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Microcirculatory perfusion-based approach to the critically ill patient: bringing a research tool technology to the bedside

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"Rifiutate di accedere ad una carriera solo perché vi assicura una pensione. La migliore pensione è il possesso di un cervello in piena attività, che vi permetta di continuare a pensare usque ad finem, fino alla fine."

Rita Levi Montalcini

A me stessa,

alla mia testardaggine,

alla mia perseveranza.

CONTENTS

Chapter 1

Introduction and outline of the thesis

INTRODUCTION

The microcirculation is defined as the intricated net of vessels smaller then 100 μm, including thus arterioles, venules and capillaries. This is an essential functional unit of the organs, representing the site of gasses and nutrients exchange between blood and tissues.

After pioneer studies carried out by Leeuwenhoek and Krogh between 1700 and 1950 about the physiologic aspects of microcirculation, especially in animals [1,2], the research in this field gained an enthusiast attention.

The study of microcirculation in human beings by using techniques of intravital microscopy is almost 20 years old: in 1999 Groner et al [3] gave a first insight into this fascinating and extremely complex world, giving the birth to the first non invasive imaging technique to observe microcirculation in vivo and real-time: the orthogonal polarization spectral imaging (OPS). This technique allowed to image the microcirculation in a large diversity of organs and tissues surfaces in multiple surgical and clinical settings by condensing the mechanical and optical components in microscopes of smaller dimensions, capable to configure an instrument usable at the bedside. [4,5] In 2007 the second-generation technology, the Sidestream Dark Field (SDF), takes its place, with the purpose of solving some of the major pitfalls of the OPS system. [6] A system of green light LED (530 nm wavelength), corresponding to the adsorbing spectrum of the hemoglobin, disposed in a ring form, produce a pulsatile illumination and an analogic video camera captures the reflecting light and produces video imaging of the microcirculation, which could be directly send to a monitor and visualized in real time. Brightness and focus can be manually adjusted, such in a conventional camera. The main advancements of this technique were in better video quality (ameliorated optical system which leads to less disturbances in signal) and maneuverability (including smaller size and inner battery) both in experimental and clinical settings. The probe of this device, covered by a sterile disposable cap, can be gently applied to organs or mucosal surfaces to observe the microcirculation.

The sublingual region is the most investigated by researchers: it is a site covered by a thin lay of epithelial cells and its perfusion derives from extern carotid artery and lingual and sublingual arteries and share the embryogenetic origin with intestine and splanchnic circulation [7] and warrants a rapid and easily accessible window to evaluate human microcirculation, also in critical conditions, without performing invasive maneuvers. However, many other sites have been investigated such as conjunctiva, mucosa of the intestinal tract, stomas, vaginal mucosae and, in surgical settings, also cerebral cortex [8-11].

Next to in vivo videomicroscopy, other technologies give information about the status of microcirculation. Near Infrared Spectroscopy (NIRS) is one of them. Apply in conjunction with a vascular occlusion test of the forearm, it enables the assessment of tissue oxygenation and microvascular reactivity on the skeletal muscle [12].

The development of such instruments of direct in vivo visualization of the microcirculation, lead to a rapid increasing in production of research studies aiming to clarify the mechanisms underlying shock, hemodynamic impairment and many other conditions of organ dysfunction and VO2/DO2 mismatch. Seen the high complexity and severity, the critically ill patient represents the most investigated subject in the field of microcirculation.

In recent studies, many selected subpopulations of critically ill patients showed a consistent incidence of microcirculatory abnormalities when observed with the previously described techniques and were associated with unfavorable clinical evolution, higher morbidity and mortality. [13-22]

The recent microSOAP study, provides the largest prospective observation of the point-prevalence of microcirculatory alterations in a mixed population of 501 adult critically ill patients, involving 36 ICUs all over the world. [23] In such an heterogeneous population, observed in a single time-point in all the participating centers, 17% of the patients showed abnormal microvascular flow (identified as a Microvascular Flow Index inferior to 2.6 AU); even if it did not resulted to be associated to adverse outcome in the overall population, it seems to maintain prognostic significance in a subgroup of high risk patients, identified by the presence of tachycardia (heart rate > 90 beats per minute).

The most extensively investigated clinical condition is sepsis. Oxidative stress, inflammatory mediators and cytokines during systemic inflammation induce alterations in the physiologic endothelial function: disruption in the glycocalyx barrier, increased vascular permeability with generation of tissue oedema, enhanced endothelium-leucocyte interaction, formation of red blood cell aggregates and microthrombi with disseminated intravascular coagulation, resulting in impaired microvascular blood flow and hypoperfusion [24-25]. This leads to the typical pattern of sublingual microcirculation during sepsis: an extremely heterogeneous flow due to a patchy distribution of capillaries with no flow next to areas of normal flow associated with a varying vessel density causing an important impairment of convective and diffusive oxygen transport to the cells and, ultimately, organ dysfunction.

These alterations are barely detected by macro-hemodynamic variables and, conversely, the normalization of macro-hemodynamic variables with standards treatment (such as blood products [26], vasopressors [27], inotropes [28], etc) cannot always restore the microcirculatory perfusion,

thus failing to achieve the final goal of restoring tissue oxygenation and configurating the loss of hemodynamic coherence [29], especially in septic patients. For all these reasons, it is important to understand the microvascular response to commonly applied interventions, as well as the potential role of the microcirculation as a therapeutic target.

Many efforts have been made to bring this more physiologic approach to the bedside, especially within the intensive care units, but the step has not been made yet due to a series of technical limitations, time-consuming off-line analysis of the videos to obtain most of the microcirculatory variables of flow and vessel density, and concerns about the reproducibility of the results because of inter-observer variability. Moreover, many studies intended to monitoring the effects of common treatments on microcirculation, shown conflicting results, partly due to great variability in patients' selection and stratification, quantification of responses and timing. Finally, it remains to be elucidated weather an improvement of microcirculatory dysfunction can lead to an improvement of patient outcome.

OUTLINE OF THE THESIS

This research project aimed to explore the advances in present technology for microcirculatory imaging, depicting its advantages and pitfalls in developing knowledges in this fields and in connecting research to clinical practice. For this purpose, all the work was focused on trying to answer these questions: How could microcirculatory monitoring improve the management (diagnostics, treatment and, thus, prognosis) of our critically ill patients? Are we ready to transform a pure research tools in a clinical instrument to use at the bedside in the daily practice?

