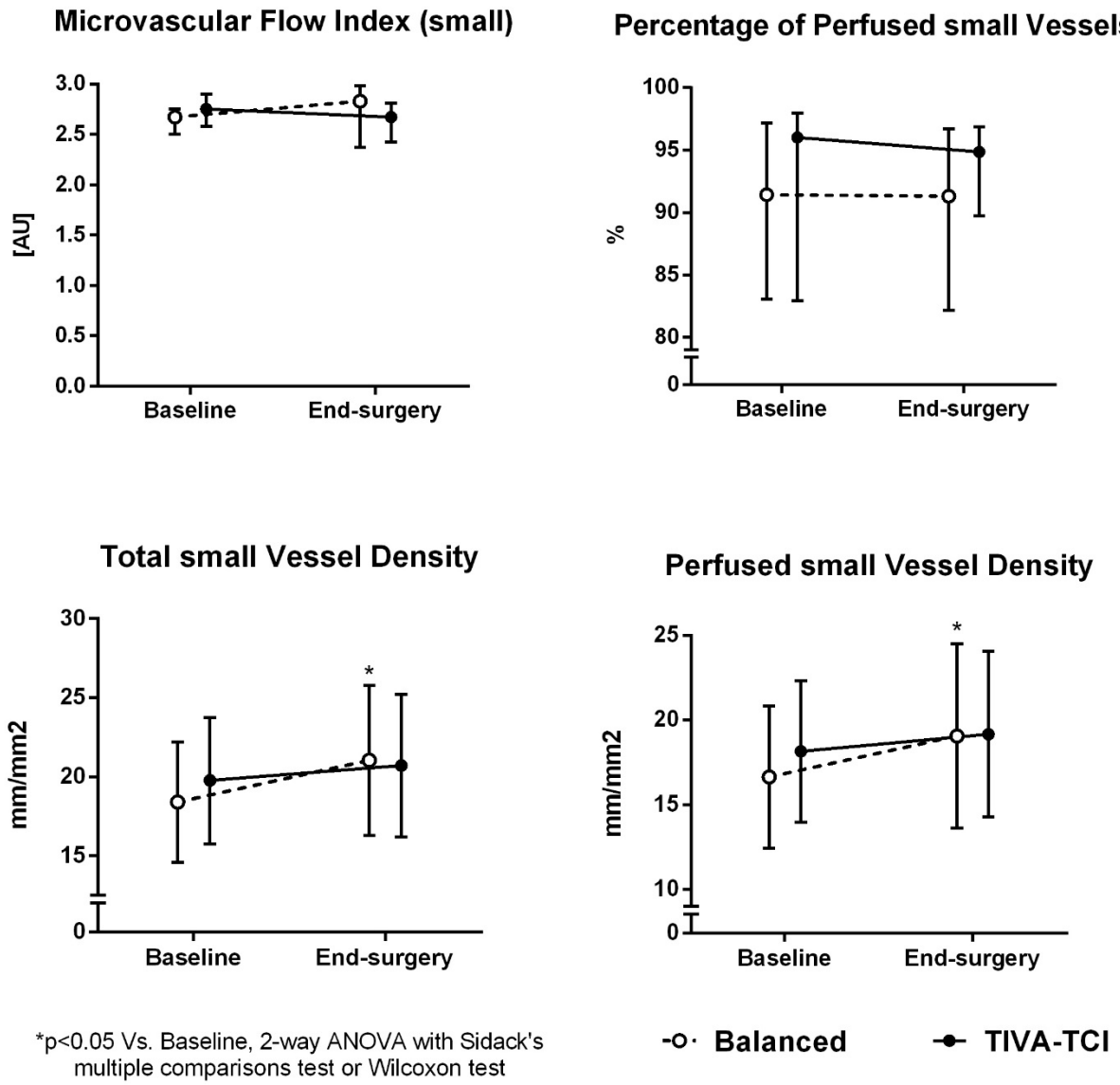
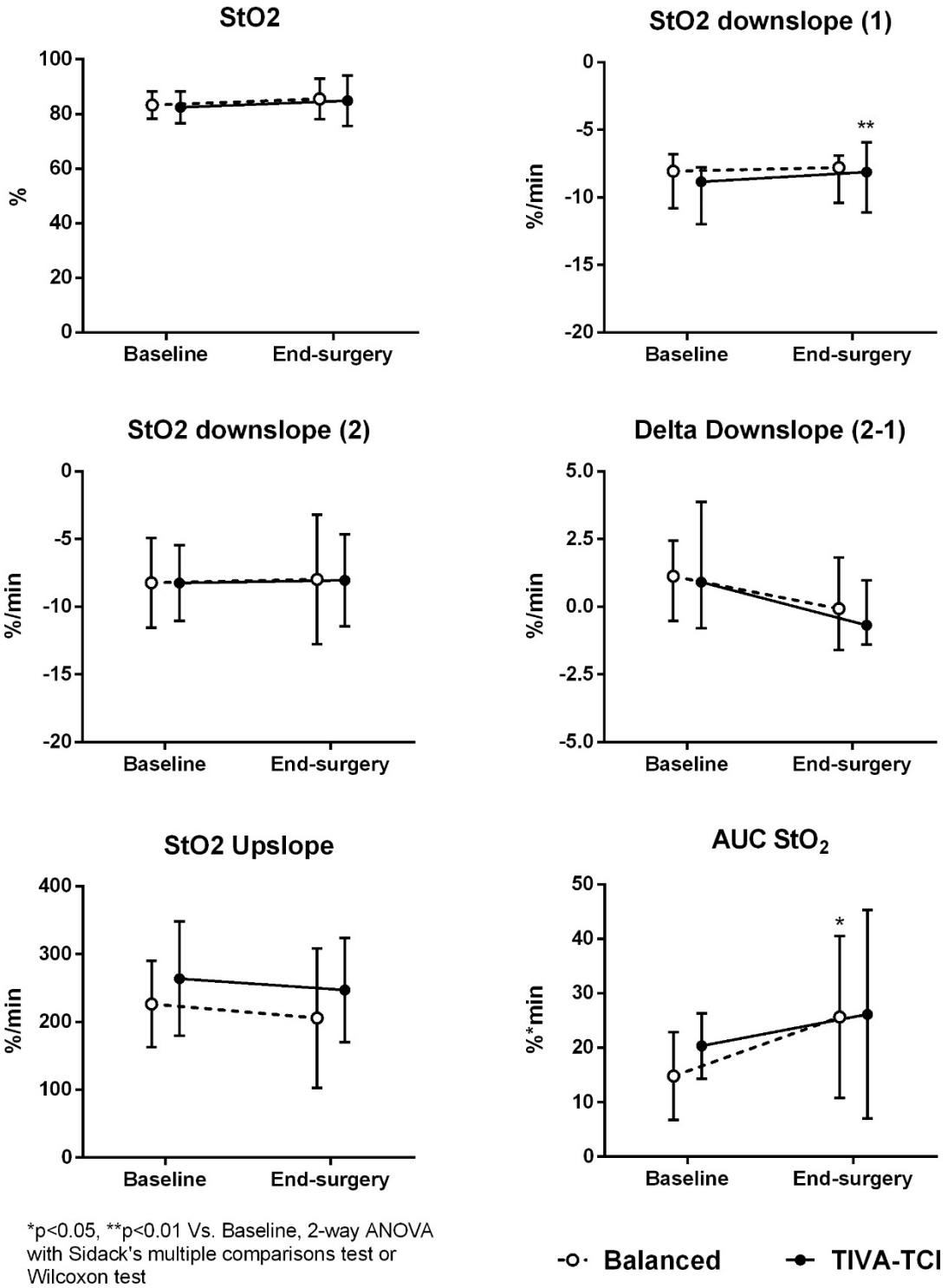


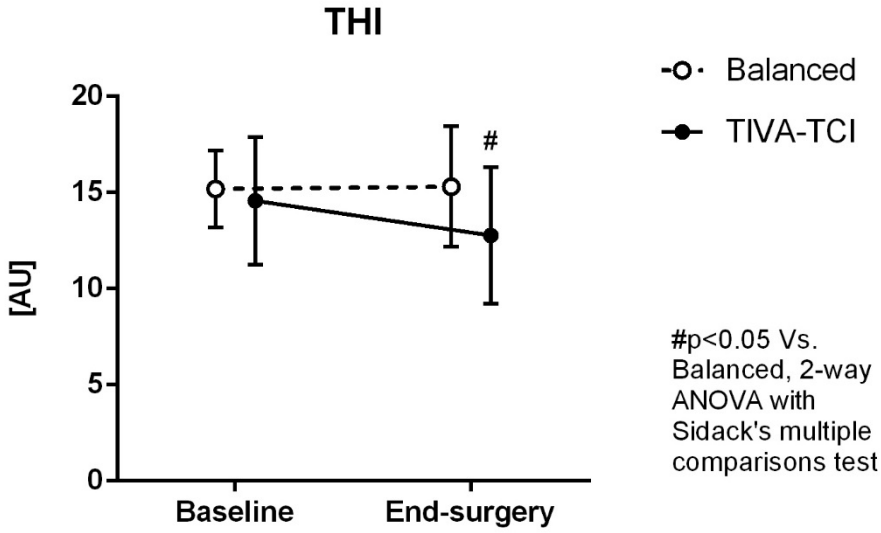
Figure 3 – Changes in NIRS-derived parameters from baseline to end-surgery in the two groups of patients receiving balanced anaesthesia or total intravenous anaesthesia (TIVA).

StO_2 tissue oxygen saturation, *AUC StO_2* area under the curve of StO_2 (area of hyperaemia).



*p<0.05, **p<0.01 Vs. Baseline, 2-way ANOVA with Sidack's multiple comparisons test or Wilcoxon test

Figure 4 – Changes in the tissue haemoglobin index (THI) from baseline to end-surgery in the two groups of patients receiving balanced anaesthesia or total intravenous anaesthesia (TIVA).



Chapter 4

Mid-regional proadrenomedullin (MR-proADM) and microcirculation in monitoring organ dysfunction of critical care patients with infection: prospective observational study

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Abstract:

Introduction: Organ failure is one of the main challenges in critical care management of infection-related illness. Mid-regional proadrenomedullin (MR-proADM) may potentially predict organ damage and may relate to its evolution. Microvascular alterations are strongly implicated in the development of organ injury. Therefore, the main objective of this study was to evaluate if a correlation existed between MR-proADM and Microvascular Flow Index (MFI) at admission in ICU in 20 adult patients diagnosed with infection, sepsis or septic shock. Further objective of the study was to assess the prognostic value of MR-proADM in term of mortality and its correlation with SOFA score

Methods: Prospective observational study. Inclusion criteria: consecutive patients admitted to ICU for or with infection-related illness, with a Length of Stay (LOS) in ICU before inclusion inferior to 24 hours. Daily measurement of MR-proADM and calculation of SOFA score, from day of admission in ICU to day 5. Repeated evaluation of sublingual microcirculation at day 1, 2 and 5, collection of clinical data and laboratory tests.

Results: the correlation between MR-proADM and MFI of small or total vessel was not statistically significant at admission in ICU. A clearance of MR-proADM of 20% or more in the first 24hours of admission was related to a percentage of improvement of MFI (Mann-Whitney U test, median increase 12.35% versus 2.23%, p=0.005). MR-proADM was related to SOFA score at admission in ICU, at day 2, 4 and 5 but the relation was not significant at day 3. MR-proADM at admission in ICU was related to ICU-mortality (AUC 0.941 [0.819-1]; p=0.017).

Conclusions: The study could not demonstrate a correlation of the single admission value of MR-proADM with MFI at admission in ICU. However, it showed a good inverse correlation of the trend in evolution of the two variables in the first two days. It also supported the prognostic value of MR-proADM. Further study may improve the reproducibility of our data

Introduction:

Sepsis is a life-threatening syndrome, characterized by a widespread tissue and microvascular injury. [1-2]

Organ failure is one of the main challenges of critical care septic patients and hemodynamic optimization is a cornerstone of adequate organ perfusion for prevention and treatment of organ dysfunction. [3]

However, organ sufferance may occur even after apparent restoration of systemic hemodynamics. The mechanism underlying it are multifactorial and not completely clear, but there is increasing evidence that alterations in microvascular blood flow are strongly implicated. Sepsis affects endothelial cell function; the endothelial barrier disruption and leakage lead to microcirculatory alteration and directly contributes to organ dysfunction. [4-7]

Adrenomedullin (ADM) is an endogenous peptide hormone of 52 amino acids synthetized widely through tissues (including bone, adrenal cortex, kidney, lung, blood vessels and heart). ADM is biologically active and its effects include vasodilator, positive inotropic, diuretic, natriuretic and bronchodilator actions, it is an inhibitor of secretion of insulin, aldosterone and adrenocorticotropic hormone. [8-9] Previous studies show that ADM increases in inflammatory diseases, including sepsis and septic shock, in order to stabilize the microcirculation and to protect against endothelial hyper-permeability, and it represents a marker of endothelial damage. [10-16]

Mid-regional proadrenomedullin (MR-proADM) is a fragment of Adrenomedullin (ADM) with no known function. It is produced in ratio 1:1 to ADM. Its half-life is several hours and it proportionally represents the levels and activity of Adrenomedullin. [8]

It was already described as biomarker in Community Acquired Pneumonia, and has been recently proposed as prognostic marker with potential clinical role in sepsis. [17-19] In the study of Valenzuela-Sánchez et al, MR-proADM showed good correlation with organ dysfunction related to

infection and to mortality in critical care patients. They also evaluated the clearance of MR-proADM demonstrating an enhanced clearance in survivor patients. [20]

In this study, we aimed to investigate if MR-proADM, as biomarker of organ failure, could be associated with microvascular alterations in critical care patients admitted in Intensive Care Unit (ICU) with different degrees of infection-related illness.

Methods

Population, enrolment and data collection:

This is a prospective observational study performed in the 14-bed General and Traumatic ICU of Azienda Ospedaliero -Universitaria Ospedali Riuniti of Ancona.
It included 20 adult patients consecutively admitted in ICU for or with different degrees of infection-related illness, with a Length of Stay (LOS) in ICU before inclusion inferior to 24 hours.
Exclusion Criteria to enrollment were: age inferior to 18 years old, LOS in hospital longer than 48 hours, conditions that prevented adequate monitoring of sublingual microcirculation, end-of-life care, or refusal to consent.

The primary objective of this study was to research a correlation between plasma levels of MR-proADM and Microcirculatory Flow Index (MFI) at admission in ICU. It was also purpose of the study to evaluate the relationship between MR-proADM and other microvascular variables (Total Vessel Density (TVD), Perfused Vessel Density (PVD) and Proportion of Perfused Vessels (PPV)), and to verify the association of MR-proADM with SOFA score (as marker of organ dysfunction), the severity scores at admission in ICU and with ICU-mortality.

The study was articulated in five days of monitoring (Time-point T1 to Time-point T5) from admission in ICU.

Plasmatic levels of ProADM were dosed for all timepoints. The Simplified Acute Physiology Score (SAPS) II and Acute Physiology and Chronic Health Disease Classification System II (APACHE) scores were calculated at admission in ICU. The Sequential Organ Failure Assessment (SOFA) score was evaluated at admission, and daily T1 to T5.

Anthropometric and demographic data were collected at baseline, including age, sex, weight and height.

Microbiological parameters were assessed to categorize patients (infection, sepsis or septic shock; source of infection, presence of MDR).

For each of the five timepoints we recorded clinical, hemodynamic and laboratory parameters (systolic, diastolic and mean arterial pressure, heart rate, cardiac output where available, respiratory parameters and mechanical ventilation, venous and arterial blood gases, vasoactive therapy, main parameters for renal, hepatic and hematological function, procalcitonin).

At T1 (<24hours from ICU admission), T2 (24 hours after the first assessment) and T5 (day five) the sublingual microcirculation was assessed using Incident Dark Field technology.

Microvascular assessment:

Sublingual microcirculation was assesses by well-trained, experienced operators using high-resolution video microscopy camera (CytoCam, Braedius Medical B.V., Huizen, the Netherlands) with incident dark-field (IDF) technology. Microcirculatory parameters were, then, calculated offline with the Automated Vascular Analysis software (AVA v3.2, Microvision Medical, Amsterdam, NL), Microvascular assessment and analysis were performed according to the "second consensus on the assessment of sublingual microcirculation in critically ill patients" and "the microcirculation image quality score". [21-22]

The microvascular flow index (MFI), Total vessel density (TVD), the Perfused Vessel Vensity (PVD), the Proportion of Perfused Vessels (PPV) were calculated for both small-size vessels and medium-size ones in all the videos analyzed. The De Backer score was measured for total vessels.

Measurement of MR-proADM:

Arterial blood samples were collected and immediately centrifuged. Plasma and serum samples were, then, stored at -80°C for subsequent measurement of MR-proADM.

Plasmatic levels of MR-proADM were measured using TRACE technology (Time Resolved Amplified Cryptate Emission) with thermo Scientific™ B·R·A·H·M·S™ KRYPTOR compact PLUS (Dasit). The reference limit for this method was 0.55 nmol/L.

Ethics:

In compliance with national applicable laws, informed consent was obtained from the subject or his next of kin before inclusion, by signing the appropriate informed consent paperwork. The study protocol was approved by the Local Ethics Committee (Comitato Etico Regione Marche—CERM; protocol number 212639, clinicaltrial.gov NCT03931967) and it conformed to the principles of Helsinki declaration (last revision, Edinburgh 2000).

Sample size calculation

Sample size calculation was computed on the basis of the primary end-point of the study (correlation between plasma levels of MR-proADM and MFI at admission in ICU-T1). A total of 19 patients was shown to be sufficient to detect a statistically significant correlation coefficient (higher than 0.6) with a power of 80% and an alpha error of 0.05.

Statistical Analysis:

Statistical analysis was performed by using IBM SPSS statistic software (Version 17.0).

According to the distribution of the main variables (assessed with Kolmogorov-Smirnov test) and to the limited sample size, non-parametric statistics predominated. Data are presented as median and interquartile ranges (IQR) for continuous variables, number and percentage for discrete variables.

Spearman's Rank correlation coefficient was used to summarize the strength and direction (negative or positive) of a relationship between MR-proADM and MFI as primary outcome measure, with further parameters of microcirculation and with severity scales. Non-parametric Mann–Whitney U was used for comparison between independent samples. The area under the Receiver Operating Characteristic (ROC) curve was calculated to confirm the ability of MR-proADM to discriminate the severity of patients and the differences in terms of microcirculation. Differences were considered significant at p values <0.05

Results

Descriptive of the sample:

From November 2018 to June 2019, 29 patients were screened for the study and 20 of them were enrolled after obtaining the informed consent.

Patients were predominantly males (65%), with a median age of 70 [51-74] years. At admission in ICU SAPS II score was 52.5 [35.50-75.05], APACHE II score was 19.5 [12.25-30.00]. SOFA score 11 [8-14]. SOFA at admission corresponded to SOFA score at time of enrollment, as all patients were enrolled in the first 24 hours of ICU stay. [Table 1]

Half of the patients were in septic shock at recruitment and 5 on 20 patients were septic. The origin of infection was respiratory in the vast majority of them (65%). On 13 patients with low respiratory tract infections, 5 were diagnosed with type 1 influenza virus. Mortality rate was 12.5%, all those patients were in septic shock (30% of septic shock patients died, all of them for multi-organ dysfunction syndrome,). The LOS in our ICU was 12.50 [9.00-16.75] days, but 11 on 20 patients

were transferred to other ICUs for further treatments. [Table 1] Median LOS for patients who did not survive was 21 [9-21] days.

In Table 2 we report the median values of MR-proADM and of Procalcitonin at admission in ICU (day 1 of enrollment) according to the diagnosis (infection, sepsis and septic shock) and in the subgroup of patients who deceased in ICU. [Table 2]

The values of MR-proADM in our sample were respectively 1.44 [0.94-1.84]nmol/l in patients diagnosed with infections, 2.16 [1.35-4.00] nmol/l in septic patients, 3.99 [3.58-7.04] nmol/l in patients with septic shock. Procalcitonin showed very wide interquartile ranges in all the subgroups analyzed. MR-proADM and Procalcitonin showed a moderate correlation in the population, when evaluated at day 1 (Spearman coefficient 0.672, p=0.001). The correlation was more solid in the subgroup of patients with septic shock (Spearman coefficient 0.758, p=0.011).

MR-proADM and sublingual microcirculation:

Table 3 presents median values of the parameters of microcirculation in the general population. [Table 3]

The MFI of small vessels at admission in ICU was lower in the subgroup of septic shock patients (2.72 [2.5-2.85]), but not statistically different from that of septic (2.83[2.83-2.96]) and infected (2.83 [2.58-2.87] patients.

Spearman's Rank correlation coefficient for MR-proADM with MFI of total and small vessels was not statistically significant at admission in ICU both in the general population, and in the three subgroups of patients (infected, septic, and shocked).

We calculated the clearance of MR-proADM (in percentage) in the first 24 hours of recruitment; we used a cut-off of 20% of clearance, dividing 2 groups: patients who showed a clearance of MR-

proADM higher and lower to 20%. We measured the percentage of variation of MFI of small vessels in the same timeframe. Both groups showed an improvement of MFIs in the first 24hours of intensive care treatment, but it was significantly higher in patients that showed a clearance of MR-proADM>20%. (Mann-Whitney U test, median increase 12.35% versus 2.23%, p=0.005).

Patients with reduced clearance of MR-proADM showed lower clearance also at T5 (clearance T5 to T2 19.2% versus 24.3%) and deterioration in MFI (negative variation of -6.1% [-10.5-0] versus 0% [-6.33-(+3.1)].

MR-proADM, severity scores and mortality in ICU:

Median values of MR-proADM were significantly higher in (ICU) non-survivors (Mann-Whitney U test) in the first 4 days of evaluation, in particular at day 1, where median values of MR-proADM in survivors were 2.16 (1.41-3.85) nmol/l, p= 0.012. The Area Under the Curve (AUC) of the ROC curve for mortality, showed that MR-proADM was able to discriminate non-survivors at admission in ICU (AUC 0.941 [0.819-1]; p=0.017, optimal cut-off 3.99 nmol/l SE 100%, SP 82%).

In our sample, the absolute value of clearance of MR-proADM at day 2 or day 5 was not related to mortality.

MR-proADM was only lightly correlated with SAPS II score (Spearman correlation coefficient of 0.57, p=0.009) and with APACHE II score (Spearman correlation coefficient 0.52, p=0.018) at admission in ICU.