The clinical studies presented in this manuscript represent part of the research work within the Hemodynamic Research Group, operating in the Clinic of General, Respiratory and Major Trauma Intensive Care of AOU Ospedali Riuniti di Ancona. Two studies are products of the cooperation with the researchers of the Intensive Care Unit of Medisch Centrum in Leeuwarden, the Netherlands. during a year stage.

Chapter 2 presents the validation study for the newest technology for microcirculatory imaging, the Incident Dark Field illumination (IDF), providing a detailed comparison with the gold standard Sidestream Dark Field illumination (SDF) in a population of healthy volunteers.

Chapter 3 discusses the technical and executional issues which potentially affect the microcirculatory imaging and the interpretation of derived data. This represents the first

systematical analysis on SDF videomicroscopy video quality and describe the potential influence of this aspect in evaluating and interpreting microvascular density and perfusion.

Chapter 4 shows the results of a prospective observational study where NIRS monitoring was performed daily in a population of critically ill patients along their entire admission to the Intensive Care Unit, exploring the relationship between NIRS-derived parameters and outcome.

The MicroDAIMON study, is currently the largest prospective longitudinal observational study to describe the incidence of microcirculatory derangements among a mixed group of critically ill patients, offering a day-by-day follow-up. In Chapter 5, this study, shows the results of a microcirculatory daily monitoring and investigate the relationship between microcirculatory abnormalities and outcome, exploring the integration of data about capillary density and perfusion into a set of macroscopic clinical variables to predict mortality.

In a subgroup analysis of the MicroDAIMON study, Chapter 6 focused the attention on microcirculatory monitoring in multiple trauma patients, correlating microvascular variables and tissue perfusion to the evolution of organ dysfunction in the first 4 days of admission to the Intensive Care Unit. This analysis aimed to explore if adequately resuscitated traumatic patients, who still present impaired microvascular perfusion or altered tissues oxygenation, could develop more severe organ dysfunction or unfavorable prognosis.

In previous studies, many pharmacologic approaches have been proposed to improve microvascular density and perfusion. More recently, some vasodilatory agents have been investigated for their capacity to recruit impaired microcirculation. Chapter 7 explore in an open-label pilot study, the effects of ketanserin, a serotonin receptor antagonist, which represent an interesting molecule, seen its vasodilatory, antithrombotic and anti-inflammatory properties which could produce pleiotropic effects on microcirculation.

Chapter 8 gives an overview on the entire research project and describes the future perspectives in research in the field of microcirculation.

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Chapter 2

Cytocam-IDF (incident dark field illumination) imaging for bed-side monitoring of the microcirculation

Cytocam-IDF (incident dark field illumination) imaging for bed-side monitoring of the microcirculation.

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ABSTRACT:

Background: *Orthogonal polarized spectral (OPS) and sidestream dark field (SDF) imaging video microscope devices were introduced for observation of the microcirculation but, due to technical limitations, have remained as research tools. Recently, a novel handheld microscope based on incident dark field illumination (IDF) has been introduced for clinical use. The Cytocam-IDF imaging device consists of a pen-like probe incorporating IDF illumination with a set of high-resolution lenses projecting images on to a computer controlled image sensor synchronized with very short pulsed illumination light. This study was performed to validate Cytocam-IDF imaging by comparison to SDF imaging in volunteers.*

Methods: *This study is a prospective, observational study. The subjects consist of 25 volunteers.*

Results: *Sublingual microcirculation was evaluated using both techniques. The main result was that Cytocam-IDF imaging provided better quality images and was able to detect 30% more capillaries than SDF imaging (total vessels density Cytocam-IDF: 21.60 ± 4.30 mm/mm^2 vs SDF: 16.35 ± 2.78 mm/mm^2, $p < 0.0001$). Comparison of the images showed increased contrast, sharpness, and image quality of both venules and capillaries.*

Conclusions: *Cytocam-IDF imaging detected more capillaries and provided better image quality than SDF imaging. It is concluded that Cytocam-IDF imaging may provide a new improved imaging modality for clinical assessment of microcirculatory alterations.*

KEY WORDS:

Microcirculation, SDF imaging, Cytocam-IDF imaging, intra vital microscopy, sepsis.

BACKGROUND

Microcirculation is the main means of oxygen delivery to tissue cells and is essential for the maintenance of cellular life and function. Its function relies on the complex interaction of its component cellular systems, including red and white blood cells, endothelial, smooth muscle, and parenchymal cells. The function of the organs is directly dependent on the function of their respective microcirculation, and achievement of good microcirculatory function can be considered to be the prime goal of the cardiovascular system and of particular importance to critically ill patients, especially ones who are in shock [1]. Many studies have demonstrated that persistent microcirculation alterations that are unresponsive to therapy are independently associated with adverse outcome, especially in septic patients [1-5]. Additionally, these microcirculatory alterations have been shown in various studies to be independent of systemic hemodynamic variables, making the observation of microcirculation a potentially important extension of the conventional systemic hemodynamic monitoring of critically ill patients [3,4].

In the early 20th century, direct intravital observation of human microcirculation was limited to the use of bulky capillary microscopes, which were mainly applied to the nailfold capillary bed. In 1964, Krahl made use of incident light directed at an oblique angle to the study tissue surfaces [6]. In 1971, Sherman et al. introduced a new method of microcirculation observations called incident dark field illumination (IDF) microscopy. This method enabled observations of organ surface microcirculation using epi-illumination, without the need for transillumination of the tissue from below [7]. An alternative method to observe microcirculation using epi-illumination was introduced by Slaaf et al., enabling the imaging of subsurfaces using cross polarized light microscopy [8]. In the late 1990s, Groner et al. adapted the Slaaf et al. technique to a handheld video microscope [9]. This method was called orthogonal polarization spectral (OPS) imaging. We validated and introduced this technique to patients and were able for the first time to produce organ surface microcirculation images in surgical patients [10,11]. This technique opened up the field of studying human microcirculation in organ and tissue surfaces at the bedside especially in critically ill patients.

OPS imaging can be regarded as the first generation handheld bedside imaging instrument to be applied to critically ill patients, resulting in general recognition that microcirculation is an important physiological process that is compromised during critical illness and needs to be monitored in a clinical environment [12,13]. OPS imaging was improved upon by our development of a second generation handheld analogue video microscopes based on sidestream dark field (SDF) imaging [14]. Its advancement was that it provided better images than OPS imaging and allowed

battery operation, making the device more mobile than its predecessor. A device similar to SDF imaging device fitted with a USB extension called the Capiscope was also recently introduced [15]. These devices, however, remained research tools mainly due to the technological limitations preventing operator independent reproducible measurements and the inability to achieve automatic microcirculation analysis for quantification needed for clinical decision making [16-18]. Analysis of the images to extract relevant functional microcirculatory parameters required time-consuming off-line analysis [16] precluding their use in bedside clinical decision making and in titrating therapy to reach microcirculatory end points [19].

Cytocam-IDF imaging can be regarded as third generation handheld microscope because it employs a completely new hardware platform where a high density pixel- based imaging chip and short pulsed illumination source under computer control synchronizes and controls illumination and image acquisition. The device consists of a pen-like probe incorporating IDF illumination with a set of high-resolution lenses projecting images on to a computer controlled high-density image sensor synchronized to an illumination unit. The probe is covered by a sterilizable cap. Cytocam-IDF imaging is based on the IDF, a principle originally introduced by Sherman and Cook [7]. It further incorporates a stepping motor for quantitative focusing as well as high-resolution optics.

In the first part of this study, Cytocam-IDF imaging is validated by quantitative comparison of microcirculatory parameters to SDF imaging in sublingual tissue using specialized image processing software developed earlier by us [20]. In addition, Cytocam-IDF and SDF images of one and the same sublingual microcirculatory area were obtained to directly compare the image quality to each other in the second part of the study. This feature allows serial measurements to be made without the need to refocus, an important feature with respect to previous generation devices which require time-consuming manual adjustment of focus dials.

Subjects:

Twenty-five healthy volunteers (8 male and 17 female) between the ages 23 and 55 were recruited. None of the subjects had history or evidence of disease or were taking drugs that are known to affect microcirculatory function.

METHODS

SDF imaging:

In SDF imaging (Microscan, MicroVision Medical, Amsterdam, The Netherlands), illumination is provided by surrounding a central light guide with concentrically placed light-emitting diodes

(LEDs) to provide sidestream dark field illumination [14]. The magnification lens in the core of the light guide is optically isolated from the illuminating outer ring, thus preventing tissue surface reflections. Light from the illuminating outer core of the SDF probe penetrates the tissue and illuminates the tissue-embedded microcirculation by scattering. The LEDs use green light (530 nm wavelength) corresponding to an isobestic point in the absorption spectra of oxyhemoglobin and de-oxyhemoglobin. The LEDs provide pulsed illumination to overcome the inter- lacing of the analogue video cameras used. The SDF device with a total weight of 320 g is fitted with a 5× objective lens. It is based on an analogue video camera which allows its output to be directly connected to a television monitor. For analysis of the video sequences, images need to be digitized using an external analogue to digital converting device and then analyzed off-line using specialized image-processing software [20]. Illumination intensity and image focus are adjusted manually by a dial on the devices. The probe, covered by a sterile disposable cap, can be placed on organ and tissue surfaces to observe the microcirculation.

Cytocam-IDF imaging:

Cytocam-IDF imaging (Braedius Medical, Huizen, The Netherlands) consists of a combination of IDF illumination with optical and technical features optimized for visualization of the microcirculation on organ surfaces. It uses incident dark field illumination [7] with high-brightness LEDs with a very short illumination pulse time of 2 ms. The image acquisition and sensor are under computer control and electronically synchronized to the illumination pulses. This feature, in combination with a specialized set of lenses, projects images onto a computer controlled image sensor and results in high penetration sharp contour visualization of the microcirculation showing flowing red and white blood cells. The device is constructed of aluminum and titanium, resulting in a lightweight (120 g) and pen-like instrument (length 220 mm, diameter 23 mm). The cam- era is fully digital with a high-resolution sensor, which is used in binning mode, resulting in a 3.5 megapixel frame size. The combination of an optical magnification factor of 4 and the large image area of the sensor provides a field of view of 1.55 × 1.16 mm about three times larger than the field of view of previous devices (see Figure 1).

The optical system provides an optical resolution of more than 300 lines/mm.

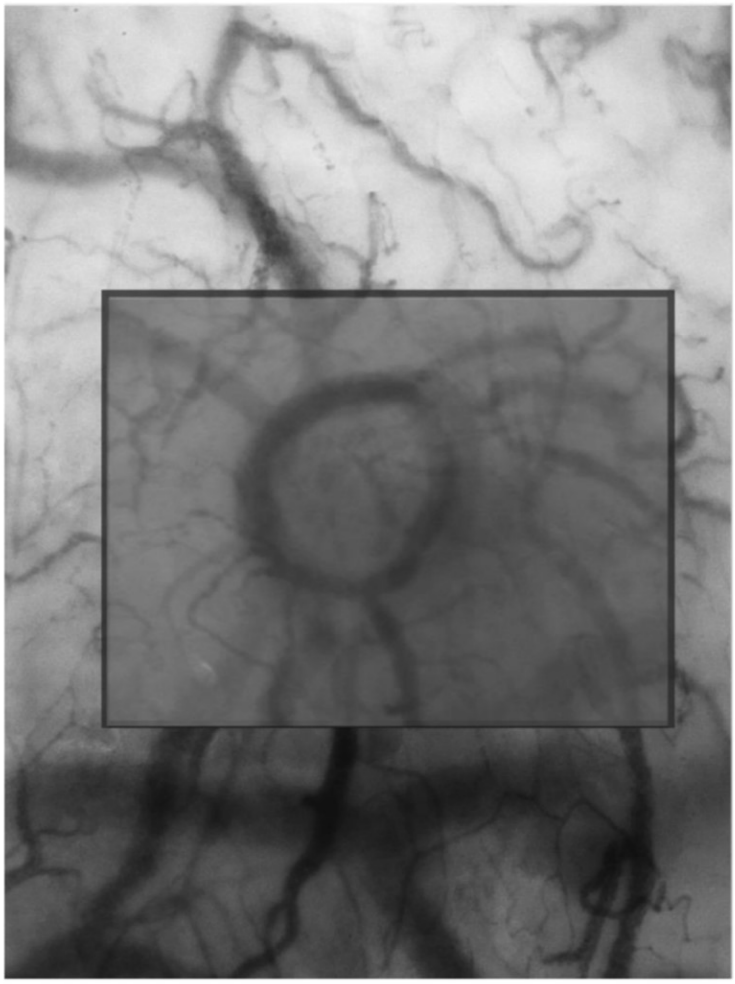

Figure 1 Smaller SDF image in larger Cytocam-IDF image. This figure shows the field of view of SDF and Cytocam-IDF imaging superimposed on each other showing the larger field of view offered by the larger image sensor used by the later technique.