MR-proADM and organ dysfunction:

Organ dysfunction was monitored throughout the 5 days of enrollment with a daily SOFA score calculation. A cut-off of MR-proADM>1.42nmol/l at admission in ICU was able to discriminate higher SOFA score at admission, at day 2, day 4 and day 5, but not at day 3. [Figure 2]

A clearance of MR-proADM≤20% in the first 24 hours of ICU stay was able to predict worsening of SOFA score from day2 to day 5 (AUC 0.938, CI 0.776-1, p=0.025), with a median SOFA score at day-5 of 13 [9-15] versus 8 [6-11]. SOFA score was not related to MFI and sublingual microcirculation in the three time points of assessment.

Discussion:

In this study, we recruited 20 adult patients admitted in ICU with/for infection, sepsis or septic shock. We monitored them for 5 consecutive days analyzing the plasmatic levels of MR-proADM, and the main clinical parameters and scores of severity. We evaluated the sublingual microcirculation to understand if MR-proADM, as biomarker of organ failure could be associated or predict alterations of the variables of microcirculation and in particular to MFI at admission in ICU. Although the study could not demonstrate a significant correlation between MR-proADM ando MFI at admission in ICU, the reduction of plasmatic levels of MR-proADM in the first 24 hours of intensive care treatment was associated with an improvement of MFI, that was more evident than in patients with reduced or no clearance of the biomarker. Patients in which MR-proADM cleared, showed a substantial stability of MFI towards the first five days and an improvement in SOFA score, while the opposite group suffered a deterioration of sublingual microcirculation in terms of MFI and showed a statistically higher SOFA score at day five.

Interestingly, most of the patients admitted to ICU showed deranged MFI, and this alteration was more evident in septic shock patients. This result is consistent with the main results of the MicroDAIMON study (Scorcella C et al), where the authors of our group reported an abnormal MFI on day 1 of admission in 20.6%, and in 55.7% of cases during ICU stay. [23]

In consideration to the limited half-life of MR-proADM, we could suggest that patients who showed enhanced clearance of MR-proADM could be considered as patients who controlled the inflammatory

source that triggered ADM's production (ratio 1:1 with MR-proADM) and in which the endothelial damage of microcirculation resolved (or was more controlled) if compared to the other group.

Although this is study a negative study, and the primary hypothesis is not satisfied, it may justify further studies that could evaluate if the trend of the plasmatic values of MR-proADM toward days could be able to relate to the evolution of microvascular dysfunction. MR-proADM may play a clinical role as biomarker in predicting the microvascular response to infection sepsis or septic shock.

Our results also support the hypothesis that MR-proADM is a valuable prognostic marker in critical care patients and that it may able to identify predicted non-survivors at admission in ICU. In fact, in our sample, MR-proADM at admission in ICU was associated with mortality and was correlated with SOFA scale as score of organ dysfunction. However, the relevance of this result is limited because the mortality of the sample was particularly low, impeding to reduce the bias in the main variable of outcome.

One of the main limitations of this study is the sample size that was not powered to control all the clinical features and variables that our patients presented, having wide different degrees of infection-related illness, from infection itself, to septic shock. Moreover, some of the patients presented sepsis related to viral origin in which MR-proADM performs within given limits, if considered just as single value (admission value) without examining a trend.

Lastly, the study was not correctly powered to evaluate trends of MR-proADM during ICU admission. Therefore, further studies in larger patient populations may be important to analyze these important points.

There are limited publications on the usefulness of MR-proADM in the field of critical care, while the majority of the studies analyzed isolated MR-proADM levels in the emergency department (ED) and at admission in ICU; we suggest this will be a field of interest.

Conclusions:

In our study, MR-proADM did not demonstrate a statistically significant correlation with the Microvascular Flow Index at admission in ICU, however it highlighted a good inverse correlation between the clearance of the biomarker and the improvement of the MFI in the first 24 hours of treatment. Moreover MRproADM performed as valuable prognostic biomarker, supporting previous literature on critical care infected patients. The clearance of MR-proADM could be a variable of interest for further studies and for a comprehensive evaluation of its correlation with the microvascular dysfunction.

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Table1: Descriptive of the study population. Median [iqr], n (%), AU.

SAPS II score = Simplified Acute Physiology Score; APACHE II score = acute physiology and chronic health evaluation; SOFA score = Sequential Organ Failure Assessment.

Age, years		70 [51-74]
Males, n (%)		13 (65)
SAPS II score, AU		52.50 [35.50-75.05]
APACHE II score, AU		19.50 [12.25-30.00]
SOFA score, AU		11 [8-14]
Source of infection, n (%)		
	Respiratory	13 (65)
	Abdominal	3 (15)
	Genito-urinary	2 (10)
	CNS	2 (10)
Septic shock, n (%)		10 (50)
Sepsis, n (%)		5 (25)
Infection without sepsis, n (%)		5 (25)
LOS in ICU, days		12.50 [9.00-16.75]
Mortality, n(%)		3 (15)

Table 2: Day 1 plasmatic values of MR-proADM and Procalcitonin. Median [iqr].

ICU= Intensive Care Unit; PCT= Procalcitonin.

At admission in ICU (day 1)		
	MR-proADM, *nmol/l*	**PCT, *ng/ml***
Infection, median [iqr]	1.44 [0.94-1.84]	1.20 [0.52-6.93]
Sepsis, median [iqr]	2.16 [1.35-4.00]	0.8 [0.59-3.73]
Septic shock, median [iqr]	3.99 [3.58-7.04]	25.95 [1.79-86.62]
Non-survivors, median [iqr]	8.86 [4.08-8-86]	79.10 [29.70-79.10]

Table 3: Descriptive of microvascular parameters at day 1, day 2 and day 5. Median {iqr]. MFI = Microvascular Flow Index of small vessels; TVDs Total small Vessel Density; PVDs= Perfused small Vessel Density; PPVs= Proportion of Perfused small Vessels.

	Day 1	**Day 2**	**Day 5**
MFIs, AU	2.83 [2.67-2.83]	2.92 [2.75-2.00]	2.75 [2.58-2.92]
TVDs, mm/mm2	21.20 [17.07-23.85]	18.98 [16.99-23.11]	20.31 [18.29-23.17]
PVDs, mm/mm2	20.10 [16.55-23.08]	18.90 [16.89-22.01]	18.73 [17.52-22.73]
PPVs, %	97.01 [95.53-98.06]	98.69 [96.28-99.38]	96.86 [95.31-98.71]
De Backer score, 1/mm	12.99 [10.29-13.96]	11.28 [10.18-13.27]	11.77 [11.14-13.83]

Figure 1: Percentage of variation for MFI of small vessels in the first 24 hours of evaluation, in the 2 groups of patients (clearance of MR-proADM inferior-to-equal or higher than 20%). P=0.005

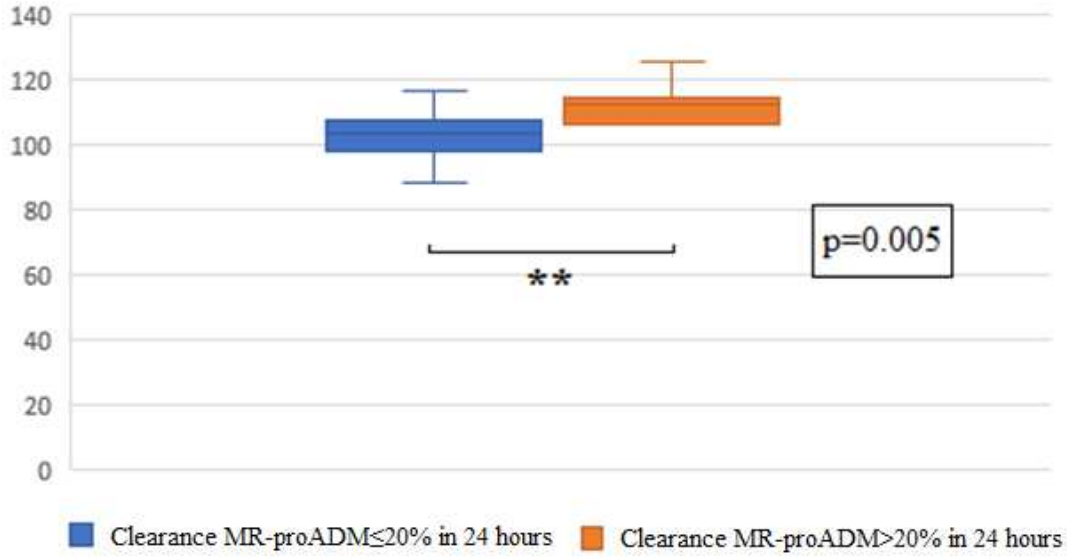
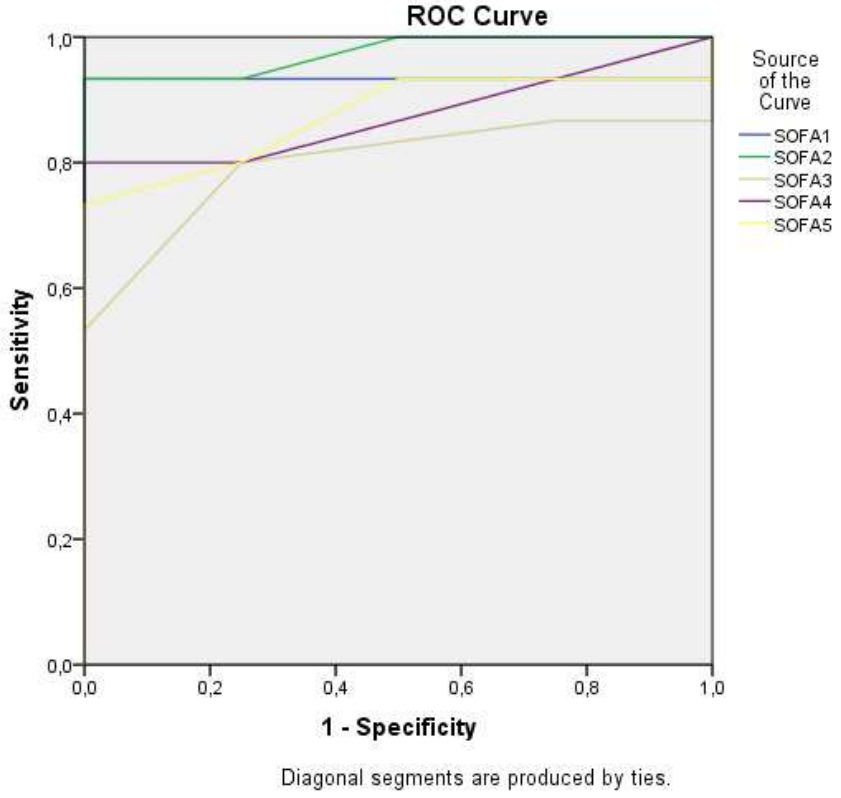


Figure 2: ROC curves. SOFA score day1-to-day5 in patients with MR-proADM >1.42 nmol/l at admission in ICU. SOFA-D1: AUC 0.933 [0.807-1], *p=0.009;* SOFA-D2: AUC 0.975 [0.911-1], *p=0.004;* SOFA-D3: AUC 0.8 [0.601-0.99], p=0.072; SOFA-D4: AUC 0.875 [0.718-1], *p=0.024;* SOFA-D5: AUC 0.875 [0.716-1], *p=0.024.* SOFA score = Sequential Organ Failure Assessment score.



Chapter 5

IgM-enriched immunoglobulins (Pentaglobin) may improve the microcirculation in sepsis: a pilot randomized trial.

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Abstract

Background: Polyclonal or IgM-enriched immunoglobulins may be beneficial during sepsis as an adjuvant immunomodulatory therapy. We aimed to test whether the infusion of IgM-enriched immunoglobulins improves microvascular perfusion during sepsis.

Methods: Single-centre, randomized, double-blind, placebo-controlled phase II trial including adult patients with a diagnosis of sepsis or septic shock for less than 24 hours. Patients received an intravenous infusion of 250 mg/kg (5 ml/kg) per day of IgM-enriched immunoglobulins (Pentaglobin, n=10) for 72 hours or placebo (NaCl 0.9%, n=9). At baseline and after 24 and 72 hours of infusion, the sublingual microcirculation was assessed with Incident Dark Field videomicroscopy. Thenar Near Infarared Spectroscopy (NIRS) was applied with a vascular occlusion test to assess tissue oxygenation and microvascular reactivity. Levels of interleukin (IL) 1-beta, IL-6, IL-8, IL-10 and tumor necrosis factor alpha were measured in the serum.

Results: The perfused vessel density (PVD) for small vessels (diameter <20 micron) increased in the Pentaglobin group (from 21.7±4.7 to 25.5±5.1 mm/mm^2) and decreased in the placebo group (from 25±5.8 to 20.7±4.1 mm/mm^2, p for interaction <0.001, two-way analysis of variance). The absolute between-group difference at 72 hours was 4.77 (standard error 2.34), p=0.140. The microvascular flow index for small vessels increased at 24 hours in the Pentaglobin group (from 2.68 [2.38-2.78] to 2.93 [2.82-3], p<0.01) and decreased at 72 hours in the placebo group (from 2.83 [2.60-2.97] to 2.67 [2.48-2.73], p<0.05). Changes in general parameters, cytokines and NIRS-derived parameters were similar between the two groups, except for IL-6 and IL-10 that significantly decreased at 72 hours only in the Pentaglobin group.

Conclusions: A 72-hour infusion of IgM-enriched immunoglobulins (Pentaglobin) in patients with sepsis or septic shock may be associated with an increase in sublingual microvascular perfusion. Further studies are needed to confirm our findings.

Background

Sepsis is a major healthcare problem, with high mortality and morbidity: even if some reports showed a decline in crude hospital mortality in the last decade [1], sepsis survivors remain at higher risk for infections, cardiovascular events, acute renal failure or the development of new physical disability or cognitive impairment [2]. At present, sepsis treatment is non-specific and mainly based on antibiotics and hemodynamic support [3].

Sepsis is characterized by a dysregulated host response to an infection, with uncontrolled activation of both pro- and anti-inflammatory pathways [4]. Increasing evidence suggests that a state of immunoparalysis is the main responsible for adverse outcome. A recent meta-analysis showed a significant reduction in circulating B cells and immunoglobulin M (IgM) levels in sepsis non-survivors as compared to survivors [5]. The administration of polyclonal or IgM-enriched immunoglobulins as an adjuvant immunomodulatory therapy gave encouraging results in both pre-clinical and clinical studies [6], although the evidence supporting a reduction in mortality is still too weak to justify a widespread use in septic patients [7]. The potential benefits of immunoglobulins (especially IgM-enriched preparations) are related not only to their anti-inflammatory activity (pathogen recognition and clearance, toxin scavenging, inhibition of inflammatory mediators production, cytokine neutralization, complement-scavenging properties) but also to their anti-apoptotic effects on immune cells [8]. Preclinical studies showed a potential role in the regulation of endothelial cell function, leukocyte adhesion and capillary perfusion [9, 10]. Nonetheless, no clinical studies exist that evaluated the microvascular effects of immunoglobulins in septic patients.

We hypothesized that the intravenous administration of IgM-enriched immunoglobulins in patients with sepsis as an adjunctive therapy could improve microvascular perfusion. This may result in better tissue oxygenation and preserved organ function. The primary goal of this study was to evaluate whether the infusion of IgM-enriched immunoglobulins was able to increase the sublingual perfused vessel density (PVD) after 72 hours as compared to a placebo. Secondary endpoints were parameters

of microcirculatory flow quality, peripheral (skeletal muscle) tissue oxygenation and microvascular reactivity.

Methods

This single-centre randomized, double-blind, placebo-controlled phase-II trial was conducted in the Intensive Care Unit of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona in Italy. The study protocol was approved by the local ethics committee (Comitato Etico Regionale Marche) and registered in www.ClinicalTrials.gov (Identifier: NCT02655133, date of registration 7th January 2016, https://www.clinicaltrials.gov/ct2/show/NCT02655133). Written informed consent was obtained before enrolment from all patients or their legal representatives in accordance with current Italian legislation. A deferred consent procedure was applied in case of temporary inability.

This manuscript adheres to the 2010 Consolidated Standards of Reporting Trials statement (please see the CONSORT checklist in Additional File 1).

Participants

We included adult (≥18 years old) patients with severe sepsis or septic shock according to the 2001 International Sepsis Definition Conference criteria [11], as the original study protocol was approved before the publication of the Sepsis-3 definitions [12]. Severe sepsis was defined by the presence of at least one sepsis-induced organ dysfunction; septic shock was defined as persistent hypotension despite adequate fluid resuscitation, requiring vasopressor infusion [11]. Nonetheless, the term "sepsis" (instead of severe sepsis) will be used hereafter, as the current concept of sepsis now includes the presence of an organ dysfunction induced by a dysregulated response to infection [12]. In addition, the term "septic shock" will refer to a condition of persistent arterial hypotension despite adequate fluid resuscitation and lactate levels >2 mmol/l, based on the current definition [12]. All patients were

enrolled within the first 24 hours of sepsis development. Exclusion criteria were: contraindications to immunoglobulin treatment; sepsis/septic shock for more than 24 hours; history of chronic renal failure; life expectancy <24 hours; lack of informed consent; pregnancy; factors impeding the sublingual microcirculation evaluation (recent oral surgery or maxillo-facial trauma); inclusion in other interventional studies. Patients with a history of chronic renal failure were excluded since previous studies showed a higher risk of osmotic-induced renal damage following intravenous immunoglobulins infusion in those with pre-existing renal insufficiency [13].

Interventions

Patients were randomly assigned to one of two study groups. Patients in the Pentaglobin group received 250 mg/kg (5 ml/kg) per day of IgM-enriched immunoglobulins (Pentaglobin, Biotest Pharma GmbH, Dreieich, Germany) as a continuous intravenous infusion for 72 hours. Patients in the placebo group received the same volume of normal saline solution (NaCl 0.9%) within a period of 72 hours. Saline solution was chosen as the placebo treatment as being an inert substance with no expected biological effect at the volume infused in this study. A simple randomization was performed by a pharmacist through sealed envelopes with a 1:1 allocation ratio. The study treatment or placebo were then provided by the Hospital Pharmacy in identical bottles masked in opaque green plastic bags: neither the attending physicians nor the investigators nor the patient were aware of the study group. All other therapies (including fluids and vasoactive agents) were provided according to individual needs and based on the Surviving Sepsis Campaign guidelines [3].

Measurements

All measurements were performed at baseline, 24 hours after starting Pentaglobin/placebo infusion and at the end of infusion (72 hours). Mean arterial pressure (MAP), heart rate (HR) and

norepinephrine requirements were recorded. Arterial and central venous blood samples were collected in order to measure blood gases, arterial lactate, haemoglobin (Hb), white blood cell (WBC) count, procalcitonin, interleukin (IL) 1 beta, IL-6, IL-8, IL-10, tumour necrosis factor alpha (TNF-α). The Simplified Acute Physiology Score (SAPS) II, Acute Physiology and Chronic Health Evaluation (APACHE) II score were obtained at admission and Sequential Organ Failure Assessment (SOFA) score at the study time-points.

The sublingual microcirculation was evaluated by means of Incident Dark Field (IDF) videomicroscopy (Cytocam, Braedius Medical, Amsterdam, The Netherlands). The Cytocam-IDF is a third-generation handheld microscope that enables real-time in vivo visualization of the microcirculation. It consists of an illumination unit based on IDF imaging with a × 4 magnification lens. The illumination light is emitted with a short pulse time of 2 ms (synchronized with a computer-controlled image sensor) and a wavelength of 548 nm, ensuring the highest absorption of oxyhemoglobin and deoxyhemoglobin, whereby flowing red blood cells are visible within the vessels as dark moving globules against a clear background [14]. After removal of secretions with a gauze, the probe was gently applied on the sublingual mucosa. At least 5 videos from different areas were recorded with adequate contrast and focus and all efforts were made to avoid pressure artifacts. The image quality was checked offline [15], and videos of inadequate quality were discarded. Three videos per time-point were analysed offline with a dedicated software (Automated Vascular Analysis 3.2, Microvision Medical, Amsterdam, NL) [16]. In brief, the image was divided by three equidistant horizontal and three equidistant vertical lines; the De Backer Score was calculated as the number of vessels crossing the lines divided by the total length of the lines. For each vessel crossing the lines, perfusion was categorized as continuous, sluggish, intermittent or absent. The percentage of perfused vessels (PPV) was estimated as follows: 100 * [(total number of grid crossings - [no flow + intermittent flow])/total number of grid crossings] [16]. The total vessel density (TVD) was calculated as the total length of vessels divided by the total area of the image. The perfused vessel density (PVD) was estimated by multiplying TVD by PPV as estimated with the De Backer method. The

microvascular flow index (MFI) was calculated semiquantitatively as described elsewhere [16]. The flow heterogeneity index (FHI) was also calculated as the highest MFI minus the lowest MFI, divided by the mean MFI, providing an index of heterogeneous microcirculatory perfusion [16]. The analysis was focused on small vessels (≤20 μ in diameter).

Near-infrared spectroscopy (NIRS) (InSpectra™ Model 650; Hutchinson Technology Inc., Hutchinson, MN, USA) with a 15 mm-sized probe was used to measure microvascular oxygen saturation (StO_2) and tissue Hb index (THI) on the thenar eminence at baseline and during a vascular occlusion test (VOT) [17].The StO_2 downslope (%/minute) was calculated as an index of oxygen consumption, while the StO_2 upslope (%/minute) and the area under the curve (AUC) of the hyperemic response were obtained to assess microvascular reactivity [17].

Sample size calculation

The sample size was calculated based on the primary outcome of the study, i.e. we hypothesized that the infusion of Pentaglobin would be able to induce an increase in the PVD at 72 hours. We calculated that the inclusion of 9 patients per group would be sufficient to detect a difference of at least 4 mm/mm^2 (standard deviation: 3 mm/mm^2) [18] between the two groups at 72 hours with a power of 80% and an alpha error of 0.05. Ten patients per group were enrolled in total to allow for dropouts.

Statistical analysis

This was performed using GraphPad Prism Version 6 (GraphPad Software, La Jolla, CA, USA) and IBM Statistical Package for Social Science version 21 (Armonk, NY: IBM Corp.). Normality of distribution was checked using the Kolmogorov-Smirnov test. The data were expressed as mean ± standard deviation (SD) for normally distributed variables or median [1st-3rd quartiles] for non-normally distributed variables. For normally distributed variables, we applied a two-way analysis of

variance (ANOVA) for repeated measures to test the effect of treatment (Pentaglobin versus Placebo) and time on the variables of interest. A Sidack's post hoc test was used for multiple comparisons. For non-normally distributed variables, the Mann-Whitney U test was applied to evaluate differences between the two groups at each time-point and the Friedman test with the Dunn's test for multiple comparisons was applied to evaluate differences between time-points in each group. Since we found significant inter-individual variability in microcirculatory parameters at baseline, we performed a secondary analysis by calculating the delta values (changes from baseline at 24 and 72 hours) in each group and performed an analysis of covariance (ANCOVA) for repeated measures to evaluate the interaction between the factors "time" and "treatment" by controlling for the baseline value of the outcome of interest (in order to correct for the "regression to the mean" phenomenon), with the Bonferroni post hoc test to assess differences between the two group at each time-point. The Pearson r (or the Spearman rho) was calculated to evaluate correlation between variables. A two-tailed $p<0.05$ was used to define statistical significance.