The camera is connected to a device controller based on a powerful medical grade computer that is used for image storage and analysis. The device controller includes a camera adapter with a dedicated microprocessor for controlling the camera. Additionally, the camera adapter enables high-speed data transfer between the camera and controller. The Cytocam-IDF imaging device is supplied with an analysis application for quantification of microcirculatory parameters. The digitally recorded images can be analyzed automatically. It is also possible to analyze the recorded files off-line, as we did for this study. A novel feature of the device is its quantitative focusing mechanism, using a piezo linear motor with an integrated distance measuring system to position the sensor within 2 μm. Investigation has shown that each person has a characteristic depth of focus, which allows serial measurements to be made by pre-setting the characteristic focused depth [21].

Protocol:

Comparison of microcirculatory parameters:

The volunteers were evaluated in a supine, 30° head-up position. Room temperature was kept between 19°C and 22°C. Demographic data (age, gender, weight and length), blood pressure, and heart rate were recorded. Blood pressure was measured noninvasively, and heart rate was recorded by plethysmography. The microvascular measurements were obtained as a single measurement in the sublingual mucosa in three different areas with SDF and Cytocam-IDF imaging without special preparation of the mouth. The probe was handheld and adjusted by experienced operators (GA and GV) to obtain optimal image quality. With the SDF technique, after adequate focus and contrast adjustment, steady images of at least 15 s were acquired and recorded on a digital videotape (Sony Video Walkman GV-D 1000E; Sony, Tokyo, Japan), which digitizes the analogue SDF images prior to video storage. The images were captured in representative AVI format video clips (Sony DVgate; Sony, Tokyo, Japan) to allow off-line computerized image analysis using specialized software we had previously developed for this purpose [20]. To use the Cytocam-IDF imaging device, the optimal focus depth and contrast were first adjusted. Steady images of 6 s were acquired and computer stored. The image clips were exported for analysis using the same image analysis software used for the SDF images [20]. Analysis took into account the different magnification of the images by the two different techniques (4× magnification in Cytocam-IDF imaging versus 5× magnification in SDF imaging.

Analysis:

Comparison of microcirculatory parameters:

The perfusion of a tissue depends on the number, distribution, and diameters of the capillaries in combination with blood viscosity and driving pressure across the capillaries. There are two main hemodynamic principles governing how oxygen in red blood cells reaches the tissue cells; the first is the convection based on red blood cell flow, and the second is the diffusion distance oxygen must travel from the red blood cells in the capillaries to the parenchymal cells [19]. Convection is quantified by measurement of flow in the microvessels, and diffusion is quantified by the density of the perfused microvessels (functional capillary density).

Subsequent image analysis was performed using microvascular density (total or perfused vessel density) and microvascular perfusion (proportion of perfused vessels and microcirculatory flow index) parameters in line with international consensus [22]. Software assisted analysis (AVA 3.0; Automated Vascular Analysis, Academic Medical Center, University of Amsterdam) was used on

the images [20]. The analysis of the microvascular density was restricted to vessels with a diameter <20 μm.

The total vessel density (TVD; mm/mm^2) was determined using the AVA soft- ware. A semiquantitative analysis previously validated [23] but assisted by the AVA software was performed in individual vessels that distinguished among no flow (0), intermittent flow (1), sluggish flow (2), and continuous flow (3). A value was assigned for each vessel. The overall score, called the microvascular flow index (MFI), is the average of the individual values [24]. The proportion of perfused vessels (PPV) was calculated as the number of vessels with flow values of 2 and 3 divided by the total number of vessels. Perfused vessel density (PVD) was determined as the total vessel density multiplied by the fraction of perfused vessels [22]. Analyses of all images were done off-line and blinded to the investigators.

Sublingual microcirculatory image contrast and sharpness analysis:

In the same way we had compared OPS imaging to SDF imaging [14], we evaluated image contrast and sharpness using image analysis software (ImageJ; developed at the US National Institutes of Health, www.nih.gov). Five venules and six capillaries found in one sublingual location, measured sublingually by the two cameras; the capillary and venular contrast, sharpness, and quality were calculated. To determine capillary contrast with respect to the surrounding tissue, cross-sectional grayscale histograms (grayscale value 0 corresponds to black, and 255 corresponds to white) were obtained. The lowest gray value in the capillaries (I min) and the highest gray value in the tissue left (I max, left) and right (I max, right) of the capillaries were measured. The increase of the maximum slope angles α left and α right of the slopes of the gray value at the capillary-tissue interfaces was calculated. Histogram points taken for analyses are presented in Figure 2.

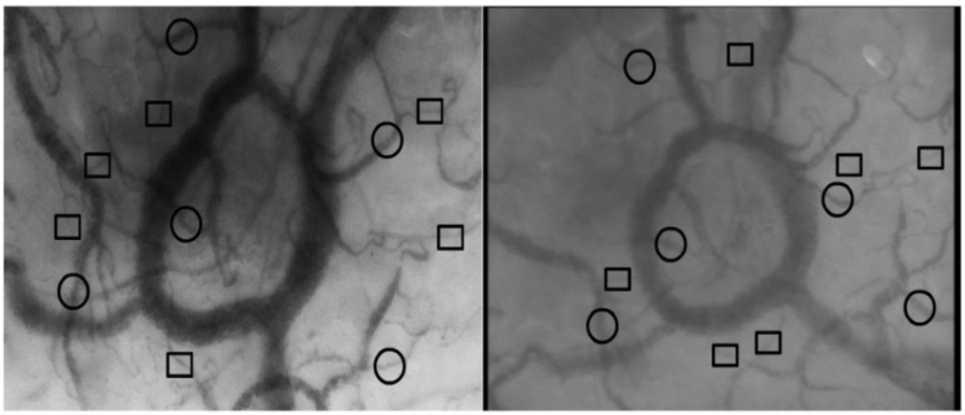

Figure 2 Histogram points taken for analyses; square capillary; circle venule; left Cytocam-IDF imaging; right SDF-imaging.

Statistical analysis

Statistical analyses were performed using SPSS statistical software, version 21 (version 18/21, SPSS Inc., USA). The Kolmogorov-Smirnov test was used to test whether the data were distributed normally. After identifying a normal distribution, the density parameters were compared by the student's t test. As the perfusion parameters did not show a normal distribution, a nonparametric test (Mann-Whitney U) was used to compare these parameters. Data are presented as the mean and standard deviation unless otherwise specified. A p value < 0.05 was considered statistically significant.

RESULTS:

Baseline characteristics are presented in Table 1. Tests for normality showed that TVD and PVD had normal distribution. The vascular density parameters TVD and PVD were significantly higher with Cytocam-IDF imaging than with SDF imaging (TVD-Cytocam-IDF: 21.60 mm/mm^2 ± 4.30 mm/mm^2 vs TVD-SDF: 16.35 mm/mm^2 ± 2.78 mm/ mm^2, $p < 0.0001$ and PVD-Cytocam-IDF: 21.50 mm/mm^2 ± 4,38 mm/mm^2 vs PVD-SDF: 16.24 mm/mm^2 ± 2.81 mm/mm^2, $p < 0.0001$). Boxplots are presented in Figure 3.

The perfusion parameters MFI and PPV did not differ significantly between the two techniques (Table 2), and the Bland-Altman plot showed no clinically significant bias. Bland-Altman plots are included in the Additional file 1.

Table 1: Baseline caracteristics.

Variable	Results
Age, [years]	33 [27.5-46.0]
Gender, male [n]	8
Systolic blood pressure, [mm Hg]	130 [119-141]
Diastolic blood pressure, [mm Hg]	74 [66-87]
Mean arterial blood pressure, [mmHg]	92 [84-102]
Heart rate, [beats per minute]	70 [65-77]
Weight, [kg]	73 [63-78]
Height, [cm]	173 [170-180]

Sublingual microcirculatory image contrast and sharpness analysis.

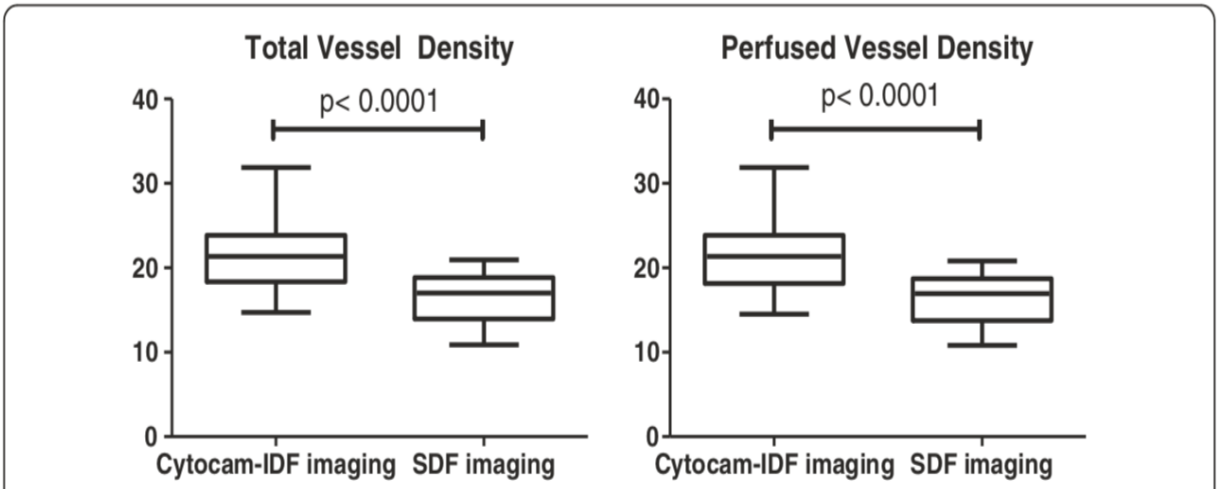

Figure 3 Boxplots of TVD and PVD.

Cytocam-IDF image quality from the sublingual area is significantly better in the capillaries and venules than the SDF image quality. (Table 3). The quality score was obtained based on contrast and sharpness, both of which are significantly improved with the Cytocam-IDF imaging in both capillaries and venules.

Table 2: Microcirculatory parameters

	Cytocam	SDF	p
MFI small	3.0 [3.0-3.0]	3.0 [2.96-3.00]	0.289
MFI large	3.0 [3.0-3.0]	3.0 [2.96-3.00]	0.494
TVD, mm/mm^2	21.60 ± 4.30	16.35 ± 2,78	< 0.0001 *
PPV, %	100 [99-100]	99 [99-100]	0.368
PVD, mm/mm^2	21.50 ± 4.38	16.24 ± 2.81	< 0.0001 *

*MFI small: < 20 um; MFI large: > 20um. * p < 0.05 is the cut off value for statistical significance.*

DISCUSSION:

In this study, we validated Cytocam-IDF imaging, a third generation novel lightweight computer-controlled imaging sensor-based handheld microscope, by comparing it to a second generation device, SDF imaging. Our results showed that Cytocam-IDF imaging visualized more (30%) microvessels as quantified by measurement of total vascular density parameters in the sublingual microcirculation than did SDF imaging. In addition, our study showed that Cytocam-IDF imaging provided improved image quality with respect to SDF imaging in terms of contrast and image sharpness. Similar results were found in a recent different preliminary validation study comparing Cytocam-IDF imaging to SDF imaging in neonates [25].

It is likely that the significantly higher vascular density, as observed with the new Cytocam-IDF technique in comparison to SDF imaging, is the direct result of the observed increase in contrast and sharpness, due to the improved magnification lens and high-resolution sensor in combination with more precise quantitative focusing. Furthermore, since the new device is fully digitalized, there is no loss of image quality in the conversion process from analogue to digital. Analogue cameras have the disadvantage of alternatively scanning odd and even video lines, resulting in a loss of resolution in the time domain as is the case in the SDF camera. An alternative explanation could be the reduction in pressure artefacts in the lighter Cytocam-IDF device in comparison to the much heavier SDF device resulting in compressed microvessels becoming now more visible. We think, however, that this is unlikely since pressure artefacts are mainly reflected in a reduction in red blood cell velocity characteristically in the larger, venular, vessels. Since this study was performed in healthy individuals, such flow abnormalities should be absent, as is the case in the observations in both systems. Therefore, it seems likely that the observed difference in capillary density is not related to reduction pressure artefacts.