Results

From January 2016 to December 2017, 20 patients were enrolled in the study and randomized to receive Pentaglobin or placebo. One patient in the placebo group died 18 hours after randomization, leaving 19 patients in total for the final analysis (Figure 1).

Baseline characteristics for the two study groups are reported in Table 1.

Changes in sublingual microvascular and NIRS-derived parameters are shown in Figure 2 and Table 2. A Two-way ANOVA showed a significant interaction effect of treatment and time on the PVD with an F ratio of F (degree of freedom=2, error=34) = 9.84 ($p<0.001$). The Sidack's post hoc test showed that the PVD was increased at 72 hours in the Pentaglobin group ($p<0.05$ versus baseline),

while it was reduced in the Placebo group ($p<0.01$ versus baseline). The between-group comparison at 72 hours showed an absolute difference of 4.77 (standard error= 2.34), p=0.140 (Student's t test, p=0.039). A comparison of delta-values (adjusted for the baseline value) showed opposite variations of the PVD at 72 hours (Pentaglobin: +3.8 ±3.8, versus Placebo: -4.2 ±4.7, p=0.003, Additional File 2, Figure 3). The MFI was increased at 24 hours in the Pentaglobin group, while it decreased at 72 hours in the placebo group (Figure 2, Table 2) and the comparison of delta values showed divergent changes at 72 hours (+0.2 ±0.2 versus -0.2 ±0.2, p=0.035, Additional File 2). The PPV was higher in the Pentaglobin group as compared to the placebo group at 72 hours (Figure 2), however variations from baseline did not differ between the two groups (Additional File 2). An example of sublingual microcirculation before and 72 hours after Pentaglobin infusion is shown in Figure 4.

Two-way ANOVA showed significant effects of both time (with an F (2, 34) 6.29, p=0.005) and treatment (with an F (1, 17) 4.51, p=0.049) on the StO_2 upslope, with no significant interaction (p=0.681). Changes from baseline were similar between the two groups (Additional File 2). We found a significant effect of time on the Area of hyperemia with an F (2, 34) 7.19 (p=0.002), but variations over time did not differ between the two groups (Figure 2, Additional File 2). No other differences were observed for microvascular and NIRS-derived parameters.

The administration of Pentaglobin did not induce any significant variation in MAP or HR, while norepinephrine dosage was decreased in the placebo group at 72 hours (Table 3). A greater decrease in central venous O_2 saturation ($ScvO_2$) was found in the placebo group compared with the Pentaglobin group at 24 hours (Table 3). Changes in the other parameters and SOFA score were similar between the two groups. Similarly, changes in WBC count, procalcitonin and cytokine levels in the Pentaglobin group did not differ from those in the placebo group, although a significant decrease in IL-6 and IL-10 at 72 hours was only found in the Pentaglobin group (Table 4). ICU-mortality was similar between the two groups (20% in the Pentaglobin group and 22% in the placebo

group, p=0.999), as well as the ICU length-of-stay (19±13 days in the Pentaglobin group versus 16±12 days in the placebo group, p=0.649).

No correlation was found between changes (delta 72h-baseline) in PVDs and changes in MAP (Pearson r = -0.073, p=0.765), norepinephrine dose (r = 0.325, p=0.175), $ScvO_2$ (r = 0.171, p=0.483) and cytokine levels (Spearman rho for TNF-alpha = -0.125, p=0.610; Il-6 = -0.040, p=0.870; Il-10 = -0.146, p=0.552).

No unintended effects were reported for any of the two study groups.

Discussion

Microcirculatory dysfunction plays a key role in the pathogenesis of sepsis [19-22]. Persistent microcirculatory alterations during septic shock are associated with organ failure and death [23, 24]. In this single-centre randomized, double-blind, placebo-controlled phase II trial, we showed that a 72-hour infusion of IgM-enriched immunoglobulins (Pentaglobin) as an adjunctive therapy during sepsis may be associated with an increase in the sublingual microvascular density and blood flow quality. These changes did not correlate with variations in macro-hemodynamic parameters or cytokine levels. Although exploratory, these data would support a potential role of Pentaglobin therapy in favouring microvascular recruitment and tissue perfusion during sepsis.

A number of clinical studies suggested a beneficial effect of IgM-enriched immunoglobulins in sepsis, however the quality of the available evidence remains low [7]. The use of immunoglobulins was introduced with the rationale of modulating the inflammatory reaction and supporting the immune system in the fight against pathogens [25]. In septic pigs, the infusion of Pentaglobin was able to shift the inflammatory response toward an anti-inflammatory profile [26]. In experimental sepsis models, Pentaglobin normalized capillary perfusion at 24 hours by reducing venular leukocyte adhesion [10] and alleviated the histopathological injury in the lungs and small intestine [27, 28]. In

a rat model of pneumonia, IgM-enriched immunoglobulins enhanced the anti-inflammatory response by increasing blood IL-10 levels and reducing TNF-alpha in bronchoalveolar lavage fluid [29]. In this study, we failed to detect a clear impact of Pentaglobin on the cytokine profile. The heterogeneity of sepsis syndrome likely influenced the variation in cytokine levels. In addition, we could have missed changes in cytokines occurring earlier than the first 24 hours of treatment. Our study was not powered to detect changes in cytokine levels, which are extremely variable during sepsis.

Although it cannot be excluded that macro-hemodynamic changes unrelated to immunoglobulin therapy were responsible for the observed variations in the microcirculation, we could not find any correlation between changes in microvascular perfusion and variations of macro-hemodynamic parameters. While the infusion of Pentaglobin was able to improve the sublingual microcirculation without inducing any significant change in MAP or vasopressor dose, in the placebo group the PVD and MFI were reduced at 72 hours despite a decrease in norepinephrine requirements. This loss of coherence between the macro- and the microvascular responses has been described during sepsis and shock states [30] and emphasizes that targeting systemic hemodynamic parameters may not be sufficient to ensure an optimization of tissue perfusion.

Pentaglobin had no consistent impact on tissue oxygenation and microvascular reactivity as assessed by NIRS. Thenar NIRS with a VOT enables to investigate alterations in tissue oxygen delivery and consumption, and to test the microvascular reserve capacity following a short period of ischemia. Reduced StO_2 and slower StO_2 downslopes and upslopes are generally found during sepsis and are associated with worse outcome [31]. Nonetheless, NIRS shows alterations in peripheral (skeletal muscle) oxygenation and may not be sensitive enough to detect a hypoperfusion in inner organs, whereas the capillary perfusion of the sublingual mucosa is generally explored as a window to the splanchnic microcirculation [32].

Our study has several limitations. First, the small sample size carries a high risk of type-I error. The study may be underpowered to detect differences in some parameters (including cytokine levels).

Moreover, the two groups may be unbalanced for some baseline characteristics (e.g. norepinephrine dose and lactate levels). Therefore, our results should be considered as exploratory and need confirmation by future studies. No differences were observed in the SOFA score between the groups: unfortunately however, our study was not powered to detect differences in mortality or other major outcomes (organ failures, shock reversal, ICU length of stay). Second, the comparison of absolute PVD values at 72 hours (as per pre-planned statistical analysis) did not reach statistical significance. However, we believe that this is (at least partly) due to a between-group variability at baseline. In order to control for this confounder, we included a comparison of delta values (adjusted for baseline) that confirmed the different trend observed in the two groups. Third, most of the enrolled patients were already hemodynamically stable and less than 30% were in shock based on the Sepsis-3 definitions [12]. Consistently, we did not find severe microvascular alterations at baseline: an MFI <2.6 [16] was observed only in 5 patients out of 19 and the PPV was <90% only in 4 cases. As the infusion of Pentaglobin could produce a bigger effect in patients with more severe microcirculatory dysfunction, the presence of an altered microcirculation should be among the inclusion criteria in future studies. Fourth, we did not measure baseline immunoglobulin levels. The infusion of IgM-enriched immunoglobulins could have been more effective in patients with more severe hypo-IgG or hypo-IgM, who may represent the best target for this immunomodulatory therapy [33]. Lastly, data of cardiac output were not evaluated as available only for a small number of patients.

Conclusions

In this single-centre randomized, double-blind, placebo-controlled, phase II trial, a 72-hour infusion of IgM-enriched immunoglobulins (Pentaglobin) in patients with sepsis or septic shock was associated with an increase in sublingual microvascular perfusion. Given the small sample size, these results must be seen as exploratory and need to be confirmed by other studies.

List of abbreviations

IgM immunoglobulins M, *PVD* perfused vessel density, *MAP* mean arterial pressure, *HR* heart rate, *Hb* haemoglobin, *WBC* white blood cells, IL interleukin, *TNF-α* tumour necrosis factor alpha, *SAPS* Simplified Acute Physiology Score, *APACHE* Acute Physiology And Chronic Health Evaluation, *SOFA* Sequential Organ Failure Assessment, *IDF* Incident Dark Field, *TVD* total vessel density, *PPV* percentage of perfused vessels, *MFI* microvascular flow index, *FHI* flow heterogeneity index, *NIRS* near infrared spectroscopy, StO_2 tissue O_2 saturation, *THI* tissue haemoglobin index, *VOT* vascular occlusion test, *AUC* area under the curve, *ANCOVA* analysis of covariance, $ScvO_2$ central venous O_2 saturation.

Trial registration: NCT02655133, www.ClinicalTrials.gov, date of registration 7th January 2016, https://www.clinicaltrials.gov/ct2/show/NCT02655133.

Keywords: immunoglobulins; Pentaglobin; sepsis; microcirculation; immunomodulation

Declarations

Ethics approval and consent to participate

The study protocol was approved by the local ethics committee (Comitato Etico Regionale Marche) and registered in www.ClinicalTrials.gov (Identifier: NCT02655133, date of registration 7th January 2016). Written informed consent was obtained before enrolment from all patients or their legal representatives in accordance with current Italian legislation. A deferred consent procedure was applied in case of temporary inability.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RD and ED collected the data, performed the statistical analysis, interpreted the data and drafted the manuscript. EA designed the study and revised the manuscript. CS, AC, PG, EC, VG, SP, SC participated in the collection and analysis of the data. EL contributed to the study drug preparation and randomization procedure and revised the manuscript. AD designed the study, participated in the statistical analysis and interpretation of the data and revised the manuscript. All authors approved the

submitted version of the manuscript and agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Table 1 – Baseline characteristics.

Patient Demographics	Pentaglobin (n=10)	Placebo (n=9)	p
Age (years)	62±20	67±16	0.545
Gender (nr of males, %)	7 (70%)	8 (89%)	0.582
Comorbidities (n, %)			0.659
arterial hypertension	4 (40%)	3 (33%)	
Cardiopathy	1 (10%)	1 (11%)	
Diabetes	2 (20%)	1 (11%)	
Malignancy	3 (30%)	2 (22%)	
SAPS II (admission)	42±17	45±12	0.690
APACHE II (admission)	17±8	21±7	0.243
SOFA (admission)	9±4	10±3	0.663
Source of sepsis (n, %)			0.532
Abdominal	4 (40%)	2 (22%)	
Pulmonary	3 (30%)	2 (22%)	
uro-genital	1 (10%)	3 (33%)	
soft tissues	1 (10%)	2 (22%)	
Other	1 (10%)	0 (0%)	
Multi-drug resistant pathogen (n, %)	4 (40%)	5 (56%)	0.656
Shock* (n, %)	3 (30%)	2 (22%)	0.999

*Persistent arterial hypotension despite adequate fluid resuscitation and hyperlactatemia (lactate levels >2 mmol/l).

SAPS Simplified Acute Physiology Score, *APACHE* Acute Physiology and Chronic Health Evaluation, *SOFA* Sequential Organ Failure Assessment

Table 2 – Changes in sublingual microcirculation and NIRS-derived parameters.

	Baseline	24 hours	72 hours	p (time)[a]	p (interaction)[b]
PVDs (mm/mm^2)				0.869	<0.001
Pentaglobin (n=10)	21.7±4.7	23.4±6.0	25.5±5.1*		
Placebo (n=9)	25.0±5.8	23.8±4.4	20.7±4.1**		
MFIs (AU)					-
Pentaglobin (n=10)	2.68 [2.38-2.78]	2.93 [2.82-3.00]**	2.82 [2.65-2.95]	0.002	
Placebo (n=9)	2.83 [2.60-2.97]	2.93 [2.62-2.93]	2.67 [2.48-2.73]*	0.016	
TVDs (mm/mm^2)					-
Pentaglobin (n=10)	25.5 [17.9-27.1]	24.9 [18.7-27.6]	27.0 [19.3-29.6]	0.436	
Placebo (n=9)	26.6 [21.1-30.3]	25.2 [20.5-28.1]	22.5 [17.8-26.6]	0.154	
De Backer score (n/mm)				0.887	0.144
Pentaglobin (n=10)	13.1±2.2	13.3±3.4	14.2±2.7		
Placebo (n=9)	13.9±2.8	13.2±2.4	12.8±2.7		
PPVs (%)					-
Pentaglobin (n=10)	96 [87-98]	99 [98-100]	98 [97-100]	0.050	
Placebo (n=9)	98 [96-99]	98 [97-99]	96 [94-98]#	0.154	
FHI (AU)					-
Pentaglobin (n=10)	0.31 [0.16-0.45]	0.03 [0-0.13]	0.07 [0-0.18]	0.032	
Placebo (n=9)	0.18 [0.03-0.30]	0.07 [0.07-0.18]	0.11 [0.03-0.26]	0.495	
StO_2 (%)					-
Pentaglobin (n=10)	80 [79-84]	84 [79-89]	84 [77-88]	0.316	
Placebo (n=9)	85 [72-86]	81 [78-84]	84 [75-87]	0.658	
StO_2 Downslope (%/min)					-
Pentaglobin (n=10)	-8.4 [-10.8, -6.1]	-10.9 [-11.9, -8.2]	-11.5 [-16.3, -9.1]	0.367	
Placebo (n=9)	-8.0 [-14.2, -5.7]	-6.6 [-13.9, -6.1]	-10.2 [-12.3, -6.7]	0.813	
StO_2 Upslope (%/min)				0.005	0.681
Pentaglobin (n=10)	157±37	224±82**	207±77		
Placebo (n=9)	120±57	162±68	146±73		
Area of hyperemia (%*min)				0.002	0.544
Pentaglobin (n=10)	16.7±10.5	12.7±8.3	10.3±5.9*		
Placebo (n=9)	15.3±10.3	13.9±7.4	7.1±6.8*		
THI (AU)					-
Pentaglobin (n=10)	12 [9-15]	11 [8-12]	10 [8-14]	0.601	
Placebo (n=9)	11 [7-13]	10 [7-13]	8 [7-12]	0.654	

Data are expressed as mean ± standard deviation or median [1st-3rd quartile], as appropriate.

[a] Two-way analysis of variance for repeated measures (testing the effect of time) or Friedman test, as appropriate.

[b] Two-way analysis of variance for repeated measures (testing for the interaction between time and treatment), when applicable.

*p<0.05, **p<0.01 versus baseline, Two-way analysis of variance for repeated measures with Sidack's post hoc test or Friedman test with Dunn's post hoc test for multiple comparisons, as appropriate.

#p<0.05 versus Pentaglobin group, Mann-Whitney U test.

NIRS Near Infrared Spectroscopy, *PVD* perfused vessel density, *MFI* microvascular flow index, *TVD* total vessel density, *PPV* percentage of perfused vessels, *FHI* flow heterogeneity index, *StO2* tissue oxygen saturation, *THI* tissue haemoglobin index, *AU* arbitrary units

Table 3 – Changes in hemodynamic, blood gas parameters and organ function.

	Baseline	24 hours	72 hours	p (time)[a]	p (interaction)[b]
MAP (mmHg)				0.083	0.309
Pentaglobin (n=10)	80±14	82±9	86±15		
Placebo (n=9)	85±15	75±17	89±13		
HR (bpm)				0.662	0.169
Pentaglobin (n=10)	84±23	85±11	90±21		
Placebo (n=9)	95±19	89±30	80±25		
Norepinephrine tartrate (n, mcg/kg/min)					-
Pentaglobin (n=10)	8, 0.13 [0.03-0.39]	8, 0.12 [0.03-0.23]	7, 0.11 [0-0.38]	0.616	
Placebo (n=9)	8, 0.38 [0.23-0.70]	8, 0.50 [0.14-0.70]	6, 0.10 [0-0.40]*	0.004	
Arterial pH				0.110	0.377
Pentaglobin (n=10)	7.43±0.07	7.42±0.06	7.45±0.07		
Placebo (n=9)	7.45±0.03	7.47±0.05	7.49±0.07		
PaO2/FiO2 (mmHg)				0.875	0.474
Pentaglobin (n=10)	300±89	311±151	320±89		
Placebo (n=9)	311±89	280±74	262±85		
ScvO2 (%)				<0.001	0.053
Pentaglobin (n=10)	80±5	78±10	72±9*		
Placebo (n=9)	79±9	68±9**#	71±7*		
Base Excess (mEq/l)				<0.001	0.659
Pentaglobin (n=10)	0.4±4.9	2.5±3.8	5.6±4.9**		
Placebo (n=9)	1.2±4.7	4.0±4.8	5.1±4.4*		
Arterial Lactate (mmol/l)					-
Pentaglobin (n=10)	1.7 [1.3-3.5]	1.5 [1.0-2.2]	1.9 [0.9-2.7]	0.682	
Placebo (n=9)	1.6 [1.0-2.2]	1.4 [1.0-2.2]	1.2 [1.1-1.7]	0.755	
Hemoglobin (g/dl)				0.460	0.356
Pentaglobin (n=10)	10.4±1.5	10.5±0.9	9.6±0.9		
Placebo (n=9)	9.8±1.8	10.1±1.3	10.0±1.1		
Platelets (*10^3/mmc)				0.170	0.036
Pentaglobin (n=10)	158±98	141±80	153±83		
Placebo (n=9)	163±86	168±84	136±67		
Creatinine (mg/dl)				0.284	0.522
Pentaglobin (n=10)	1.1±0.7	1.0±0.5	0.9±0.4		
Placebo (n=9)	1.9±1.0	2.1±1.6	1.8±1.2		
Bilirubin (mg/dl)					-
Pentaglobin (n=10)	0.7 [0.5-1.7]	0.8 [0.5-1.6]	0.9 [0.5-1.7]	0.356	
Placebo (n=9)	1.0 [0.5-1.3]	0.9 [0.5-2.3]	0.7 [0.6-2.4]	0.515	
Glasgow Coma Scale					-

Pentaglobin (n=10)	14 [13-15]	15 [13-15]	15 [11-15]	0.999	
Placebo (n=9)	14 [10-14]	13 [9-14]	12 [10-14]	0.999	
SOFA score					-
Pentaglobin (n=10)	9 [7-11]	9 [5-10]	7 [6-8]	0.229	
Placebo (n=9)	10 [7-13]	12 [6-12]	10 [6-12]	0.544	
Propofol (mg/kg/h, n)					-
Pentaglobin (n=10)	1.2 [0-2.4], 7	0 [0-2.6], 4	0 [0-2.6], 4	0.790	
Placebo (n=9)	0.8 [0-1.6], 5	0 [0-1.1], 4	0 [0-1.6], 3	0.518	
Remifentanil (mcg/kg/min, n)					-
Pentaglobin (n=10)	0.08 [0.04-0.09], 8	0.05 [0-0.08], 7	0.06 [0-0.10], 7	0.366	
Placebo (n=9)	0.06 [0-0.10], 8	0.05 [0-0.08], 7	0.06 [0-0.10], 7	0.991	

Data are expressed as mean ± standard deviation or median [1st-3rd quartile], as appropriate.

[a] Two-way analysis of variance for repeated measures (testing the effect of time) or Friedman test, as appropriate.

[b] Two-way analysis of variance for repeated measures (testing for the interaction between time and treatment), when applicable.

*p<0.05, **p<0.01 versus baseline, Two-way analysis of variance for repeated measures with Sidack's post hoc test or Friedman test with Dunn's post hoc test for multiple comparisons, as appropriate.

#p<0.05 versus Pentaglobin group, Two-way analysis of variance for repeated measures with Sidack's post hoc test or Mann-Whitney U test, as appriopriate.

MAP mean arterial pressure, *HR* heart rate, $ScvO_2$ central venous oxygen saturation, *SOFA* Sequential Organ Failure Assessment

Table 4 – White blood cells, procalcitonin and cytokine levels.

	Baseline	24 hours	72 hours	p (Friedman test)
White Blood Cell count (n/mmc)				
Pentaglobin (n=10)	9070 [5560-18660]	10710 [6260-13790]	10100 [7388-11720]	0.763
Placebo (n=9)	12000 [6145-23300]	12560 [8185-25090]	12990 [7275-26220]	0.569
Procalcitonin (ng/ml)				
Pentaglobin (n=10)	14.4 [3.4-48.6]	14.6 [4.8, 28.8]	7.1 [3.2-14.9]*	0.026
Placebo (n=9)	20.0 [4.5-95.1]	19.7 [4.6-79.9]	5.2 [2.4-33]**	<0.001
Interleukin-1 Beta (pg/ml)				
Pentaglobin (n=10)	5.3 [4-12.8]	4.5 [4-6.7]	4 [4-5.6]	0.057
Placebo (n=9)	4 [4-5.6]	4 [4-5]	4 [4-5.8]	0.376
Tumor Necrosis Factor Alpha (pg/ml)				
Pentaglobin (n=10)	32 [20-84]	18 [14-38]	16 [12-28]	0.078
Placebo (n=9)	30 [24-77]	39 [25-51]#	19 [16-42]	0.010
Interleukin-6 (pg/ml)				
Pentaglobin (n=10)	350 [104-1418]	166 [61-781]	151 [41-296]*	0.030
Placebo (n=9)	212 [52-971]	98 [36-217]	69 [21-141]	0.154
Interleukin-8 (pg/ml)				
Pentaglobin (n=10)	138 [52-1268]	74 [29-177]	75 [49-108]	0.262
Placebo (n=9)	146 [66-302]	62 [32-79]	57 [45-115]	0.278
Interleukin-10 (pg/ml)				
Pentaglobin (n=10)	30 [10-118]	9 [5-15]*	8 [6-13]**	0.003
Placebo (n=9)	20 [8-71]	19 [6-29]	10 [6-13]	0.685

Data are expressed as median [1st-3rd quartile].

*p<0.05, **p<0.01 versus baseline, Friedman test with Dunn's test for multiple comparisons

Figure 1 – Study flow chart.

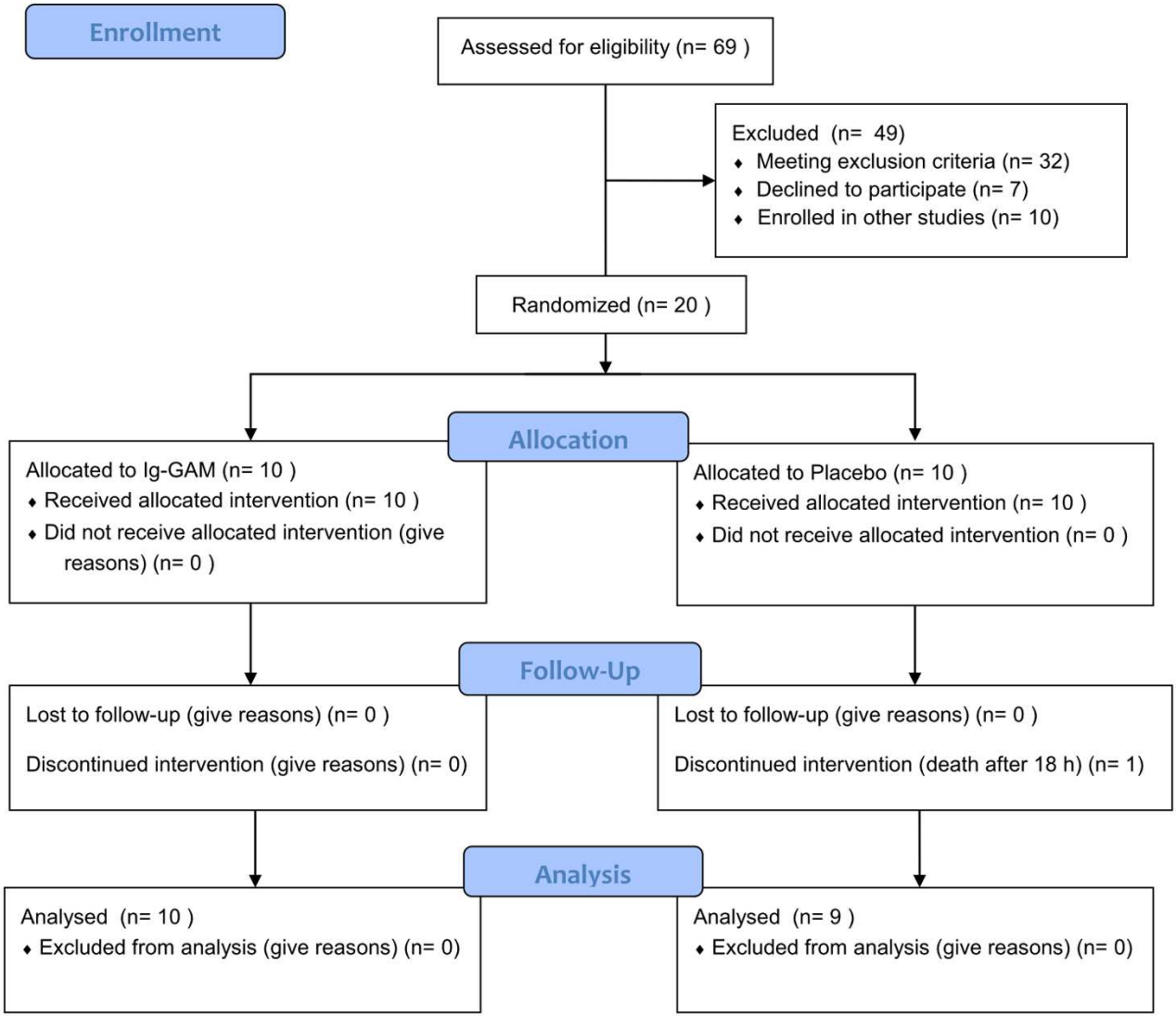


Figure 2 – Comparison of microcirculatory and NIRS-derived parameters.

Data are expressed as mean and standard deviation of median [interquartile range], lines indicate individual changes. *p<0.05, **p<0.01 versus baseline, Two-way ANOVA for repeated measures with Sidack's post hoc test or Friedman test with Bonferroni post hoc test, as appropriate. #p<0.05 versus placebo, Two-way ANOVA for repeated measures with Sidack's post hoc test or Mann-Whitney U test, as appropriate.

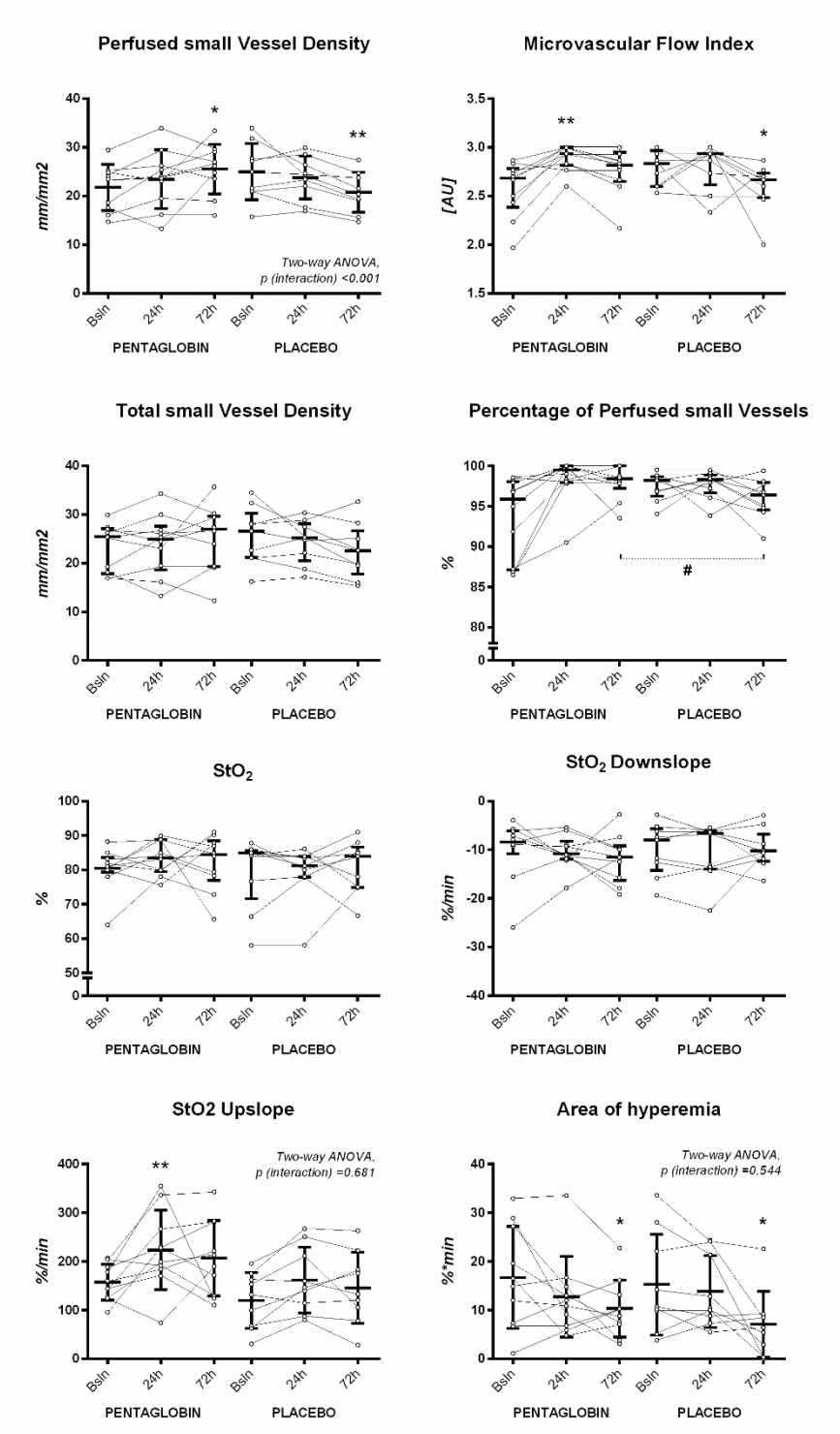


Figure 3 – Comparison of the delta values (variations from baseline) for the perfused vessel density.

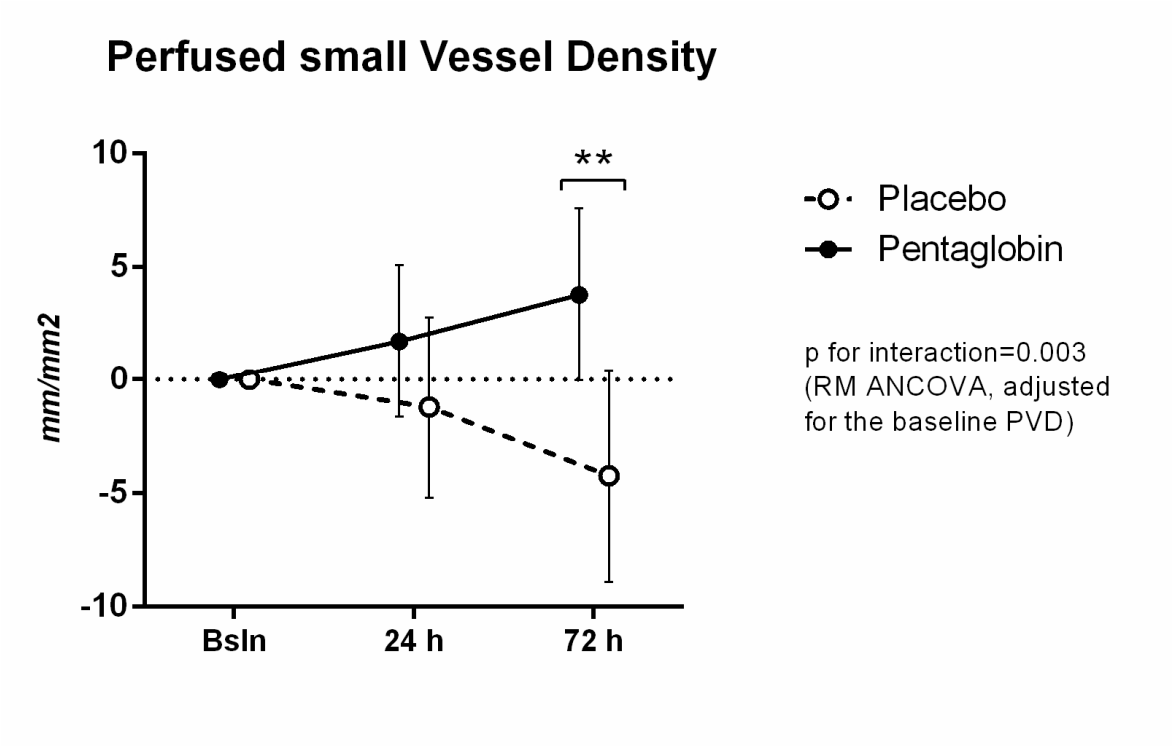
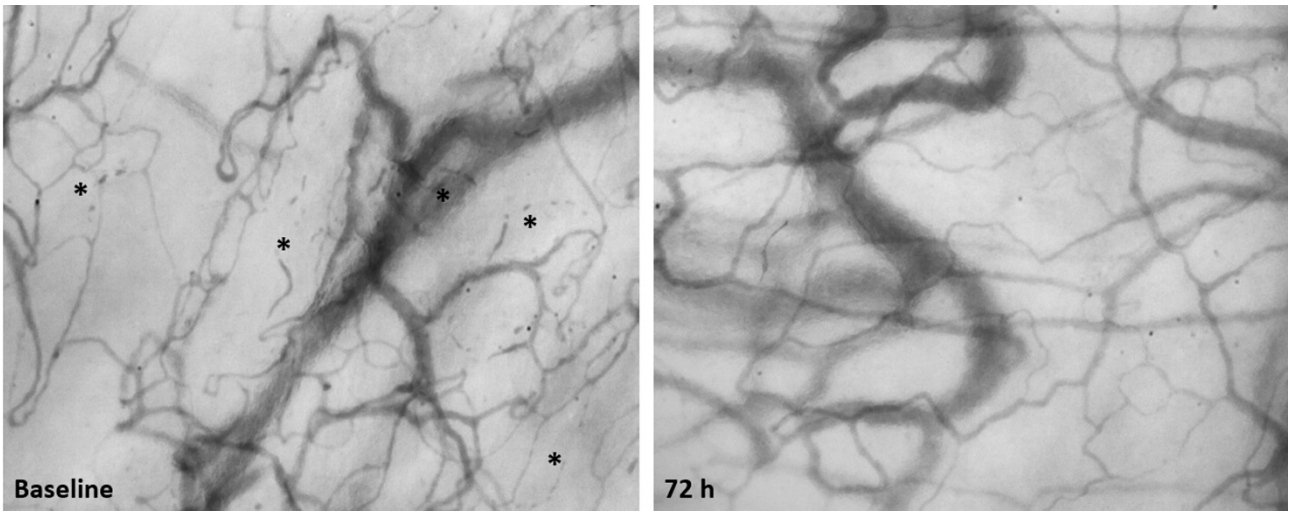


Figure 4 – Images of the sublingual microcirculation of a patients at baseline and after 72 hours of Pentaglobin infusion.

Non-perfused vessels are indicated with stars.



Additional File 1 (.pdf) - 2010 Consolidated Standards of Reporting Trials (CONSORT) checklist.

Additional File 2 (.doc) - Comparison of changes in sublingual microcirculation and NIRS-derived parameters.

Chapter 6

Changes in cytokines, haemodynamics and microcirculation in patients with sepsis/septic shock undergoing continuous renal replacement therapy and blood purification with CytoSorb

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Keywords: blood purification; cytokines; haemoadsorption, sepsis; septic shock; sorbents; sublingual microcirculation.

Abstract

Background: Extracorporeal blood purification therapies have been proposed as a strategy to remove inflammatory mediators during sepsis, thus improving outcome.

Objectives: We aimed to evaluate changes in cytokines, haemodynamics and microcirculation during blood purification with Cytosorb adsorber in septic patients.

Methods: Prospective observational study on critically ill adult patients with sepsis/septic shock underwent renal replacement therapy (RRT) for acute renal failure and haemoadsorption with Cytosorb as adjunctive therapy for 24 hours. Measurements were taken at baseline, after 6 and 24 hours: haemodynamic parameters, arterial and central venous blood gases, plasma levels of tumor necrosis factor-α, interleukin (IL) 1-beta, IL-6, IL-8 and IL-10. The sublingual microcirculation was assessed with sidestream dark field videomicroscopy to evaluate the perfused vessel density (PVD) and microvascular flow quality. Tissue oxygenation and microvascular reactivity were assessed with thenar near infrared spectroscopy (NIRS) with a vascular occlusion test.

Results: 9 patients; plasma levels of IL-8 decreased at 24 hours ($p<0.05$ versus 6 hours); no significant variation was found for other cytokines. Haemodynamic remained stable throughout the observation. Microvascular perfusion improved over time, with an increase in PVDs at 6 and 24 hours (from 13.9 [13.3-16.4] to 15.7 [15-17.3] and 17 [14.8-18.6] mm/mm^2 respectively, $p=0.003$) and TVDs at 24 hours (14.9 [13.9-16.9] vs 17.9 [15.3-20], $p=0.0015$). No significant variation was detected in NIRS-derived parameters. The Sequential Organ Failure Assessment score decreased from 12±3 to 10±1 at 24 hours ($p=0.039$).

Conclusions: In septic patients undergoing RRT, haemoadsorption with Cytosorb seems to determine a decreasing in plasma levels of IL-8, although levels of other cytokines did not vary significantly, and an improving of microcirculation despite no significant variation in macro-haemodynamics.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. This involves an early activation of pro- and anti-inflammatory pathways, with a dual mechanism of inducing both cytokine-mediated cell damage and a state of severely impaired immunity (immunoparalysis) [2]. Systemic inflammation is the leading cause of microcirculatory alterations observed during sepsis, which play a key role in the pathogenesis of tissue hypoperfusion and organ dysfunction [3]. Pro-inflammatory cytokines are the main responsible for leukocyte activation, oxidative stress, endothelial glycocalyx dysfunction and impaired nitric oxide pathway, resulting in reduced red blood cell deformability and impaired hemorheology, loss of microvascular tone, microcirculatory shunting, tissue oedema and oxygen extraction deficit [4, 5].

Extracorporeal blood purification therapies have been proposed as a strategy to improve outcome of septic patients, attenuating the systemic expression of pro- and anti-inflammatory mediators and restoring the immune homeostasis [6]. Among these techniques, haemoadsorption places sorbents in direct contact with blood in an extracorporeal circuit: solutes are attracted by the sorbent through hydrophobic interactions, ionic attractions, hydrogen bonding and Van der Waals interactions [6]. Haemoadsorption acts through a non-specific removal of a broad spectrum of inflammatory mediators, which can also include microbial toxins [7]. The CytoSorb ® technology (CytoSorbents Corporation, Monmouth Junction, NJ, USA) is based on cartridges containing biocompatible polystyrene divinyl benzene copolymer beads that can remove several cytokines both *in vitro* and *in vivo* [8]. Preclinical data in rat models of sepsis are encouraging, showing a reduction in circulating cytokines, increased blood pressure and improved short-term survival with CytoSorb haemoadsorption [9].

We hypothesize that haemoadsorption with CytoSorb used as an adjunctive therapy during sepsis, by removing circulating inflammatory mediators, may contribute to restore microvascular perfusion.

Materials and methods

This prospective observational study was approved by the local Ethics Review Committee (Comitato Etico Regione Marche, reference number CE150164; NCT03456180, www.clinicaltrials.gov). Written informed consent was obtained from the patients or their next of kin. All the adult patients admitted to the 14-bed medical-surgical Intensive Care Unit of Azienda Ospedaliera Ospedali Riuniti of Ancona (Italy) between December 2016 and December 2017 with diagnosis of sepsis or septic shock following the Sepsis-3 definition [1] and requiring continuous renal replacement therapy (CRRT) for acute kidney failure were eligible to participate. Exclusion criteria were: hypersensitivity or allergy to any component of the CytoSorb adsorber; pregnancy; factors impeding the evaluation of the sublingual microcirculation (oral surgery or maxillo-facial trauma); indication to undergo a blood purification technique other than haemoadsorption with CytoSorb; enrolment in another trial evaluating the microcirculation and lack of informed consent.

Patients were managed according to the 2016 Surviving Sepsis Campaign guidelines [10]. Acute renal failure requiring CRRT had to be present despite maximum standard therapy including adequate fluid resuscitation and norepinephrine administration targeted to achieve a mean arterial pressure (MAP) >65 mmHg [10]. CRRT was performed in continuous veno-venous haemodialysis (CVVHD) mode with citrate-based anticoagulation. The CytoSorb adsorber was integrated in the CVVHD circuit. Blood flow rates (100-150 ml/min) and dialysis doses (20-30 ml/kg/h) were applied according to standard care. All patients had a hemodynamic monitoring performed with transpulmonary thermodilution technique with a PICCO2 monitoring device (Pulsion Medical Systems, Munich, Germany).

Measurements:

Measurements were taken at baseline and after 6 and 24 hours after the beginning of treatment with CytoSorb. These included heart rate, MAP, arterial and central venous blood gases, haemodynamic variables, vasoactive drugs doses. Arterial blood samples were collected at each time point in order to assess plasma levels of interleukin (IL) 1-beta, IL-6, IL-8, IL-10 and tumor necrosis factor alpha

(TNF-alpha). Procalcitonin levels were measured at baseline and after 24 hours. The Sequential Organ Failure Assessment (SOFA) score was calculated at baseline and after 24 hours.

Evaluation of microvascular perfusion:

The sublingual microcirculation was evaluated at each timepoint with sidestream dark field (SDF) videomicroscopy (Microscan, Microvision Medical, Amsterdam, NL). Details on the SDF imaging technique have been extensively described elsewhere [11]. Three videos of adequate quality were selected for each timepoint and analysed offline with the Automated Vascular Analysis software version 3.2 (Microvision Medical, Amsterdam, NL). The following parameters were obtained for small vessels (diameter ≤20 micro meters) as described elsewhere [12]: total vessel density (TVDs), perfused vessel density (PVDs), percentage of perfused vessels (PPVs) and microvascular flow index (MFIs).

Tissue oxygenation and microvascular reactivity:

At each timepoint Near-infrared spectroscopy (NIRS) (InSpectra^{TM} Model 650, Hutchinson Technology Inc, Hutchinson, MN, USA) was performed with a 15-mm sized probe to measure peripheral muscle tissue oxygen saturation (StO2) and tissue haemoglobin index on the thenar eminence [13] before and during a vascular occlusion test [14]. This was performed by inflating a sphygmomanometer cuff placed on the forearm to 50 mmHg above the systolic blood pressure. Arterial inflow was thus arrested until the StO2 decreased to 40%. StO2 was recorded during the ischemic and the reperfusion phases until stabilization [14]. The StO2 downslope was calculated from the regression line of the first part of StO2 decay after occlusion, providing an index of tissue oxygen extraction rate [14]. The StO2 upslope during the reperfusion phase and the area under the curve of the hyperaemic response (area of hyperaemia) were calculated as indices of microcirculation reactivity [14].

Statistical analysis:

Statistical analysis was performed using Graphpad Prism 6 (GraphPad Software, La Jolla, CA, USA). The data distribution was explored using a Kolmogrov-Smirnov test. Data were expressed as mean

and standard deviation for normally distributed variables or median and interquartile range for non-normally distributed variables. One-way analysis of variance for repeated measurements with Bonferroni post-hoc test or the Friedmann test with Dunn's post hoc test for repeated measurements test were used as appropriate to evaluate changes over time. A paired t test or the Wilcoxon test were used as appropriate to evaluate changes between two time points. The alpha level of significance was set a priori at 0.05. We calculated a sample size of 10 patients, sufficient to detect a 20% increase in the PVDs after 24 hours of treatment with a power >80% and an alpha error of 0.05.

Results

A total of 446 patients were screened in total. Ten patients (5 males and 5 females) were enrolled. Plotting clinical and laboratory variables, one out of ten patients was discarded for the final analysis because acting as an outlier, resulting in a sample of 9 patients. Mean age was 63±18 years. The mean Simplified Acute Physiology (SAPS) II score was 62[24-71]. The sources of infection were respiratory (2 cases), abdominal (2 cases), soft tissues (2 cases), urinary tract (2 cases), mediastinitis (1 case). Microbiological details are shown in Supplemental Material 1.