This conclusion is in line with the second important observation of the study, the absence of difference in variables of red blood cell velocity, such as MFI and PPV. Previous publications report a ‘normal’ MFI in healthy volunteers, equal or close to 3, and a PPV close to 100% [4,5,26,27]. Therefore, the absence of difference in MFI and PPV between the two methods can be considered as an important quality check of the observations found in the present study.

Although the visualization of vessels with the new device is based on the same physical principal of indirect background illumination applied in all such devices, there are clearly new features with relevance to the development of research in this field. The high-resolution sensor combined with lenses especially made for microcirculatory imaging makes the optical resolution higher than the SDF system. As a result, more capillaries become visible (Figure 2), with substantial implications

for the observation of the microcirculation in several disease states. This also has the potential to observe smaller structures such as vessel wall abnormalities and possibly the endothelial glycocalyx. The new quantitative focusing mechanism not only allows more precise focusing but also maintains optimal focusing depth throughout a measurement allowing multiple observations to be made over time without the need to refocus each time a measurement is made. Focus of ongoing research, i.e., measurements for extended periods of time on one and the same spot, is currently not possible, especially in the non-sedated patients. As such, the potential to maintain optimal focusing depth reduces the variability in observations. The potential for development of treatment based on the on- line analysis of the fully digitalized images and increase in frame rate are outside the scope of this article.

Two studies [4,27] have reported sublingual vascular density values in mm/mm^2 in volunteers using SDF imaging. Ours are in exact agreement with the values found by Edul et al. who used similar AVA software. However, SDF-derived TVD in our experiment is lower than in comparison to those found by Hubble et al., but this may be explained by a difference in software analysis (Capiscope, KK Technology, Axminster, UK). Agreement was found however between the three studies on the finding that in volunteers almost all vessels exhibit flow.

Clearly, the results of this study are limited to healthy volunteers, and further validation is needed in the clinical setting. However, heterogeneity of blood flow within the catchment area of the device has now been recognized as a key characteristic in many disease states [5]. To this end, the consensus paper decided to obtain three to five video clips per observation and report the average of the variables [28]. It was key to our experiment to exclude this heterogeneity.

Secondly, our data do not deal with the potential influence of intra-observer variability. Although a substantial variability has been reported in the jejunal mucosal microcirculation of pigs [29], multiple studies have confirmed the excellent reproducibility in the human sublingual area [4,5,26].

CONCLUSION:

In this study, we validated a third generation novel lightweight computer-controlled imaging sensor-based handheld microscope called the Cytocam-IDF imaging by comparing it to a second generation device, SDF imaging. Our results showed that Cytocam-IDF imaging was able to detect more capillaries in terms of density and provided improved quality image in the sublingual microcirculation. Considering the improved image quality along with its light weight and ability

to automatically analyze images, we expect it to contribute to the clinical assessment of microcirculation alterations in various clinical scenarios.

Competing interests

Dr Ince has developed SDF imaging and is listed as inventor on related patents commercialized by MicroVision Medical (MVM) under a license from the Academic Medical Center (AMC). He has been a consultant for MVM in the past, but has not been involved with this company for more than five years now, except that he still holds shares. Braedius Medical, a company owned by a relative of Dr Ince, has developed and designed a hand-held microscope called CytoCam-IDF imaging. Dr Ince has no financial relation with Braedius Medical of any sort, i.e., never owned shares, or received consultancy or speaker fees from Braedius Medical. The other authors have no conflict of interest.

Authors' contributions

GA and GV coordinated the study, performed the SDF and IDF imaging, performed analysis and participated in the draft of the manuscript. CI and CB participated in the design of the study and the draft of the manuscript.

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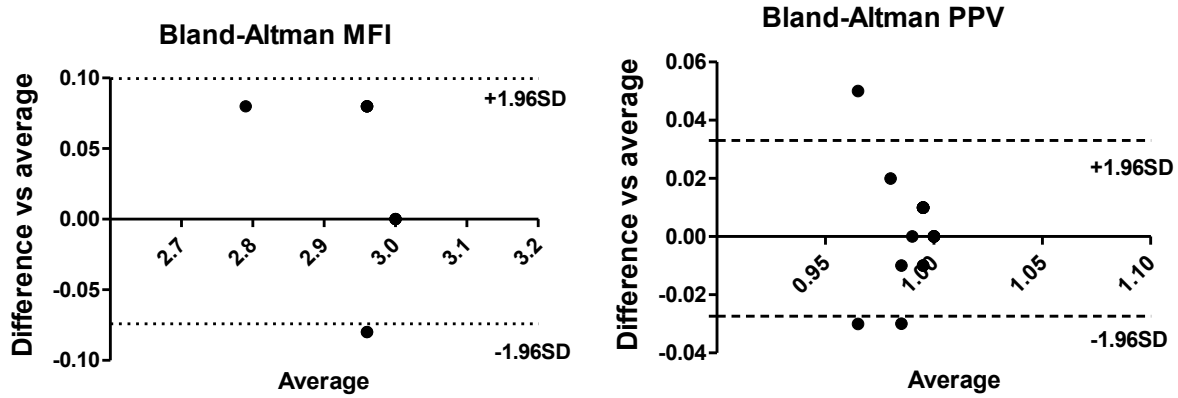
Additional file 1: Bland-Altman MFI and PPV. Due to substantial overlap multiple observations may be represented as one dot.



Chapter 3

Impact of microcirculatory video quality on the evaluation of sublingual microcirculation in critically ill patients

Impact of microcirculatory video quality on the evaluation of sublingual microcirculation in critically ill patients Cytocam-IDF

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ABSTRACT

Purpose: *To assess the impact of image quality on microcirculatory evaluation with sidestream dark-field (SDF) videomicroscopy in critically ill patients and explore factors associated with low video quality.*

Methods: *Retrospective analysis of a single-centre prospective observational study. Videos of the sublingual microcirculation were recorded using SDF videomicroscopy in 100 adult patients within 12 hours from admittance in the Intensive Care Unit and every 24 hours until discharge/death. Parameters of vessel density and perfusion were calculated offline for small vessels. For all videos, a quality score (-12=unacceptable, 1=suboptimal, 2=optimal) was assigned for brightness, focus, content, stability, pressure and duration. Videos with a total score ≤8 were deemed as unacceptable.*