The SOFA score was 12±3 at baseline and decreased significantly to 10±1 after 24 hours of treatment with CytoSorb (p=0.039).

No significant change was observed over time in any haemodynamic or blood gas variables during the observation (Table 1). All patients required norepinephrine infusion throughout the 24-hour study period and no significant reduction in norepinephrine dosage were observed over time.

Changes in plasma levels of cytokines are shown in Fig 1. A significant decrease was only found for IL-8 from 6 hours to 24 hours of CytoSorb treatment, while levels of the other cytokines did not vary significantly. Procalcitonin levels tended to decrease after 24 hours of treatment (34[6-157] vs 6[3-176] ng/ml, p=0.074).

All sublingual microvascular variables tended to improve over time and a significant increase was found for PVDs after 6 and 24 hours of treatment (13.9 [13.3-16.4] vs 15.7 [15-17.3] and vs 17 [14.8-

18.6] respectively, p=0.003) and for TVDs after 24 hours (14.9 [13.9-16.9] vs 17.9 [15.3-20], p=0.0015) as shown in Fig 2. NIRS-derived variables did not change significantly over time (Table 2).

Discussion

This small pilot prospective observational study in nine septic patients undergoing CVVHD and a 24-hour treatment with CytoSorb, showed a reduction in IL-8 plasma levels, whereas TNF-alpha, IL-1beta, IL-6 and IL-10 did not decrease significantly. Haemodynamic parameters and vasopressor requirement remained substantially stable during the 24-hours treatment; nonetheless an increase in sublingual microcirculatory density was observed and microvascular flow quality tended to improve over time.

Given the central role of the overwhelming systemic inflammation in the pathophysiology of sepsis-induced organ failure, several attempts have been recently made to develop therapies aimed to damper the cytokines storm. Nonetheless, specific antagonists for pro-inflammatory cytokines and treatments designed to control the early cytokines activation during sepsis failed to improve outcome [2]. Blood purification therapy is a non-selective and broad-spectrum strategy able to remove both pro- and anti-inflammatory mediators from the bloodstream, thus restoring the immune homeostasis [15]. In addition, by performing a concentration-dependent removal of molecules, blood purification acts as a self-tailored therapy. Haemoadsorption with CytoSorb was able to reduce plasma levels of IL-6 and IL-10, increase blood pressure and improve survival in rat models of sepsis [8, 9]. In recent years, multiple reports described a successful use of CytoSorb treatment in different disease categories, including patients with non-infectious systemic inflammatory response [16], acute respiratory distress syndrome [17] or those undergoing cardiopulmonary bypass for cardiothoracic surgery [18-21]. A number of case reports and case series suggested a beneficial effect of CytoSorb during sepsis, with attenuation of the inflammatory reaction, haemodynamic stabilization and potential survival benefit

[22-24]. In refractory septic shock, the use of CytoSorb as a rescue therapy was associated with improved lactate clearance and shock reversal in two-thirds of patients [25].

In this study, a significant reduction in plasma levels of IL-8 during the 24-hour CytoSorb treatment was detected. However, levels of the other measured cytokines remained substantially unaltered; a non-significant trend towards a reduction was observed for TNF-alpha. These results seem to contrast with data from some preclinical studies [8, 9] and clinical reports [21]. In a multicentre randomized controlled trial in patients with severe sepsis/septic shock, haemoadsorption with CytoSorb was able to remove IL-6 with a 5-18% elimination per blood pass through the filter (calculated by dosing IL-6 levels from the arterial [inlet] and the venous [outlet] blood), however this did not lead to lower plasma IL-6 levels in treated patients as compared to controls [26]. The stability of plasma cytokines levels despite effective extraction from the blood may be explained by a cytokines' shift from the interstitium into the blood compartment (cytokinetic theory [27]), exacerbated by a continuous production during the treatment period. Unfortunately, the elimination rate of cytokines in order to demonstrate the effective removal of the haemoadsorption device, has not been measured in the present study. Moreover, the inability of this study to reproduce the effectiveness of Cytosorb in reducing cytokines levels could suggest other positive effects of this filter on septic patients leading to a microvascular improvement. Peng et al already postulated a different potential beneficial effect of sorbents, other than cytokines removal, in experimental model of sepsis. [28] A plausible hypothesis is the capability of Cytosorb to remove free haemoglobin, a well-known nitric oxide scavenger which can induce vasoconstriction and a reduction in microvascular density [29].

All the enrolled patients had sepsis/septic shock with high SAPS II and SOFA scores. They received standard resuscitation therapies according to current guidelines [10] including antibiotics, fluids, vasopressors and/or inotropes before enrolment. The indication for CRRT was based on persisting acute renal failure with oliguria/anuria despite maximal treatment and CytoSorb adsorber was applied as a rescue therapy. Initial haemodynamic stabilization had been achieved in most patients at enrolment, as indicated by a baseline MAP ≥65 mmHg in eight patients out of nine and a central

venous O2 saturation >70% in eight patients out of nine. Despite this, microvascular perfusion was significantly impaired at the time of enrolment, with seven patients out of nine showing an MFIs <2.6, a cut off already used in previous study to identify patients with microvascular impairment [12, 30]. This may reflect a loss of haemodynamic coherence, a condition that frequently occurs during sepsis and is a predictor of adverse outcome [31]. During the 24-hour treatment with CVVHD and CytoSorb, haemodynamic parameters remained stable, as well as vasopressor and/or inotrope requirements. Nonetheless, microcirculatory alterations were attenuated over time, with a significant increase in microvascular density and a trend towards an improvement in blood flow quality. These findings support the idea that extracorporeal cytokines removal during sepsis may produce beneficial effects by preventing (or ameliorating) microvascular dysfunction. David et al. evaluated the effects of extracorporeal cytokines removal on vascular barrier integrity *in vitro* using human umbilical vein endothelial cells (HUVECs): alterations in endothelial morphology and function were prevented when HUVECs were challenged with serum from a septic shock patient collected after CytoSorb treatment, suggesting a protective effect [32]. The use of a CytoSorb adsorber in an isolated kidney perfusion system was able to improve mean renal blood flow and reduce the inflammatory response due to ischemia/reperfusion injury, although this did not lead to better renal function [33]. The improvement in microcirculatory perfusion in the present study was not accompanied by a significant decrease in lactate levels during the treatment, although substantial inter-individual variability was seen. We cannot exclude that a longer follow-up period was necessary to detect improvements in lactate clearance as a sign of restored aerobic metabolism, as well as to record a reduction in norepinephrine requirement.

No variations in NIRS-derived parameters that could reflect changes in tissue oxygenation or microvascular reactivity during CytoSorb treatment were observed. This discrepancy with the improvement seen sublingually may be explained by physiological differences between the two microvascular beds examined. The sublingual mucosa has been selected for the assessment of microvascular blood flow as an easy-accessible that shares the embryologic origin with the gut, and

is thus seen as a possible window to splanchnic microcirculatory perfusion [34], although a dissociation with the gut microcirculation was found in some studies [35, 36]. On the contrary, thenar-NIRS evaluates peripheral oxygenation in the skeletal muscle tissue, which is likely to respond differently to cardiovascular insults or vasoactive stimuli such as norepinephrine infusion [14].

The study has several limitations. First, the observational design and the lack of a control group prevent to demonstrate any cause-effect relationship between CytoSorb treatment and microvascular changes: we cannot exclude that the improvement in microcirculatory perfusion was a result of other treatments. Second, the small sample size may have been responsible for a type-II error: the study may be underpowered to detect significant changes in some variables. However, this was conceived as an exploratory observational study aimed to detect any improvement in the microcirculation during haemoadsorption therapy in sepsis and these findings warrant further investigations. Third, a longer follow-up period could have been required to observe changes in some variables, such as haemodynamic parameters and arterial lactate. Lastly, the CytoSorb filter elimination rate of cytokines by sampling blood from the arterial and venous branches of the circuit, in order to verify the methodological efficacy of the technique, was not assessed.

Concluding, even with some limitations, the present pilot study suggests a potential beneficial effect of treatment with CytoSorb on microcirculatory perfusion of septic patients going beyond the cytokines' removal. These preliminary data warrant further investigations to clarify the mechanisms of this effect in a wider setting.

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Statement of ethics

This study was conducted ethically in respect of the principles of World Medical Association Declaration of Helsinki and was approved by the local Ethics Review Committee (Comitato Etico Regione Marche, reference number CE150164; registered in ClinicalTrials.gov with the number NCT03456180, www.clinicaltrials.gov). Written informed consent was obtained from all the enrolled patients or their next of kin.

Disclosure statement

The authors have no conflicts of interest to declare.

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Authors contribution

SZ, AD, ED, CS, RD designed the study, contributed to the interpretation of the results and critically revised the manuscript. RD and ED performed the statistical analysis, drafted the manuscript, and interpreted the data. SZ, CS, RD, SV made a substantial contribution to the acquisition of the data and the analysis of SDF videos and revised the manuscript for important intellectual content. MD, SP, AR, AC, EA made a substantial contribution in the study design, critically revised the manuscript for important intellectual contents. EC gave an important contribution in statistical revision and data interpretation. All authors had full access to the data, take responsibility for the integrity of the data and the accuracy of the analysis, and have read and approved the final manuscript.

Table 1 – Haemodynamic and blood gas variables.

All data are reported as median [1st-3rd quartile]. *Friedman test; corrected p for repeated measures significance <0.017.

	Baseline	6 hours	24 hours	p*
Heart rate (bpm)	86 [74-94]	98 [74-107]	79 [75-97]	0.143
Mean arterial pressure (mmHg)	70 [65-80]	75 [71-85]	73 [65-79]	0.569
Cardiac Index (L/min/m^2)	3.62 [2.48-4.05]	3.45 [2.25-4.58]	3.13 [2.67-3.52]	0.971
Global End Diastolic Index (ml/m^2)	861 [655-1002]	746 [643-964]	763 [650-977]	0.531
Systemic Vascular Resistance Index (dynes*sec/cm^5/m^2)	1690 [1413-2008]	1824 [1255-2450]	1922 [1562-2081]	0.813
Extravascular Lung Water Index (ml/kg)	14 [8-19]	11 [9-13]	11 [9-16]	0.734
Norepinephrine dose (mcg/kg/min)	0.50 [0.16-0.87]	0.50 [0.18-1.25]	0.50 [0.11-1.05]	0.078
Dobutamine dose (mcg/kg/min, nr of patients)	3 [1-4.53], 5	3.33 [2.5-4.53], 5	3.57 [2.66-4.5], 5	0.999
pH	7.38 [7.35-7.40]	7.41 [7.35-7.48]	7.43 [7.30-7.51]	0.118
PaO_2 (mmHg)	98 [75-116]	106 [93-157]	116 [83-128]	0.741
$PaCO_2$ (mmHg)	38 [33-44]	40 [34-41]	38 [33-40]	0.813
PaO_2/FiO_2 (mmHg)	218 [163-305]	238 [224-363]	270 [248-372]	0.187
Base excess (mmol/l)	-3.2 [-5.9, 2.4]	-2.5 [-4, 4.35]	-1.1 [-4.6, 6]	0.262
Arterial lactate (mmol/l)	2.6 [1.7-6.65]	2.7 [1.25-4.45]	2 [1-3.8]	0.026
$ScvO_2$ (%)	81 [73-86]	86 [75-89]	83 [72-86]	0.645

Table 2 – Sublingual microcirculation and NIRS-derived variables.

All data are reported as median [1st-3rd quartile]. *Friedman test; corrected p for repeated measures <0.017. # p<0.05 versus Baseline; ## p<0.01 versus Baseline. AU: arbitrary units.

	Baseline	**6 hours**	**24 hours**	**p***
Total small Vessel Density (mm/mm^2)	14.9 [13.9-16.9]	17 [16-18.7]	17.9 [15.3-20]#	0.015
Perfused small Vessel Density (mm/mm^2)	13.9 [13.3-16.4]	15.7 [15-17.3]#	17 [14.8-18.6]##	0.003
Microvascular Flow Index [AU]	2.50 [2.37-2.62]	2.67 [2.62-2.75]	2.83 [2.58-3.00]	0.046
Percentage of Perfused small Vessels (%)	89 [86-92]	92 [91-93]	93 [92-94]	0.048
StO_2 (%)	83 [74-91]	87 [82-90]	85 [83-88]	0.528
StO_2 Downslope (%/min)	-6 [-10.8, -5.3]	-7.7 [-10.4, -3.3]	-9 [-13.9, -7.5]	0.442
StO_2 Upslope (%/min)	153 [106-186]	164 [118-253]	144 [58-194]	0.654
Area of hyperemia (%*min)	13.8 [5.3-15.2]	10.7 [5.1-17.8]	10.6 [6.7-18.4]	0.764
Tissue Haemoglobin Index [AU]	10.6 [6.5-16.7]	12.9 [9.8-18.4]	11.1 [9.1-13.7]	0.236

Figure 1 – Changes in plasma levels of cytokines.

Data are expressed as median [1st – 3rd quartile]. Friedman test with Dunn's test for repeated measures; *corrected p for repeated measure significance <0.017. TNF, tumor necrosis factor; IL, interleukin.

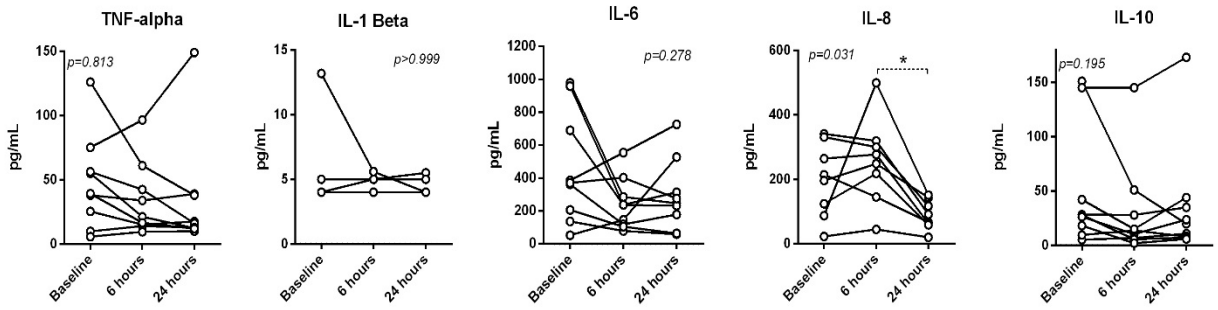
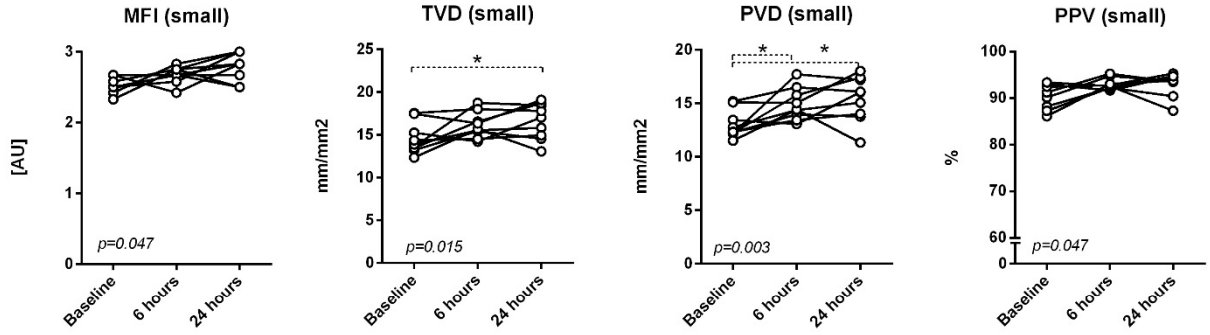


Figure 2 – Individual changes in sublingual microvascular parameters.

Data are expressed as median [1st – 3rd quartile]. Friedman test with Dunn's test for repeated measures; *corrected p for repeated measure significance <0.017. MFI, microvascular flow index; TVD, total vessel density; PVD, perfused vessel density; PPV, percentage of perfused vessels.



Supplemental material 1 Description of clinical setting and microbiologic data of enrolled patients.

	Sepsis/septic shock origin	**Microbiology data**
Patient 1	Abdomen (bowel perforation)	*Polymicrobial* (abdominal fluid, faecal peritonitis)) *Escherichia coli* (urine)
Patient 2	Respiratory (pneumonia)	No microbiological isolation in respiratory cultures
Patient 3	Respiratory (pneumonia)	*Streptococcus pneumoniae* (blood)
Patient 4	Urinary tract	*Escherichia coli* ESBL (urine) *Escherichia coli* + *Enterococcus faecalis* MDR (urine)
Patient 5	Urinary tract (renal abscess)	*Klebsiella pneumoniae* (urine and blood)
Patient 6	Soft tissues (gluteal abscess)	*Klebsiella pneumoniae* KPC (surgical sample of purulent material)
Patient 7	Mediastinitis (in peritonsillar abscess)	*Streptococcus constellatum* (surgical sample of purulent material + brochoalveolar lavage)
Patient 8	Soft tissues (Necrotizing fasciitis of abdominal wall)	*Escherichia coli* ESBL (wound culture)
Patient 9	Abdomen (cholangitis)	*Klebsiella pneumoniae* (blood)

Chapter 7

Circulating Free Hemoglobin and Microcirculation After Administration of Paracetamol in Febrile Septic Patients.

Domizi R, Damiani E, Scorcella C, Carsetti A, Giaccaglia P, Casarotta E, Graciotti L, Castaldo P, Amoroso S, Donati A

Recruitment status completed, statistical analysis under completion

Introduction

Abnormally high levels of extra-cellular free hemoglobin (free Hb) have been reported in plasma and serum of patients with different diseases, including sickle cellular disease, or undergoing invasive treatments, like coronary bypass surgery, blood transfusions and hemodialysis. In all these conditions, high levels of free hemoglobin were associated with poor outcome in term of mortality and with serious complications such as acute renal failure or myocardial infarction. [1-3]

Limited evidence is available for the role of free Hb in the systemic response to sepsis, althogh in the animal model of sepsis, increased values of plasmatic free hemoglobin has been related to increased mortality, especially if a concomitant lack of haptoglobin and hemopexin coexisted. The release of free hemoglobin during sepsis could be pathophysiologically attributable to changes in the membrane of red blood cells that produces hemolysis and unload free hb into plasma.

It has been hypothesized that the extracellular hemoglobin causes nitrate's formation and lipids peroxidation through reaction with NO, and boosts the immune response mediated by the monocyte -macrophage system. It has been also hypothesized that this phenomenon could be enhanced when the protective mechanism have been already saturated. These changes have been also recognized in humans as a source of damage of the vascular endothelium and consumption of free radical scavenger systems. [4-5]

In our previous study (Damiani E et al) we showed that septic critical care patients undergoing blood trasfusion developed higher levels of free hemoglobin post hemotransfusion and that this event resulted in a significant increase of impairment of the microcirculation, in a condition, like sepsis is, where microvascular and endothelial cell suffer proved derangement. [6] Radical oxygen species (ROS) are implicated in the damage of microcirculation, and similar effect of oxidative stress in sepsis are reported also on endothelial glycocalix, that is the thin layer that lines the surface of endothelium in contact with the blood. [7]

ROS are also responsible to up-regulation of Programmed Death-1 related molecules (PD-1/PD-L1) that render T cells susceptible to inhibitory modulation, inability to proliferate and reduced capacity to clear bacteria, inducing "immunosuppression" and more difficult recovery from septic shock. PD-1/PD-L1 system has been recently studied as potential predictor of mortality and active player in resolution of sepsis-induced organ dysfunction, Acute Lung Injury first. [8-10]

Paracetamol (or acetaminophen) is one of the most widely available and commonly used drug for its analgesic and antipyretic effects.

On animal model, it showed significant effect in prevention of oxidative damage and protection from renal damage in a context of increased levels of plasma free hb (as in Rhabdomyolysis and myoglobulinemia). This effect could be attributed to the ability of Paracetamol to cross-react with nitric oxide synthase (NOS) by increasing the synthesis of NO, and with cyclooxygenase (COX), by inhibiting the synthesis of prostaglandin I2 (PGI2-antiplatelet and vasodilator effect) without interfering with the production of Thromboxane A2 (TXA2-powerful vasoconstrictor and pro-thrombogenic agent) causing an imbalance of endovascular homeostasis. [11-12]

Recently, Janz et al demonstrated an increased mortality in septic patients with elevated values of free hb versus patients without this increase, and the efficacy of Acetaminophen to lower the concentration of F2- isoprostane (and other markers of oxidative stress). This effect was consistent with an association with lower risk of death and it lost in the cohort of septic patients without free hemoglobin registered.[13]

However, genetic variability could predispose some individuals to lower bioavailability or higher risk of acetaminophen-induced liver injury than would be predicted based on the dose administered. These eventualities could be particularly relavant in fragile ICU patients and could also alter the efficacy of paracetamol[16]

The main objective of this study is to evaluate the effect of paracetamol administration (indication on clinical judgment) on the microcirculation of pyrexial septic critical care patients. The primary endpoint is an improvement of Perfused Vessel Density (PVD) 30 minuts after the infusion of paracetamol, all the parameters of microcirculation will be studied. Further aim of the study is to evaluate the response of freeHb (measured on plasma), of oxidative stress markers, and of PD1/Pd-L1 to administration of paracetamol.

Lastly, we evaluated the plasmatic levels of paracetamol after administration and the prevalence of rs776746 e rs8330 polymorphisms in the sample population and their association with pharmacocynetic and pharmacodynamic of the drug and with the response of microcirculation to it.

Methods:

This is a prospective observational study conducted in the 14-bed General, Respiratory and Traumatic Intensive Care Unit (ICU) of Ospedali Riuniti of Ancona. It included 50 adult critical care patients.

Inclusion criterion: Patients with sepsis or septic shock with high body temperature, that need administration of Paracetamol on clinical judgment.

Exclusion Criteria: age<18 years, pregnancy, hemodialysis, hemolysis of the blood sample, use of Paracetamol in the previous 12 hours, conditions that do not allow the possibility of getting a monitoring of sublingual microcirculation (maxillofacial trauma, serious inability to jaw, copious blood loss or secretions from the mouth).

In compliance with national applicable laws, informed consent was obteined from the subject or his next of kin before inclusion, by signing the appropriate informed consent paperwork. The study protocol was approved by the Local Ethics Committee (Comitato Etico Regione Marche—CERM) and deposited as NCT02750163; it conformed to the principles of Helsinki declaration (last revision, Edinburgh 2000).