Results: *A total of 2,455 videos (853 triplets) was analysed. Quality was acceptable in 56% of videos. Lower quality was associated with worse microvascular density and perfusion. Unreliable triplets (≥1 unacceptable or missing video, 65% of total) showed lower vessel density, worse perfusion and higher flow heterogeneity as compared to reliable triplets ($p<0.001$). Quality was higher among triplets collected by an extensively-experienced investigator or in patients receiving sedation or mechanical ventilation. Perfused vessel density was higher in patients with Glasgow Coma Scale (GCS) ≤8 (18.9 ± 4.5 versus 17.0 ± 3.9 mm/mm^2 in those with GCS>8, $p<0.001$) or requiring mechanical ventilation (18.0 ± 4.5 versus 17.2 ± 3.8 mm/mm^2 in not mechanically ventilated patients, $p=0.059$).*

Conclusions: *SDF video quality depends on both the operator's experience and patient's cooperation. Low-quality videos may produce spurious data, leading to an overestimation of microvascular alterations.*

Keywords: *microcirculation, sidestream dark field imaging, microcirculatory image quality, critically ill patients*

BACKGROUND

The development of non-invasive videomicroscopy techniques, such as sidestream dark-field (SDF) imaging, has enabled the *in vivo* assessment of microcirculatory blood flow at the bedside, using the sublingual region as an accessible window to the microcirculation of inner organs [1]. SDF technology is incorporated into a hand-held video microscope system which epi-illuminates the tissue with green (530 nm) light-emitting diodes [2]. As this light is absorbed by hemoglobin, erythrocytes appear as dark globules against a grayish background. Sublingual microcirculatory alterations have been reported in different patient subsets, especially during sepsis [3, 4], and were associated with morbidity and mortality [5-7]. A number of studies have shown a dissociation between macro-hemodynamics and microcirculatory response to several interventions, including fluid infusion [8] and vasopressor administration [9]. These data would encourage the introduction of microcirculatory monitoring in the clinical practice as an additional target for therapy. Nonetheless, there are practical challenges to the widespread adoption of this technique in Intensive Care Units (ICUs), mainly the time-consuming offline video analysis and a long learning curve for good-quality image acquisition.

In 2007 a roundtable consensus conference indicated five key-points for optimal image acquisition (sampling 5 sites per organ, avoidance of pressure artefacts, elimination of secretion, adequate focus and contrast adjustment and high quality recording) [10]. However, obtaining good-quality videos may be challenging due to operator's inexperience or poor patient's cooperation. In a study by Sallisalmi et al., excellent technical quality was reported in only 30% of SDF videos [11]. Inadequate video quality may produce spurious microcirculatory data: blood flow may be artificially obstructed due to excessive pressure applied on the sublingual mucosa; inadequate focus and contrast or occluding artefacts (saliva bubbles or blood) may prevent the visualization of some blood vessels. Massey et al. have proposed a microcirculatory image quality score considering 6 domains of video quality: illumination, duration, focus, content, stability and pressure [12]. This score has never been used systematically and studies evaluating the microcirculation do not generally report any assessment of image quality. To our knowledge, no study has previously investigated to what extent a low microcirculatory image quality will affect the reliability of microcirculatory assessment.

We hypothesized that SDF video quality depends on both operator- and patient-related factors. Patient's compliance may contribute crucially to high-quality video acquisition: as a result, more severe patients requiring sedation and/or mechanical ventilation could paradoxically show an apparently better microvascular perfusion as compared to less severe patients, merely due to an easier video recording. In this study, we investigated the relationship between SDF video quality

and the microcirculatory parameters obtained, and evaluated operator- or patient-related factors potentially influencing microcirculatory video quality.

METHODS

This is a retrospective analysis of data collected in a single-centre prospective observational study, the MICROcirculatory DAIly MONitoring in the ICU (MICRODAIMON-ICU) Study (NCT02649088, www.clinicaltrails.gov). A total of 100 adult (>18-year old) patients admitted to our 12-bed ICU between 1st April and 31st December 2013 were included in the study. Exclusion criteria were factors impeding the sublingual microvascular evaluation (i.e. maxillo-facial trauma or surgery) and enrolment in the same study during a previous admission. The study protocol was approved by the local ethics committee of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona, Italy. Written informed consent was obtained by the patients or their next of kin.

Microcirculatory image acquisition

The sublingual microcirculation was assessed using SDF imaging (Microscan, Microvision Medical, Amsterdam, The Netherlands) [2] within the first 12 hours of admission to the ICU and every 24 hours until discharge or death. In order to minimize variability due to operator's experience with SDF technology, whenever possible video recording sessions were performed by one principal investigator (PI) with extensive (3 years approximately) clinical experience in SDF monitoring. In case of unavailability of the PI, video recording was performed by a group of adequately trained operators (6 to 12-month experience, on average). No additional sedation was provided during image acquisition. Secretions were gently removed with a gauze. Every effort was made to optimize contrast and focus and avoid pressure artefacts. Stable videos of at least 5 seconds' duration [12] from 5 different sites of sublingual mucosa were recorded during each session. When adequate stability was difficult to achieve without increasing the pressure on the probe, priority was given to avoidance of pressure artefacts and videos shorter than 5s were accepted.

Analysis of microvascular density and perfusion

A random number was assigned to each video sequence through a random number generator. Three videos of the best available quality were selected from each sequence and analysed using the Automated Vascular Analysis software (Microvision Medical, Amsterdam, The Netherlands) by a group of 4 experienced investigators who had not participated in the video acquisition. The image was divided by three equidistant horizontal lines and three equidistant vertical lines for the

calculation of the De Backer score: this resulted from the number of vessels crossing the lines, divided by the total length of the lines [13]. 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]. Total vessel density (TVD) was calculated as the total length of vessels divided by the total area of the image [10]. The perfused vessel density (PVD) was estimated by multiplying TVD by PPV as estimated with the De Backer method [14]. Microvascular flow index (MFI) was calculated semiquantitatively as described elsewhere [15]. For each video sequence, values obtained from 3 sites were averaged. The flow heterogeneity index (FHI) was calculated as the highest MFI minus the lowest MFI, divided by the mean MFI of all sublingual sites [10]. For this study, analyses were focused on small vessels (≤20 μ in diameter).