Before the administration of the drug (T0), 30 minuts after the infusion (T1) and 2 hours later (T2), we monitored the main parameters of the sublingual microcirculation with Incident Dark Field Imaging (IDF) technology (CytoCam, Braedius Medical B.V., Huizen, the Netherlands) and we assessed the Perfused Boundary Region of endothelial glycocalix using the Sidestream Dark Field (SDF) videomicroscope (Microscan,Microvision Medical, Amsterdam,The Netherlands) connected to a glycocalyx measurement system (GlycoCheck ICU, Maastricht University Medical Center, Maastricht,The Netherlands) and Near InfraRed (NIRS) parameters with the vascular occlusion test (VOT), with InSpectra StO2 Tissue Oxygenation Monitor (model 650; Hutchinson Technology, Hutchinson, MN, USA). Microcirculatory parameters were then calculated offline with the Automated Vascular Analysis software (AVA v3.2, Microvision Medical, Amsterdam, NL).

Clinical and laboratoristic records were collected at all the time points.

At baseline and 2 hours after the infusion of Acetaminophen, we collected arterial samples. They were immediately centrifuged to obtain plasma and serum samples that were stored at -80°C for subsequent analysis. We measured: fHb levels, markers of oxidative damage (F2-isoprastane and F2-isoflurane), of endothelial cell injury (endothelin-1) and glycocalyx damage (sindecan-1, heparasulfate), PD-1/PD-L1.

In order to identify the individual variability in drug response, polymorphisms of genes involved in the pharmacokinetic of Acetaminophen were investigated.

We focused on two polymorphisms, respectively called rs776746 and rs8330. They codify for Fase I CYP3A5 enzyme and Fase II UGT1A1 enzyme; they participate at different stages to the epatic metabolism of paracetamol. When one of the two polymorphisms is expressed (eterozygosis or homozygosis) they reduce the half-life of paracetamol and induce higher production of toxic metabolites.

Statistical analysis:

Parametric and non-parametric tests will be used as appropriate for comparison between independent samples, according with the properties of the main variables. The Kolmogorov-Smirnov test will be used to assess the normality of continuous variables. Student t-test for repeated measures will be used to assess the primary objective. A two-way analysis of variance (ANOVA) for repeated measures will be performed with Bonferroni post-hoc test in order to compare changes in the parameters of interest for the secondary objective. Differences will be considered significant at P values < 0.05.

Results:

This study enrolled 50 patients in 2 years (from july 2017 to june 2019). The offline analysis of sublingual microcicrulation and Near InfraRed Spectroscopy is now complete and definitive results will be available soon. We are now completing the immuno-enzimatic and colorimetric assays on the last 10 patients included, and we conclude the analysys if the two polymorphisms.

We performed an half-sample preliminary analysis on the first n=25 patients included in the study.

Microcirculation:

On Friedman test for repeated measures (RM) we reported a statistically significant improvement of Microvascular Flow Index (MFI, AU, p=0.002) after the infusion of paracetamol, with a Dunn's post hoc test (between times) statistically significant at T1 to baseline (T0=2.83[IQR 2.57-2.96], T1= 3[2.83-3] p= 0.027) and T2 to baseline (T0= 2.83[2.57-2.96], T2=3[2.85-3] p=0.04).

We showed similar results of Friedman test for RM on PPV (Proportion of Perfused Vessels, %, p=0.024), with Dunn's post hoc test between times T2 to T0 (T0= 92[88-95], T2= 96 [93-98] p=0,024).

Plasmatic levels of Paracetamol:

The plasmatic target of paracetamol of 10-20mcg/ml was satisfied only at T1 while it was significatively under-dosed at T2 in all the patients. Half of the patients were still pyrexial (T>38°C) at T2.

Pharmacogenomic:

40% of the patients expressed at least one polymorphism in etrozygosis (12% expressed rs:776746, 38% rs:8330). Non omozygotic patients were collected and the eterozygosis of the two polymorphism was not statistically associated to the plasmatic level of paracetamol.

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Chapter 8

Relationship between norepinephrine dose, tachycardia and outcome in septic shock: a multicentre evaluation

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ABSTRACT

Purpose: Septic shock is associated with massive release of endogenous catecholamines. Adrenergic agents may further exacerbate catecholamine toxicity and contribute to poor outcomes. We sought to determine whether an association existed between tachycardia and mortality in septic shock patient requiring norepinephrine for more than 6 hours despite adequate volume resuscitation.

Materials and methods: Multicentre retrospective observational study on 730 adult patients in septic shock consecutively admitted to eight European ICUs between 2011 and 2013. Three timepoints selected: T1 (first hour of infusion of norepinephrine), Tpeak (time of highest dose during the first 24 hours of treatment), and T24 (24 hours post-T1). Binary logistic regression models were constructed for the three time-points.

Results: Overall ICU mortality was 38.4%. Mortality was higher in those requiring high-dose (≥0.3 mcg/kg/min) versus low-dose (<0.3 mcg/kg/min) norepinephrine at T1 (53.4% vs 30.6%; p<0.001) and T24 (61.4% vs 20.4%; p<0.0001). Patients requiring high-dose norepinephrine with concurrent tachycardia had higher mortality at T1, while in the low-dose group tachycardia was not associated with mortality. Resolving tachycardia (from T1 to T24) was associated with lower mortality compared to patients whose tachycardia persisted (27.8% vs 46.4%; p=0.001).

Conclusions: High norepinephrine dose and tachycardia may contribute to poor outcomes in septic shock.

Keywords:

Septic shock; sepsis; tachycardia; norepinephrine; vasoactive; outcome**.**

Introduction:

Mortality from septic shock remains high. The combination of hypotension requiring vasopressor therapy and hyperlactatemia persisting despite adequate fluid resuscitation is associated with a mortality above 40% [1]. The 2016 Surviving Sepsis Campaign guidelines recommend catecholamines as first-line therapy for both vasoplegia (norepinephrine), and myocardial depression/low cardiac output states (dobutamine) [2]. However, an increasing literature implicates catecholamine toxicity as an important cause of pathology in critical illness and contributory to mortality [3-7]. Tachyarrhythmias, stress-related cardiomyopathy, cardiotoxicity with the characteristic finding of contraction band necrosis, digital and splanchnic ischemia, insulin resistance, immune suppression with enhanced bacterial growth, and increases in lipolysis, metabolic inefficiency, catabolism, muscle breakdown and pro-thrombotic activation are reported adverse features of either high endogenous levels of catecholamines, or their exogenous administration [3-12]. A dose-response relationship between catecholamines and mortality is well-established in septic shock [13-15]; while this may simply reflect underlying illness severity, an iatrogenic contribution cannot be excluded.

Tachycardia is also associated with poor outcomes in septic shock [16-18]. This may originate from excess adrenergic stimulation (both endogenous and exogenous) and/or autonomic dysfunction. Whether tachycardia is a direct contributor to mortality through impaired diastolic relaxation and reduced pumping efficiency is uncertain [19,20]. When heart rate control was achieved with beta-adrenergic blockade, there was a signal towards improved outcomes [21-24]. However, other than its effects on tachycardia and improving ventricular performance, beta-blockers have multiple non-cardiac effects that can counteract the catecholamine-related toxicities outlined above [7].

Against this background, we conducted a chart review of patients with septic shock receiving norepinephrine therapy in intensive care units (ICU) in four European countries to assess if there was an association between tachycardia in the setting of exogenous norepinephrine use and mortality in patients with septic shock.

Materials and Methods:

Data were collected retrospectively from hospital charts and records of consecutive adult patients admitted to one of eight participating European ICUs (4 UK, 2 Italian, 1 French, 1 Dutch) within the timeframe of January 2011 to January 2013. Inclusion criteria were: consecutive adult patients admitted to ICU within the timeframe, septic shock diagnosed on the ward or the ICU, requirement norepinephrine as first line vasopressor despite adequate volume resuscitation; exclusion criteria: infusion of norepinephrine for less than 6 hours, survival time in ICU inferior to 6 hours from the introduction of vasopressor; 100 consecutive patients were requested from any one site. Septic shock was defined using the 2001 definition of infection-related organ dysfunction with an ongoing norepinephrine [NE] requirement despite adequate volume resuscitation [25]. Norepinephrine was chosen as the first line vasopressor according to the 2012 Surviving Sepsis Campaign Guidelines¸ the adequacy of volume resuscitation, according to clinical judgment, was a specific inclusion criterion. (Further information about the study outline and data collection will be available in Additional Table 1).

Data collectors used either ICU and hospital electronic records (where available) or manual extraction from paper charts. Anonymized data (demographic, clinical, microbiological, biochemical, ICU and hospital outcomes) were entered onto a standardized database using standardized definitions.

The study was conducted in compliance with the Helsinki Declaration; since it was a retrospective data collection of normal clinical anonymised data, all national bodies waived the need for informed consent.

Clinical, hemodynamic, microbiological and laboratory data were collected at three timepoints, selected in relation to the introduction of vasoactive support: T1 (first hour of infusion of NE);

Tpeak (time of highest NE dose reached during the first 24 hours of treatment); T24 (24 hours' post-T1).

Two numerical scales were devised (Additional Tables 2a and 2b) to quantify concurrent dosing of other vasoactive therapies (Vasoactive Drug Index [VADI]) and analgosedation (Sedative Drug Index [SDI]). These scores were formulated with an empirically agreed range for low, medium and high doses of these drugs, based on the pharmacological activity of the different agents. (VADI and SDI are described in Additional Table 3).

Statistics: Kolmogorov-Smirnov, D'Agostino and Pearson, and Shapiro-Wilk tests were used to assess normality of continuous variables. Non-parametric (Mann–Whitney and χ^2 tests) and parametric (Student's t-test) tests were used as appropriate for comparison between independent samples. Differences were considered significant at p values <0.01, except for χ^2 tests where p values below 0.05 were accepted. Results are presented as median and interquartile ranges (IQR), mean and standard deviation (SD), or N (and %).

The area under the Receiver Operating Characteristic (ROC) curve was calculated to confirm the discriminative ability of NE to prognosticate ICU mortality, and to select ~~an arbitrary~~ a cut-off of NE infusion rate to separate patients into low-dose and high-dose groups.

The primary outcome was ICU mortality. Binary logistic regression models were constructed for the three time-points, and they included the following variables: age, SOFA score, source of sepsis, multi-drug resistance (MDR), temperature, mean arterial pressure (MAP), infusion rate of NE (mcg/kg/min), VADI, blood lactate level, ~~(~~need for RRT, mechanical ventilation, PaO_2, PEEP, SDI, Fluid Balance, use of steroids or beta-blockade in the first 24 hours (only for T24), and ICU LOS (length of stay) ($p<0.05$). Heart rate (HR) was included as a categorical (yes/no) variable for tachycardia determined as a heart rate ≥95 bpm. Variables included in the models were selected on

the basis of their ~~reported~~ association with outcome at univariate analysis ($p<0.05$), with major co-linearities excluded. No formal sample size calculation was performed.

Results:

Study population

Data collection was performed on 736 patients; three centers did not collect the requisite 100 patients either due to difficulties in data collection or to insufficient cases of septic shock in the time period. A further six patients were removed from this analysis as they did not satisfy the inclusion criteria because they received agents other than norepinephrine as first-line vasopressors, leaving 730 patients for the main analysis. Of these, 46 patients died within the first 24 hours of infusion of NE, allowing 684 patients to be analyzed at the T24 timepoint. None of the patients was discharged from ICU before t24.

General characteristics and main outcomes

Overall ICU mortality was 38.4% (n=280), 28-day mortality 29.6%, (n=216) and hospital mortality 44.9% (n=328). Non-survivors were older ($p=0.001$) and had a higher admission SOFA score ($p<0.001$), a higher incidence of multi-drug resistant organisms ($p<0.05$) and more respiratory infections as the source of septic shock (Table 1). On 117 patients (16%) all cultures were negative, 18% of data are missing. ICU mortality was not statistically different in culture-negative patients.

Data on hemodynamic monitoring were reported just on 145 (20%) patients; 118 (16.1%) patients were on oral beta blockers as part of their usual daily medication before admission in ICU.

Among centres, differences were evident in term of ICU mortality, that ranged from 17%to 57%, prevalence MDR species (5% to 65%), and source of sepsis. SOFA score at admission in ICU was lower in two of the eight centres. (Additional table 3) Of note, administration of sedative and

vasoactive drugs, as well as fluid balance of the first 24 hours of infusion of norepinephrine diverged among ICUs. (Additional Figures 1 and Additional Figure 2)

Univariate analysis

At all three timepoints (T1, Tpeak, T24) NE dose differed significantly between survivors and non-survivors ($p<0.01$) and HR was significantly higher in non-survivors ($p<0.01$) than in survivors (Additional Table 4). The area under the ROC curves constructed at T1, Tpeak and T24 demonstrated good discriminative ability of the NE dose to prognosticate ICU mortality with an AUC of 0.762 [0.72-0.80] at T24 (Figure 1). An infusion rate of 0.3 mcg/kg/min was chosen as cut-off (on the ROC curve for ICU-mortality at T24, with specificity of 80% and sensitivity of 62.1% to prognosticate ICU mortality) to separate patients into low and high (NE ≥0.3 mcg/kg/min) requirements. ICU mortality was 61.4% in those patients requiring high-dose NE (≥0.3 mcg/kg/min) at T24 compared versus 20.4% ($p<0.0001$) in those requiring low doses (<0.3 mcg/kg/min).

At T1 ICU mortality was significantly higher in tachycardic (HR ≥95 bpm) patients ~~within both groups~~, independently from the dose of NE receiving, but the difference was statistically more significant in the group of patients receiving high levels of norepinephrine (Table 2, Figure 2).

Patients with tachycardia at T1 (n=434) and who remained tachycardic at T24 (n=295) had a higher ICU mortality (46.4%) compared to the 27.8% mortality seen in the 108 patients who were similarly tachycardic at T1 but where tachycardia resolved at T24 ($p=0.001$).

Fluid balance (FB) at T24 was similar between survivors and non-survivors in the low-NE dose group (1813 [95%CI 900-3014] ml for survivors and 1600 [95%CI 364-3096] ml in non-survivors; $p=0.194$). Patients who receive high-dose of NE at T24 showed a more positive fluid balance in

survivors than in non-survivors (2059 [95%CI 1452-1525] ml in survivors, 1600 [95%CI 364-3096] ml in non-survivors; p=0.04)

Although sedation use and requirement for mechanical ventilation were higher in those receiving high-dose NE, there was no difference between survivors and non-survivors both in high-dose and low-dose NE groups

Binary logistic regression

The binary logistic regression model built for T1 found no predictive value for ICU mortality for tachycardia in the low NE group, whereas tachycardia in the high NE group showed good predictive value (OR 2.13, 95% CI 1.13-3.9; p<0.05) (Figure 3). A similar pattern was seen at Tpeak with no relationship between tachycardia and outcome in the low NE group, but an OR of 2.7 (95%CI 1.7-4.31; p<0.01) in the high NE group. SDI at the Tpeak timepoint was inversely associated with a poor prognosis (OR 0.823, 95%CI 0.748-0.906; p<0.01), (Table 3). Fluid balance was not independently predictive of ICU mortality.

As subgroup analysis, the OR for mortality for patients who were tachycardic at T1 and remained tachycardic at T24 was 2.23 (95%CI 1.14-4.36, p<0.05). Conversely, in those with a normal HR at T1 (n=296), no difference in mortality was seen between tachycardic and non-tachycardic patients at T24 (p=0.477).

Of the 638 patients in whom the use of beta-adrenergic blockade was reported, 40 were taking oral beta-blockade as usual medication before ICU admission and 97 received an agent within the first 24-hour period of critical care. The use of beta blockade was associated with lower mortality (OR 0.258, 95%CI 0.069-0.95; p<0.05).

Discussion:

This study performed a retrospective analysis of data, from a large cohort of septic shock patients, admitted for critical care management, in 8 European ICUs, and managed according to international guidelines. The study protocol focused on patients who required vasopressor (norepinephrine selectively) for more than 6 hours, and compared different requirements of norepinephrine after the first hour of infusion and at 24 hours. The peak level of norepinephrine reached during that period of observation was also noted.

We found that patients in septic shock who immediately required high doses (≥0.3 mcg/kg/min) of norepinephrine (despite fluid resuscitation) to maintain an adequate arterial pressure had a higher risk of ICU mortality compared to those patients requiring lower levels of vasopressor support. This risk increased further if NE doses remained high at 24h. Of note, in those patients who immediately required high doses of vasopressor, tachycardia (HR ≥95 bpm) was independently associated with a further risk of ICU mortality, while persisting tachycardia at 24 hours' post-introduction of NE was associated with poor outcomes in both groups (high-dose and low-dose NE). On the contrary, in the low-dose group, tachycardia was not associated with higher mortality at T1

Although norepinephrine usually generates a reflex bradycardic response through baroreceptor activation, tachycardia may potentially represent an adverse effect of high-dose norepinephrine, especially in patients who are not adequately fluid resuscitated. [26]

Tachycardia may also represent a clinical biomarker of adrenergic overstimulation ± autonomic dysfunction: sepsis results in downregulation of alpha-adrenergic receptors and post-receptor signaling pathways [20]. In addition, reduced myocardial sensitivity to calcium may be related to altered intracellular calcium levels during systole and diastole (affecting contraction and relaxation, respectively), and/or altered actin myosin cross-bridge formation and uncoupling [20]. In this situation, there may be toxicity rather than benefit from an exogenous infusion of catecholamines.

Patients requiring high doses of exogenous catecholamines who have a tachycardia response have likely exhausted their compensatory reflex mechanisms.

Our analysis showed that patients remaining tachycardic after 24 hours of norepinephrine infusion have a three-fold increased risk of death compared to their non-tachycardic counterparts. This finding supports previously reported literature [18]. Persistent tachycardia stimulates detrimental effects on heart increasing myocardial oxygen demand, reducing diastolic filling period, inducing direct cardiotoxicity. Beta-adrenergic blockade, either long-term use prior to admission or commenced within the first 24 hours of high dose NE, was associated with a lower mortality. Caution should be applied as this was an unselected group of patients though this finding is supportive of other studies. Macchia *et al* analysed administrative databases of Italian patients hospitalized for sepsis between 2003–2008, of whom 11.2% were prescribed ß-blocker therapy pre-admission [27]; despite a higher risk profile, these patients had a lower 28-day mortality: 17.7% compared to 22.1% in those previously untreated ($p<0.005$).

Morelli *et al* [21] reported that septic shock patients requiring high-dose norepinephrine and with persisting tachycardia (HR >95 bpm) after 24 hours showed a reduction in 28 day mortality from 80.5% in control patients compared to 49.4% in those randomized to receive esmolol. This study has been criticized for the high control group mortality (28), however it is important to stress that a particularly high-risk subset (high-dose norepinephrine requirement and ongoing tachycardia) was selected. A similar cohort of patients in our retrospective study had an ICU mortality of 65%. This also reflects other published reports. For example, Benbenishty *et al* reported a 96% probability of death in ICU patients if treated with >0.5 mcg/kg/min of norepinephrine or epinephrine [15].

Of note, fluid balance was similar between survivors and non-survivors over the first 24 hours of norepinephrine therapy and there was also no correlation between fluid balance and persistence of tachycardia. However, in the high-dose group, at T24, survivors showed more positive fluid balance than non survivors.

The main limitation of our study is its retrospective nature. Differences in practice between centers e.g. endpoints for volume resuscitation, hemodynamic targets (e.g. mean arterial pressure) and use of sedation, and patient/infection factors such as antimicrobial resistance are likely to impact upon catecholamine dose and may have confounded the findings. Limited information is available to exclude inadequate fluid resuscitation prior to initiation of vasoconstrictor (norepinephrine) and to assess persistent hypovolemia.

In conclusion, in this retrospective analysis of septic shock patients admitted to ICUs in several European countries, both early and persisting requirements for high-dose norepinephrine were associated with poor prognosis. Tachycardia was a poor prognosticator ~~but only~~ in patients requiring immediate high-dose norepinephrine, and in patients where tachycardia persisted at 24 hours, independently from the level of noradrenergic support received at that point. This suggests the possibility that, in addition to endogenous adrenergic overstimulation and/or autonomic dysfunction, norepinephrine may be contributory.

Declarations:

- Ethics approval and consent to participate: Where required by national laws, ethical approval and consent to participate was sought from the appropriate body
- Consent for publication: no individual person's data in any form (including individual details, images or videos) are included
- Availability of data and material: the data are available on reasonable request to robertadomizi@gmail.com Competing interests: no financial and non-financial competing interests
- Funding: non-sponsor driven study. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
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Conflict of interest:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Conflict of interest:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

List of abbreviations:

MDR Multi-Drug Resistance.

SOFA Sequential Organ Failure Assessment.

SAP systolic arterial pressure.

DAP diastolic arterial pressure.

MAP mean arterial pressure.

VADI vasoactive drug index.

SDI sedation drug index.

MV mechanical ventilation.

RRT renal replacement therapy.

PEEP positive end-expiratory pressure.

S survivors.

NS non-survivor.

NE norepinephrine.

HR heart rate.

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Table legends

Table 1: Baseline Characteristics of the Study Patients.

		n (%)		
Outcome		**Survivors 450 (61.6%)**	**Non-survivors 280 (38.4%)**	**p value**
Age (y)		65 (54-75)	69 (59-78)	*<0.001*
Male, n (%)		257 (57.1)	161 (57.5)	
SOFA score at admission in ICU, AU		9 (3.2)	11 (3.9)	P<0.001
Source of infection, n (%)				
	Abdominal	129 (29)	77 (27)	
	Bacteremia	37 (8)	18 (6)	
	Genito-urinary	45 (10)	10 (4)	
	Other	22 (5)	6 (2)	
	Respiratory	169 (38)	137 (49)	*<0.01*
	Skin and soft tissue	30 (7)	16 (6)	
	Unknown	18 (4)	16 (6)	
MDR, n (%)		98 (2)	82 (29)	*<0.05*
Colture-negative patients, n(%)*		65 (14)	52 (19)	
ICU length of stay (d)		9 (5-19)	7 (3-15)	*<0.001*

Table 2: Comparison of first hour (T1) data between eventual ICU survivors and non-survivors stratified by dose

	Low-dose norepinephrine group n=483			***High-dose norepinephrine group n=247***		
	Survivors	**Non-survivors**	**P**	**Survivors**	**Non-survivors**	**P**
	335 (69.4%)	**148 (30.6%)**		**115 (46.6%)**	**132 (53.4%)**	
HR (bpm)	96 (83-115)	102 (87-133)	*<0.05*	97 (85-122)	109 (94-121)	*0.01*
SAP (mmHg)	100 (91-111)	99 (90-113)		105 (95-115)	106 (95-116)	
DAP (mmHg)	51 (46-58)	51 (46-58)		52 (49-58)	53 (49-59)	
MAP (mmHg)	67 (59-75)	68 (60-74)		70 (63-76)	72 (66-76)	
Temp (°C)	36.8 (36.2-37.7)	36.7 (36-37.5)		36.7 (36-37.4)	36.7 (36-37.5)	
SOFA score	9 (8-11)	11 (10-13)	*<0.01*	9 (7-11)	11 (10-14)	*<0.01*
Other vasoactive agent, n (%)	45 (13)	19 (13)		25 (22)	37 (28)	
VADI	2.0 (1)	2.0 (2)		3.7 (1.2)	3.9 (1.4)	
SDI	2.6 (2.4)	2.3 (2.3)		3.4 (2.3)	3.1 (2.1)	
MV, n (%)	234 (70)	113 (76)		102 (89)	121 (92)	
PEEP (cmH_2O)	6.5 (5.0)	6.5 (4.5)		7.6 (4.0)	7.6 (4.3)	
PaO_2/FiO_2	211 (139-291)	174 (121-298)		216 (131-303)	190 (108-268)	
Lactate (mmol/l)	2.5 (2.3)	3.8 (3.5)	*<0.01*	3.5 (3.2)	4.5 (4.0)	*<0.05*
RRT, n (%)	18 (5)	14 (10)		9 (8)	36 (27)	*<0.01*

Median (SD), Median and IQR, n (%). SAP systolic arterial pressure, DAP diastolic arterial pressure, MAP mean arterial pressure, SOFA sepsis organ failure assessment, VADI vasoactive drug index, SDI sedation drug index, MV mechanical ventilation, RRT renal replacement therapy

Table 3. Binary logistic regression models for ICU mortality at timepoints T1, Tpeak and T24.

		ICU mortality	
NE dose mcg/kg/min		**OR (95%CI)**	**P value**
T1			
<0.3	SOFA score	1.307 (1.193-1.432)	0.000
	Lactate (mmol/l)	1.102 (1.016-1.196)	0.019
≥0.3	Tachycardia (y/n)	2.134 (1.139-3.999)	0.018
	Age (y)	1.019 (1.000-1.038)	0.049
	SOFA score	1.257 (1.131-1.399)	0.000
	RRT (y/n)	4.764 (1.989-11.407)	0.000
Tpeak			
<0.3	RRT (y/n)	2.412 (1.122-5.186)	0.024
≥0.3	Tachycardia (y/n)	2.707 (1.702-4.308)	0.000
	Age (y)	1.019 (1.005-1.033)	0.008
	MAP (mmHg)	0.964 (0.944-0.984)	0.000
	SDI	0.823 (0.748-0.906)	0.000
	MV (y/n)	4.637 (2.079-10.343)	0.000
	RRT (y/n)	1.773 (1.108-2.835)	0.017
T24			
<0.3 and tachycardia at T1	Tachycardia (y/n)	2.865 (1.11-7.072)	0.022
	Beta blockade	0.254 (0.069-0.959)	0.043
	Lactate (mmol/l)	1.556 (1.109-2.184)	0.011
>0.3 and tachycardia at T1	Tachycardia (y/n)	1.024 (0.329-3.189)	0.967
	RRT (y/n)	2.456 (1.144-5.272)	0.021
	Lactate (mmol/l)	1.153 (1.014-1.311)	0.029

Adjusted ORs (95% CI) and p values. Abbreviations: MAP mean arterial pressure; SDI sedation drug index; MV mechanical ventilation; RRT Renal Replacement Therapy.

Figure legends

Figure 1: Receiver Operating Characteristic curves of norepinephrine dose at T1, Tpeak and T2 for ICU mortality

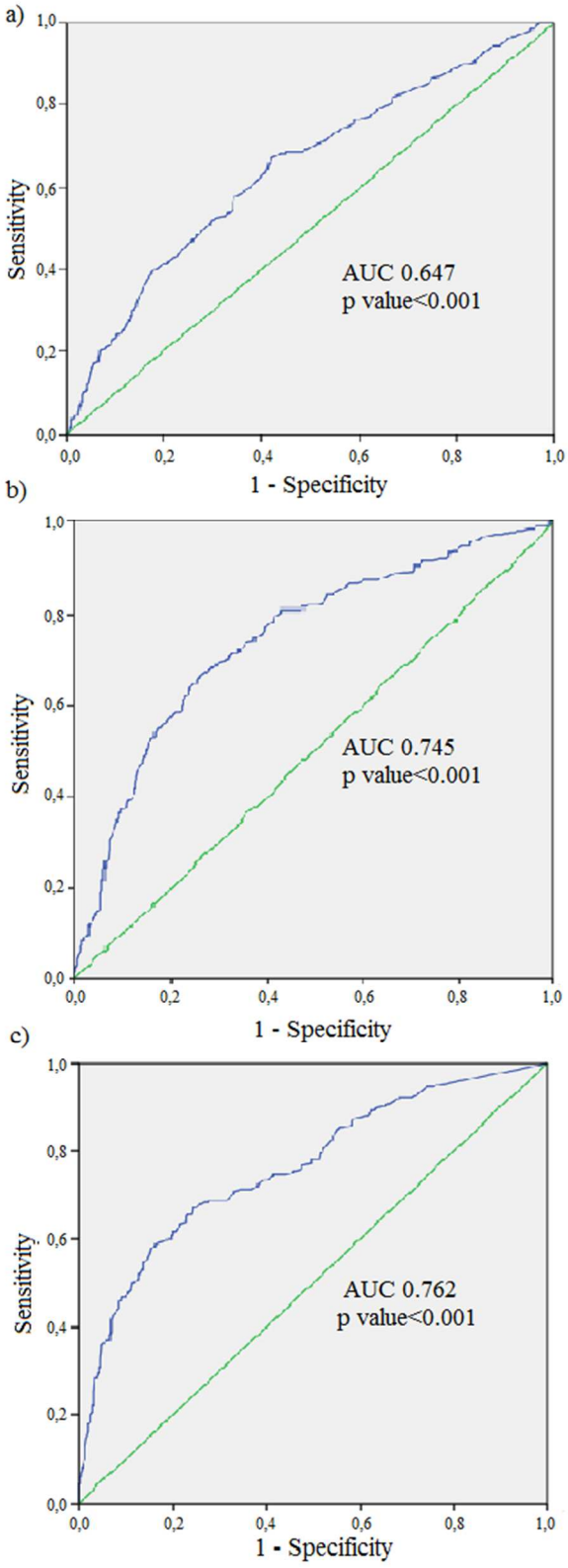


Figure 2: Tachycardia and mortality rates in low- and high-dose norepinephrine groups at T1 and T24.

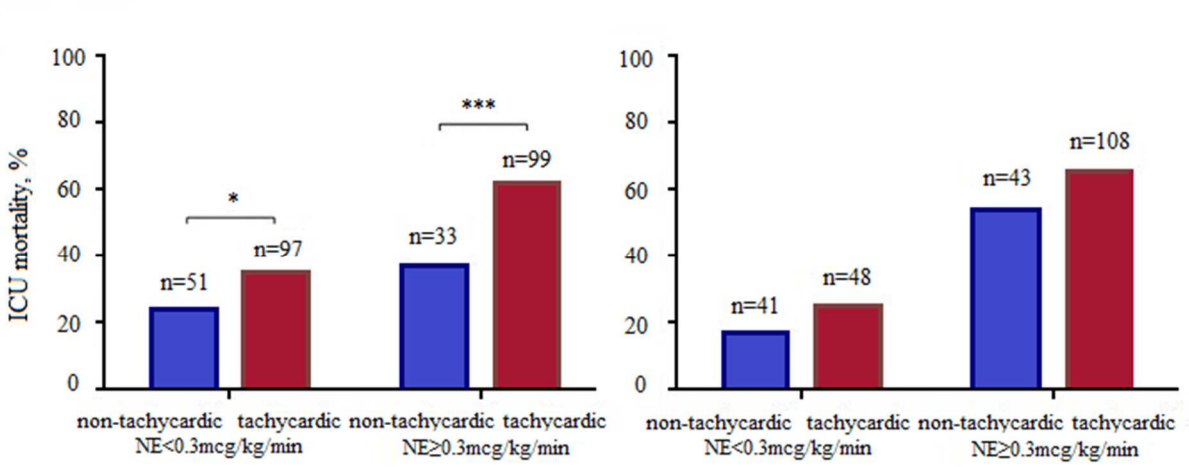
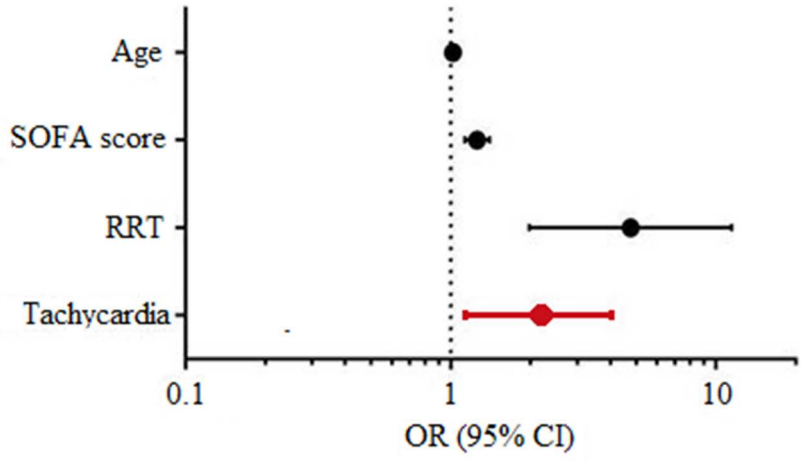


Figure 3: Odds ratio for mortality for high dose NE group (≥0.3 mcg/kg/min) at the T1 timepoint.



Additional (supplemental) files:

Additional Table 1: Study outline and data collection for the 8 participating ICUs (.doc)

Purpose of the study	To evaluate the relationship between norepinephrine infusion, heart rate and mortality in ICU septic shock patients
Design of the study	Multicentre retrospective observational study
Inclusion criteria	Adult consecutive patients, developing septic shock on the ward or the ICU, who require norepinephrine for cardiovascular support despite adequate volume resuscitation as first line vasopressor
Exclusion criteria	Less than 6 hours' infusion of norepinephrine, survival time in ICU inferior to 6 hours from the introduction of norepinephrine
Sample size	100 patients from each participating ICU
Timepoints	T1: first hour of infusion of norepinephrine
	T peak: time of highest norepinephrine dose reached during the first 24 hours of treatment
	T24: 24 hours after T1
Source of infection	Source of septic shock (respiratory, abdominal, skin and soft tissue, genito-urinary, bacteraemia, unknown, other)
Multidrug resistance	defined as: Gram positive - methicillin resistant; Gram negative - resistant to at least 3 antibiotic classes; Enterococcus - Vancomycin resistant; Candida - Fluconazole resistant
Immunosuppression	Defined as: neutrophils < 500 cells/mm3 or chemotherapy administration in the previous 2 weeks OR prednisone ≥20 mg/day or equivalent
Outcomes	ICU mortality
	28-day mortality
	Hospital mortality

Additional Tables 2a and 2b: Vasoactive Drug Index [VADI] and Sedative Drug Index [SDI]

VasoActive Drugs Index (VADI)		**0**	**1**	**2**	**3**	**4**
	Loading dose	None	LOW	MILD TO MODERATE	HIGH	VERY HIGH
Norepinephrine (mcg/kg/min)		0	<0.1	0.1-0.3	>0.3	
Dobutamine (mcg/kg/min)		0	<2.5	2.5-20	20-40	>40
Dopamine (mcg/kg/min)		0	<5	5-10	>10	
Levosimendan (mcg/kg/min)	6-12 mcg/kg	0	<0.1	0.1	0.2	>0.2
Terlipressin (mcg/h, infusion)		0	<10	10-25	>25	
Terlipressin (mcg/day, bolus)		0	≤500	501-1000	>1000	
Enoximone (mcg/kg/min)	0.5-1 mg/kg	0	<5	5-10	>10	
Phenylephrine (mcg/kg/min)	0.1-0.18 mg/min	0	<0.6	0.6-8	>8	
Epinephrine (mcg/kg/min)		0	≤0.1	0.11-0.3	>0.3	
Vasopressin (U/min)		0	≤0.01	0.011-0.04	≥0.04	
Milrinone (mcg/kg/min)		0	<0.375	0.375-0.75	>0.75	
Metaraminol (mcg/kg/min)	0.2-0.5 mg	0	<0.8	0.8-4.2	>4.2	
Nitroglycerine (glyceryl trinitrate) (mcg/min)		0	<50	50-200	>200	
Amiodarone		0				

Sedative Drug Index (SDI)		**1**	**2**	**3**	**4**
		LOW	MILD TO MODERATE	HIGH	VERY HIGH
		Spontaneous ventilation			>2 drugs or paralysis + 1-2 drugs
Propofol (mg/kg/h)		<0.3	0.3-2.15	2.16 -4	>4
Midazolam (mg/kg/h)		<0.02	0.02-0.11	0.12-0.2	>0.2
Fentanyl (mcg/kg/min)	single	<0.03	0.03-0.065	0.066-0.1	>0.1

	combined	<0.008	0.008-0.0165	0.0166-0.025	>0.025
Remifentanil (mcg/kg/min)		<0.05	0.05-1.025	1.026-2	>2
Sufentanil (mcg/kg/h)	single	<0.3	0.3-0.45	0.46-0.6	>0.6
	combined	<0.15	0.15-0.375	0.376-0.6	>0.6
Alfentanil (mg/h)		<0.5	0.5-5.25	5.26-10	>10
Alfentanil (mcg/kg/min)		<0.5	0.5-1.5	1.6-3.0	>3.0
Morphine (mg/h)		<1	1-6.5	6.6-12	>12
Dexmedetomidine (mcg/kg/h)		<0.7	0.7-1.4		
Clonidine (mcg/h)		<40	40-110	111-180	>180
Etomidate mg/kg		<0.2	0.2-0.3	>0.3	
Thiopentone mg/kg/h			3.0-5.0		

Additional Table 3: Baseline Characteristics of the Study Patients, in the eight participating centres.

Centres		**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Age (y)		63 (50-74)	70 (55-75)	64.5 (54-74)	68 (59-7.7)	62 (49-77)	66 (54-76)	68 (61-79)	66 (58-76)
Admission SOFA, AU		10 (7-12)	11 (8-14)	10 (8-12)	10 (8-12)	7 (6-8)	11 (8-13)	8 (5-11)	11 (8-11)
ICU LoS		9 (5-17)	10 (3-21)	8 (5-19)	6 (3-14)	14 (7-23)	5 (3-12)	10 (7-16)	6 (3-11)
Males, %		59.8	58	55	53.9	61	49	68.4	46.2
Source, %									
	Abdominal	27.1	35	44	23.6	30	32	11.6	10.3
	Bacteraemia	13.1	2	1	0	4	25	9.5	0
	Genito-Urinary	7.5	3	12	7.9	4	6	13.7	5.1
	Other	2.8	1	7	3.4	0	3	4.2	17.9
	Respiratory	33.6	50	23	58.4	56	22	46.3	59
	Skin and Soft Tissue	7.5	7	5	5.6	6	3	9.5	7.7
	Unknown	8.4	0	8	1.1	0	9	5.3	0
MDR, %		16.8	30	11	20.2	65	14	23.2	5.1
ICU Mortality, %		38.3	55	17	37.1	57	36	32.6	35.6

Additional Table 4: Comparison between survivors and non-survivors at timepoints T1, Tpeak and T24 (.doc)

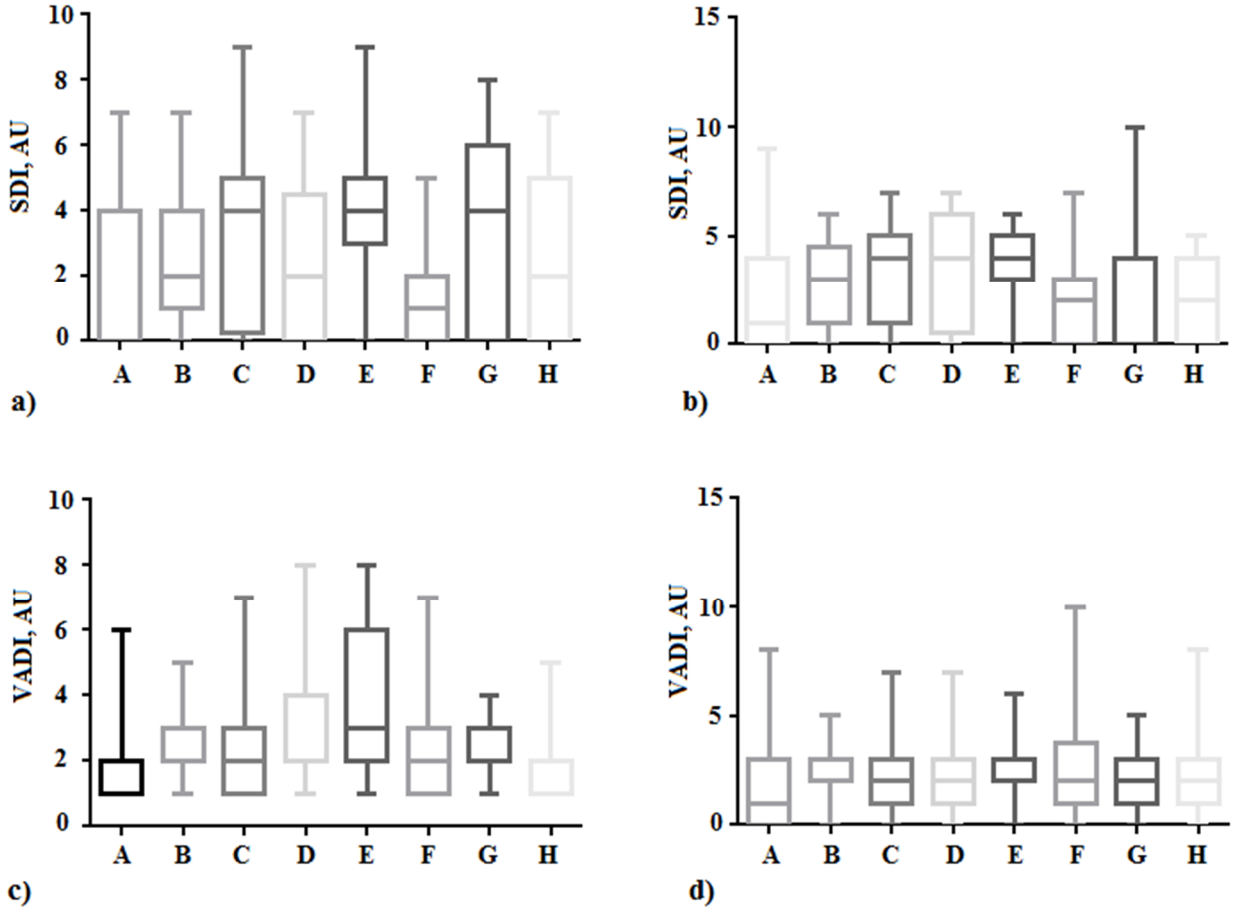
Time-points	T1			Tpeak			T24		
	S	**NS**	**p**	**S**	**NS**	**p**	**S**	**NS**	**p**
NE (mcg/kg/min)	0.15 (0.09-0.3)	0.27 (0.13-0.51)	*<0.01*	0.28 (0.15-0.54)	0.74 (0.38-0.74)	*<0.01*	0.11 (0.016-0.26)	0.49 (0.14-0.81)	*<0.01*
HR (bpm)	99 (23)	105 (22)	*<0.01*	98 (23)	108 (21)	*<0.01*	95 (20)	101 (20)	*<0.01*
SAP (mm Hg)	100 (90-112)	103 (92-114)		107 (18)	103 (21)	*<0.01*	110 (17)	109 (19)	
DAP (mm Hg)	52 (41-58)	52 (42-58)		53 (48-59)	51 (46-56)	*<0.01*	56 (51-61)	53 (49-59)	*<0.01*
MAP (mm Hg)	69 (12)	70 (11)		71 (12)	69 (11)	*<0.01*	74 (10)	72 (11)	*<0.05*
SOFA	9 (8-11)	11 (10-14)	*<0.01*	\	\		9 (7-11)	11 (10-14)	*0.01*
Other vaso-active agent, n (%)	70 (16)	56 (20)		94 (21)	86 (31)		75 (17)	59 (21)	
VADI	2 (2-3)	3 (2-3)	*<0.01*	3 (2-3)	3 (3-4)	*<0.01*	2 (1-3)	3 (2-3)	*<0.01*
SDI	2 (0-5)	2 (0-4)		4 (0-5)	3 (1-5)		2 (0-5)	3 (1-5)	*<0.05*
MV, n (%)	336 (75)	234 (84)		352 (78)	252 (90)		336 (75)	221 (79)	
PEEP (cm H_2O)	7.25 (0-10)	8 (5-10)		8 (5-10)	8 (6-10)	*<0.05*	8 (4-10)	8 (6-10)	*<0.01*
PaO_2/ FiO_2	212 (134-291)	180 (114-274)	*<0.05*	\	\		249 (95)	220 (101)	*<0.01*

Lactate (mmol/ L)	2 (1.2-3.3)	3 (1.6-5.8)	*<0.01*	\	\		1.5 (1-2.2)	2.2 (1.5-4.1)	*<0.01*
RRT, n (%)	27 (6)	50 (18)		74 (16)	86 (31)		81 (18)	98 (35)	
Fluid balance (ml)							2000 (959-3308)	1639 (1038-3250)	

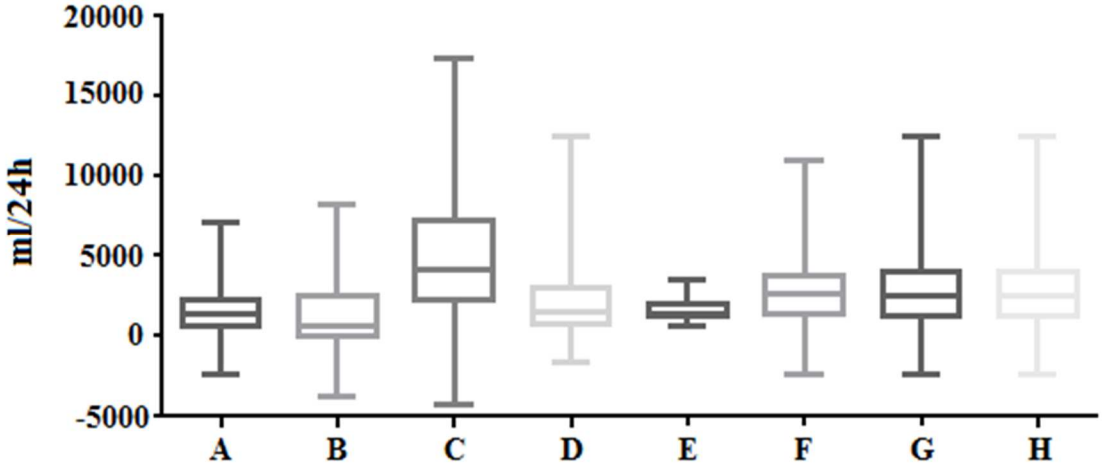
S survivors; NS non-survivor; NE norepinephrine; HR heart rate; SAP, DAP, MAP systolic, diastolic and mean arterial pressure; SOFA Sepsis Organ Failure Assessment Score; VADI Vasoactive Drug Index; SDI Sedative Drug Index; MV mechanical ventilation; PEEP positive end-expiratory pressure; RRT renal replacement therapy

Additional (supplemental) Figures:

Additional Fig 1: Sedative Drug Index [SDI] and Vasoactive Drug Index [VADI] in the eight ICUs [a) and c) show respectively SDI and VADI at T1; b) and d) show SDI and VADI at T24]



Additional Fig 2: Fluid balance of the first 24 hours of Norepinephrine infusion in the 8 centres



❖ CHAPTER 9 :

DISCUSSION AND CONCLUSIONS

Are we ready to introduce bedside assessment of microcirculation in clinical practice?

In chapter 1, we discussed the main practical aspects of bedside microvascular monitoring in critical care and we highlighted how much technology improved in the last years.

No more than 20 years ago, images were manually (semi)-quantified without any scoring system, just to identify and separate between healthy and pathological microcirculation, with a huge degree of user interaction. Afterwards, specialized software packages were developed and validated, and they increased the reproducibility of microvascular analysis and the accuracy of the results. They lowered the observer-bias and radically reduced the analysis time. The optical resolution of the video captured is now higher, and it is probably no more comparable to that of the first lenses; furthermore, the technology is constantly growing. There is, now, international consensus upon microcirculatory image acquisition and analysis, and a second consensus paper was recently published; therefore, we specifically agreed on the parameters of perfusion of microcirculation and this fact enabled the comparison of microcirculatory alterations among different studies. [1]

However, in spite of the progresses described, microvascular monitoring still represents a niche technology. Personally, I do not believe that the inter-observer bias, training requirement and costs of the equipment can be considered the responsible insurmountable obstacles to widespread of microvascular monitoring, otherwise further relevant routine technologies, like Transthoracic Echocardiography or Transcranial Doppler, could never walk through the doors of ICU! There are, however, further practical and clinical limitations to consider.

The most important practical limitation to the routine introduction of this technology is that, at the moment, there isn't any bedside device able to assess microcirculation in real-time, but it needs an offline analysis that occurs away from the patient, and does not suit for clinical therapy guidance, moreover if in emergency.

In the near future, development of novel software packages and implementation of previous algorithms will probably develop a solution for point-of-care goal-directed therapy, but no more details are available at this point. [2-3]

The main clinical limitation is that clinician feel there is uncertainty about whether the awareness of a microvascular impairment could change our clinical practice, and if treatments targeted at microcirculatory parameters could have any bearing on clinical outcomes. [4-5]

There is increasing literature describing what are the alterations of microcirculation under some critical diseases, especially in sepsis, there is consensus about the association between microvascular alterations and worse clinical outcomes, but we know very little about how to use the information.

State or art on microcirculation and recent perspectives:

From the late 1990s, when Hand-held Vital Microscopes (HVMs) were introduced in the setting of clinical research, increasing evidence was proven about the microcirculatory alterations in sepsis and septic shock and of the cause-effect correlation with some of the features of this syndrome, as already described in the introduction of this manuscript.

Nevertheless, microcirculation is not exclusively related to sepsis. Microvascular alterations, different and not completely studied, were also evident in hypovolemic and cardiogenic shock, and in other major conditions. [6-7]

In one of our previous studies (MicroDAIMON study), we performed a daily monitoring of sublingual microcirculation in 97 critically ill patients admitted in our ICU in a 9-month period of enrollment,

from April to December 2013. [8] We demonstrated that a baseline MFI < 2.6 was associated with 3 times higher ICU mortality and increased in-hospital and 90-day mortality if compared with patients with baseline MFI>2.6.

What it is extremely interesting, as collateral result, is that more than 20% of the patients showed an abnormal MFI at admission in ICU and over 55% of the patients suffered an altered MFI at least once during the admission. Our study confirmed that microvascular derangement is not rare, even in a mixed population of critically ill patients, independently from the cause of admission in ICU.

However, that study did not confirm any association of routine day-by-day microcirculatory monitoring with the main variables of outcome in terms of both organ failure (SOFA score) and mortality.

In chapter 2 we performed a subgroup analysis of the same study, on 39 (28 patients included in the main analysis) patients enrolled in MicroDIAMON and admitted in ICU for major trauma.

Differently from the main study, in this sub-population, microvascular alterations and SOFA score showed an association. Similarly to the previous paper of Tachon et al [9] that evaluated the microvascular alterations in patients with traumatic haemorrhagic shock, we focused on the first 4 days of admission in ICU and we divided the patients in two groups according with the median value of SOFA score at Day 4. The two groups did not show differences in macrohemodynamic variable (mean arterial pressure, heart rate, lactate, central venous saturation ScVO2, haemoglobin value or requirement of RBC transfusion), but the microcirculation of the group with higher SOFA score showed significantly lower parameters of vascular density (TVD and PVD) and impaired peripheral microvascular reactivity, than the group with lower SOFA score. ICU-LOS was significantly higher in patients with higher D4-SOFA score. ICU mortality was very low (three patients) and all the patients died before day 4, so we did not perform any comparison among groups.

From these results, and from previous equivalent literature, we can suggest that trauma itself is associated with microvascular impairment, that may be subsequent to the extensive tissue damage resulting from the external injury. Trauma induces release of inflammatory mediators and reactive oxygen species. The Systemic Inflammatory Response Syndrome (SIRS) probably ushers in loss of microvascular integrity, in increased vascular permeability and interstitial oedema as for SIRS from other origin, and the reduction of capillary density enhances the oxygen diffusion distance, leading to tissue hypoxemia. Therefore we can suggest that, after major trauma, microvascular dysfunction may compound other mechanisms associated too tissue damage and may deepen the risk of organ dysfunction.

These are just results from a retrospective analysis of a small sample of trauma patients, but the evidence is increased by a recent larger observational study performed by the group of Hutchings SD et al. [10] In a population of 58 traumatic hemorrhagic shocks, they demonstrated that microvascular variables acted as good prognosticators for the development of multiple organ dysfunction syndrome (MODS) and that microcirculatory hypoperfusion was associated with increased risk of organ dysfunction. They suggested microcirculation as potential endpoint of resuscitation in critical care patients admitted in ICU following traumatic hemorrhagic shock.

Hemodynamic coherence:

In the introduction of the manuscript, I described the concepts of “hemodynamic coherence” and of “loss of hemodynamic coupling”: when hemodynamic coherence is lost, the normalization of systemic hemodynamic variables does not lead to parallel improvements in microcirculation and cannot be considered as guarantee of oxygen delivery and cell oxygenation. [7]

In the study reported in chapter 2 we showed that patients with different SOFA score did not diverge in terms of main hemodynamic variables, even if they diverged in term of microcirculation.

We suggested that, in severe trauma patients, as in sepsis and other patterns of shock a “loss of coherence” between macro and micro-hemodynamic may exist, and that treatments aimed at the correction of systemic hemodynamic variables may fail to be effective on organ perfusion.

We could talk at length about the possible (multifactorial) explanations and broaden the subject to wider aspects of critical care, but I would like just to remark on one point: the patients were all stabilized at the time of assessment. What treatment did receive to reach hemodynamic stability? Patients were treated with fluids: fluid resuscitation is essential to microvascular perfusion, but not all the fluids are equal on microcirculation or to cells, fluid volume and fluid composition are important variables to be considered. [11-12] Patients were also treated with vasoactive drugs: the group on higher sofa score received significantly more vasopressors. Vasoactive drugs implicate detrimental effects at excessive dose, causing type 3 microcirculatory stasis by severe vasoconstriction. [6] Adrenergic drugs can also cause sympathetic overstimulation that is harmful for cell function (chapter 8). Third, patients required RBC transfusion: the two groups did not differ about the amount of RBC component received, and hemoglobin’s levels were similar among patients. However, in one of our previous studies (Damiani E et al) we showed that there is a large variability in the effect of blood transfusions on microcirculation in respect to quality of blood, leuco-depletion, age of RBC and storage solution. [13] Future perspective will be to implement our knowledge about these variables.

In chapter 3, we described the results of a prospective observational study where we evaluated the sublingual microcirculation of patients undergoing major surgery (elective open intrarenal abdominal aortic aneurysm repair). The patients were treated with a goal-directed hemodynamic management, and they diverged in the type of anesthesia received (balanced or totally intravenous anesthesia).

We measured both macro-hemodynamics and indices of sublingual and peripheral microvascular perfusion. We found that, differently from previous studies, the microcirculation during major surgery for aortic repair was globally preserved, independently from the type of anaesthesia and

changes in sublingual capillary density were inversely related with changes in systemic vascular resistances, reflecting a certain degree of coherence between the macro- and micro-haemodynamic responses. Literature about microvascular disturbances and hemodynamic coherence is insufficient and too heterogeneric to express any suggestions: studies focused on cardiac surgery showed a wide range of microcirculatory changes and reduced tissue oxygenation with evidence of with loss of hemodynamic coherence, while abdominal, thoracic and general surgery were associated with mixed results. [14-16]

Biomarkers of microvascular dysfunction and of organ failure

Direct visualization of the microcirculation at the bedside should be integrated to macrohemodynamic monitoring for the early recognition of loss of hemodynamic coherence in critically ill patients; but microvascular assessment is not routine, even the latest consensus cannot recommend it. [1] Is there any biomarker that can suggest when to assess for microvascular dysfunction? As previously discussed, several studies tried to identify clinical or biological marker, mainly in the setting of septic shock, without real effect.

In chapter 4 we described a very recent prospective observational study (paper still in preparation) designed to evaluate a correlation between a relatively new biomarker, named Mid-regional proAdrenomedullin (MR-proADM), and the sublingual microcirculation in patients admitted in ICU with different degrees of infection-related illness. MR-proADM is a fragment of Adrenomedullin (ADM), an endogenous peptide hormone tusked with several properties (vasodilator, positive inotropic, diuretic, natriuretic and bronchodilator, inhibitor of insulin secretion, aldosterone inhibitor and adrenocorticotropic hormone inhibitor). ADM increases in sepsis in order to stabilize the microcirculation and protect against endothelial permeability and may represent a marker of damage. MR-proADM increases consequently (1:1 ratio), and in previous studies it showed good correlation with organ dysfunction related to infection and with mortality in critical care patients.

In our study, we simultaneously evaluated MR-proADM and sublingual microcirculation at pre-determined timepoints during the first 5 days of admission in ICU. The study did not support the correlation of MR-proADM to microcirculation at admission in ICU, but it showed a good correlation of the trend in evolution of the two variables in the first 24 hours of admission in ICU. Patients where MR-proADM did not cleared in the first 24hours of treatment showed increased organ dysfunction and deterioration of the microcirculation in term of MFI. Patients in which MR-proADM cleared, showed a substantial stability of MFI and an improvement in SOFA score, while the opposite group suffered a deterioration of sublingual microcirculation in terms of MFI and showed a statistically higher SOFA score at day 5

Unfortunately, the study was not powered to correlate the three variables MR-proADM plus SOFA score plus MFI together (just 20 patients) but, for the first time, it found a correlation between the biomarker and one of the more bedside-feasible variables of microcirculation.

Future research on clinical and biological markers could guide us to understand when to monitor sublingual microcirculation in clinical practice. But thinking more optimistically, technological improvement will also help the monitoring to be also more achievable in routine clinical practice.

Immunomodulation and microcirculation in sepsis:

The management of the patient with sepsis and septic shock remains a challenge and needs to be personalized. International guidelines help in the first hours of treatment and they mainly rely on infection control and organ support. But sepsis is much more.

The systemic inflammatory response plays a central role in the pathophysiology of sepsis-induced organ failure. Understanding the importance of immune system dysregulation in the outcome of our patients, several attempts have been made in the development of immunomodulatory strategies aimed at balancing proinflammatory and anti-inflammatory mediators' responses.

Unfortunately, large randomized control trials are still lacking, and current literature provide limited evidence of the effectiveness of these therapeutic strategies. Therefore, the international guidelines cannot recommend them. [17] Specific antagonists for pro-inflammatory cytokines and treatments designed to control the early cytokines activation during sepsis have been also studied, but they failed to prove effectiveness on outcome.

Despite the low-quality level of evidence in favor of utilizing these new weapons, studies to further explore their potential applications continue to evolve.

In chapter 5 and 6 we report two studies aimed at evaluating the response of microvascular and peripheral perfusion to immunomodulatory therapies.

The first study is a prospective, randomized, double-blinded, placebo-controlled phase II trial on 20 patients with sepsis of septic shock, enrolled in the first 24 hours post diagnosis. They randomly received an intravenous infusion of IgM-enriched immunoglobulins or placebo for 72 hours and we compared pre-post evolution on microcirculation and inter-groups differences at timepoints.

The rational in administering exogenous IgM enriched immunoglobulins deepen in the pathophysiology of sepsis: immunoglobulins play important roles in sepsis because they participate to pathogen recognition and clearance, inhibition of mediator gene transcription and anti-apoptotic activities. When associated with immunosuppression with reduced circulating immunoglobulins, especially IgM, sepsis shows worse outcome. [18-19]

In our study we reported that in the group of patients treated with placebo the sublingual microcirculatory perfusion decreased in the 72 hours of inclusion (consistent with evolution of sepsis) despite a decrease of norepinephrine requirement and a substantial stability-in-time of macrovascular variables, while it increased slightly in the group of patients treated with IgM-enriched immunoglobulins.

In adding on what already covered in the limitations of the study I would like to stress that, when the study protocol was designed, there was no consensus about the target of patients where the infusion of immunoglobulins could have been effective and about the desirable plasmatic level to obtain. Based on this bias, we did not measure the plasmatic levels of immunoglobulin before infusion, nor after, so we don't know how many patients reached the target. Probably not many, if I can rely on my experience! We can also suppose we included both patients that may have taken advantage on the infusion and patients who did not need it at all. Therefore, we can suggest that e-IgM immunoglobulins affect the microcirculation favourably, but we are not allowed to definitively express the efficacy of IgM-enriched immunoglobulins on microcirculation, and further studies are needed.

Very recently, a multidisciplinary meeting of Italian Experts in Infectious Diseases, Anaesthesia and Critical Care, Respiratory Disease Physicians, Microbiology and Onco-haematology proposed a position paper on IgM-Enriched Intravenous Immunoglobulin Adjunctive Therapy in Bacterial Infections, where they created a score, The TO-PIRO SCORE, to suggest how to better use e-IgM immunoglobulins. [21] The score was titrated on differentiating between types of infections, variables of severity and aetiologies. It would be of great interest to perform a new study to replicate the one already described, taking into account the TO-PIRO score and the adjunctive and innovative knowledges about immunomodulation in sepsis. Furthermore, our group will participate on a multicentre randomized controlled trial that will compare the outcome of septic patients treated with a standard regimen of IgM enriched immunoglobulins or a personalized one.

I believe that the search for new immunomodulatory drugs will be extremely facilitated by better characterization of patients and personalization of the treatments.

In chapter 6 we report the results of a small pilot prospective observational study on the adjunctive therapy with Cytosorb (CytoSorbents Corporation, Monmouth Junction, NJ, USA). The aim of the

study was to assess if the haemoadsorption with CytoSorb, by removing circulating inflammatory mediators may contribute to restore microvascular perfusion on septic or septic shock patients.

Sepsis is associated with the release of multiple inflammatory mediators and molecules, including cytokines, chemokines, and coagulation factors. Extracorporeal blood purification techniques have evolved in recent years as a potential therapy for the purpose of immunomodulation: beyond conventional continuous renal replacement therapies, they allow high volume hemofiltration with high cut-off membranes and hemoadsorption components that target on removal of inflammatory mediators (and endotoxins in different membranes).

The sample size of our study was very low (9 patients), but we showed an increase in sublingual microcirculatory density and an improvement over time of microvascular flow of patients undergoing CVVHD plus 24-h treatment with CytoSorb hemoadsorber. The findings support the hypothesis that extracorporeal cytokines' removal during sepsis may produce beneficial effects on immuno-mediated microvascular dysfunction.

In chapter 7, I described the study protocol end preliminary results of a prospective observational study on 50 pyrexical septic patients treated with paracetamol (acetaminophen) as temperature control measure. The aim of the study is to examine the response of sublingual and peripheral perfusion to paracetamol infusion, according with the previous and subsequent plasmatic levels of cell free hemoglobin. We decided to enrich our study with a panel of biological markers of oxidative stress and endothelial damage and we also included the evaluation of PD1/Pd-L1 and of pharmacogenomic phenotypes. The study completed the recruitment and we are now analyzing the massive amount of data. Preliminary results of the ad interim analysis are descripted in chapter 7, but we are still working on the latest blood samples (on immunoessays and colorimetric techniques). What we expected and we confirmed from preliminary data is that septic patients are highly heterogeneous, not only in terms of demographic characteristics (age, sex, comorbidities), source of infection and causative microorganisms, patterns of organ dysfunction and clinical response to treatment, but they also differ

in term of biological and immunological markers and pharmacogenomic, and the degrees of the variability weights in a different way during the course of sepsis. It confirms the relevance of a wider characterization of the individuals to personalization of the therapy.

Adrenergic overstimulation in septic shock: is there any place for patient-tailored therapy?

As described in the introduction of the manuscript, the study reported in chapter 8 will not include microvascular monitoring, but I think it earns a position inside this manuscript because it evaluates the response of the organism to "stress", and researches a marker of excessive adrenergic stimulation in septic shock to learn how to recognise excessive exogenous sympathetic stimulation and reduce the iatrogenic effect on already "overstressed" patients.

Septic shock is associated with a major neuroendocrine and hormonal response, that is similar to the innate reaction of the body to stress and that is characterized by massive release of endogenous catecholamines (as part of multiple effector response). When exogenous amine's infusion compounds the endogenous component, excessive stimulation of sympathetic receptors may lead to systemic toxicity, showing higher mortality and morbidity in septic shock.

According to the Surviving Sepsis Campaign guidelines 2016 norepinephrine is the first-choice vasopressor in septic shock, [17] however it has been associated to multiple complications: tachyarrhythmia and direct cardiotoxicity, digital and splanchnic ischemia, metabolic derangement, insulin resistance, muscle catabolism, immunosuppression and enhanced bacterial growth. [21-22] We already stated that excessive vasopressor therapy can also be responsible for detrimental effect of microvascular, peripheral and organ perfusion.

When other physiological causes of tachycardia can be reasonably excluded (anxiety and agitation, fever and inadequate sedation), an increased Heart Rate (HR) related to infusion of high dose norepinephrine may represent an early marker of aminergic overstimulation.

This study aimed to evaluate if HR was associated with outcome (mortality in the ICU) in septic shock patients when evaluated during the first 24 hours of infusion of Norepinephrine (NE). It aimed to verify if there was any difference in the association between outcome and tachycardia in patients receiving high levels of exogenous norepinephrine compared to those who receive low doses. The study was a retrospective analysis of data, and we will need prospective studies to find a concrete correlation, but the population was very large, and the association was significant.

While it is not surprising that patients who immediately required high dosages of Noradrenaline demonstrated higher mortality than patients who required lower doses of support, it could be relevant that for the same rate of norepinephrine received, when the amine was infused at high dose, early tachycardia demonstrates a relevant relationship with the mortality in ICU.

There are many possible interpretations to this result: tachycardia in response to high doses of norepinephrine may represent an early prognostic factor for negative outcome in septic shock; moreover, when other main physiological causes of tachycardia can be excluded, heart rate can be considered as a clinical marker "catecholaminergic overstimulation" and excessive exposure to Catecholamines.

Patients who require high amounts of norepinephrine to obtain the target of blood pressure and that respond to the infusion with tachycardia (and if we think we corrected hypovolemia!), probably show they have already saturated all the compensation mechanisms related to adrenergic receptor with the endogenous response to stress. In that case, they will not receive any benefit from higher exogenous infusion, and they are rather showing toxicity. In that case, we could also discuss about possible alternatives to catecholamines: for example, the International Surviving Sepsis Campaign guidelines suggest vasopressin (up to 0.03 U/min) as second line vasopressor in addition to norepinephrine with the intent of reaching the target of MAP, or to decrease norepinephrine dosage. [17] We could suggest that it would not be a standardized cut-off of noradrenaline to guide the introduction of vasopressin, rather the response of the single patient to noradrenaline. β1-selective beta-blockers could be also

considered in selected patients, as they showed benefic effect to survival of septic shock, when introduced very early. [23] Independently from the possible alternatives of treatment, the strong criticism would always be to select the right patient for the right therapy.

Toward patient-tailored therapy: the role of microcirculation

This manuscript represents a comprehensive synopsis of my research project as PhD student and of my path of professional growth in the field of clinical research.

It describes clinical studies designed and performed with a common objective and interest: to evaluate the relevance of microvascular assessment in in the patient-tailored hemodynamic monitoring of critically ill patients.

A deep knowledge of the pathophysiology of critical illness represents an essential cornerstone toward the introduction of a patient-tailored approach in intensive care. Could the microvascular assessment add anything to the acquisition of the concept of patient-tailored therapy of critically ill patients?

The observations of our studies support the idea that we need to be able to "identify the right patients for the given intervention at the right time" and that some treatments may only be beneficial in certain patients and should therefore be administered on an individual patient-by-patient basis. [24]

Microvascular derangements have been identified and connected with most of the situations of critical illness. With our studies we support the idea that the microvascular monitoring in critical care, could be of clinical advantage to recognize different patterns of patients, to research for unbalances between macro and micro-hemodynamic, to recognize the loss of hemodynamic coherence, to uncover discrepancies between what we give to the patients and what the cells receive.

Providing microvascular assessment would be relevant to recognize pattern of microvascular dysfunction and to target treatments toward microvascular shock. Dysfunctioning endothelial cells or

parenchymal cells may also require tissue protective and specific therapy. [ ] Thus, it appears urgent to develop bedside devices able to assess microcirculation in real-time to better titrate the conventional therapies of hemodynamic resuscitation. [ ]

We believe that the next step toward bedside introduction of microvascular monitoring will be to perform prospective studies appropriately powered to target the microcirculation as a clinical end point of resuscitation.

Careful characterization of critical care patients, especially septic ones, would enable future research to more personalized selection of the population for clinical trial designs (including only those patients most likely to respond to the intervention under study) and would also enable clinicians to more patient-tailored treatments.

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