Analysis of microcirculatory video quality

This was performed by the same investigators before the analysis of microcirculatory parameters. The microcirculatory image quality score used in this study was adapted from the one proposed by Massey et al. [12] (Table 1). For each video, a score of -12 (unacceptable), 1 (suboptimal but acceptable) or 2 (optimal) was given for each category of quality (brightness, focus, content, stability, pressure, duration). A video was defined as unacceptable if it scored any "-12" in any category or "1" in at least 4 categories (total score ≤8). Among unacceptable videos, quality was categorized as *bad* ("-12" for at least three categories, total score ≤-30) or *poor* ("-12" in less than three categories, total score between -29 and 8). Among acceptable videos, quality was defined as *good* (total score of 9-11) or *excellent* ("2" for all categories, total score of 12). A video triplet was defined as *reliable* if it was of excellent (all videos of excellent quality) or acceptable (3 acceptable or excellent videos) quality, *unreliable* if it included at least one video of unacceptable quality or if less than three videos had been suitable for analysis.

Statistical analysis

This was performed using GraphPad Prism Version 6 (GraphPad Software, La Jolla, CA, USA). Normality of distribution was checked using the Kolmogorov-Smirnov test. Unpaired t-test or Mann Whitney U-test were used for comparisons of continuous variables, as appropriate. One-way analysis of variance (ANOVA) or Kruskal-Wallis test with Bonferroni or Dunn's post-hoc testing were used for comparisons between more than two groups, as appropriate. The chi-square

test was used for comparison of proportions. The alpha level of significance was set a priori at 0.05.

RESULTS

A median of 6 [3-12] video recording sessions per patient was performed. A total of 2,455 microcirculatory videos from 100 different patients were analyzed. This corresponded to a total of 853 video triplets. Of these, 583 (68%) had been recorded by the PI. Quality score for each single domain is reported in Table 2. Based on our predefined criteria, only 1,365 videos out of 2,455 (56%) were defined as acceptable, 27% of these being of excellent quality (Figure 1). If considering video triplets, only 301 triplets out of 853 (35%) were judged as reliable, with 4% of total being of excellent quality, while 552 (65%) were of unacceptable quality. Of these, 72 (8% of total) were incomplete triplets where less than three videos had been suitable for analysis (104 videos lacking in total). As a result, 67 [44-89] % of microcirculatory assessments performed in each patient were deemed unreliable. Only 8 patients out of 100 had reliable microcirculatory data for all the sessions performed, while we were unable to take any reliable video triplets in 20 patients.

Video quality and microcirculatory parameters

Videos of lower quality for each category were generally associated with lower vessel density, PPVs and MFIs as compared to videos of optimal quality (Electronic Supplementary Material, ESM 1 and 2). This trend was less pronounced for the category "stability", for which unacceptable videos showed slightly lower vessel densities but higher PPVs, without any differences in MFIs depending on video quality. Vessel density, PPVs and MFIs were progressively lower with decreasing overall video quality (Figure 2). All microcirculatory parameters varied significantly between reliable and unreliable video triplets, with unreliable triplets indicating worse vessel density and perfusion and higher flow heterogeneity (Electronic Supplementary Material, ESM 3).

Influence of operator and patient-related factors on video quality and microcirculatory assessment

Triplets recorded by the PI were less likely to be of unacceptable quality (60% versus 74.8% among triplets collected by other investigators, p=0.041, Electronic Supplementary Material, ESM 4) and yielded higher PVD (18.2 ± 4.1 versus 17.4 ± 4.8 mm/mm^2, p=0.001) as compared to those recorded by other operators. The vast majority of triplets recorded in non-sedated or not mechanically ventilated patients were of unacceptable quality (81% and 96% respectively), while the percentage of unreliable video triplets was lower among sedated or mechanically ventilated patients (55% and 63% respectively) (p<0.001, Electronic Supplementary Material, ESM 5). Among sedated patients, a higher video quality was associated with higher doses of propofol being administered at the time of microcirculatory assessment (Figure 3). Among non-sedated patients, a lower video quality was associated with higher values of Glasgow Coma Scale (GCS) (Figure 3), and those with GCS ≤8 showed higher PVD as compared to those with GCS >8 (18.9 ± 4.5

versus 17.0 ± 3.9 mm/mm^2, p<0.001). Similarly, mechanically ventilated patients tended to show a higher PVD as compared to those not mechanically ventilated (18.0 ± 4.5 versus 17.2 ± 3.8 mm/mm^2, p=0.059).

DISCUSSION

This is the first study that evaluated the impact of microcirculatory image quality on the assessment of sublingual microvascular density and perfusion, using one of the largest existing databases and including a heterogeneous population of 100 critically ill patients monitored in different moments over the course of their acute condition. Our first finding was that almost half of the videos collected were of unacceptable quality, resulting in 65% of video triplets being unreliable for a clinical assessment of microvascular perfusion. Secondly, parameters of microvascular density and flow varied together with image quality, with videos of lower quality suggesting lower vessel densities and a more altered perfusion. Thirdly, not only the investigator's experience in SDF technology, but also patient-related factors (mainly the neurological state) played a role in determining a good-quality video recording.

Since the first appearance of Orthogonal Polarization Spectral imaging more than 15 years ago [16], an increasing number of studies has explored the sublingual microcirculation and its response to several interventions in different patient populations [17]. Several authors supported the potential role of the microcirculation as a target for therapy [18-20]. Nonetheless, before introducing any monitoring device in the clinical practice, it is necessary to understand the potential limitations of the technique in order to obtain reliable information. Only a few studies addressed the limitations of sublingual videomicroscopy [11, 12]. Our study highlights the technical challenges to the widespread adoption of sublingual microcirculation monitoring in ICU patients. A substantial proportion of the videos recorded in each patient was of unacceptable quality. In 20 patients out of 100 we failed to perform any reliable microcirculatory assessment during their stay in the ICU. These data add to the results of Sallisalmi et al. [11], who reported a success rate as low as 8.5% of video recording sessions in creating video triplets of adequate quality. More importantly, our study demonstrates that low-quality videos can introduce a substantial bias in microcirculatory assessment. The analysis of videos with defects in focus or brightness, as well as artefacts such as secretions or blood, yielded significantly lower vessel densities, PPV and MFI. Similarly, the presence of pressure artefacts was associated with lower vessel density and worse perfusion. Even if they can be recognized and distinguished from real flow alterations by a well-trained investigator during video analysis, pressure artefacts preclude a reliable evaluation of microvascular flow. The first step for the introduction of the microcirculation

as a guidance in clinical practice [18-20], but also for its use as a surrogate endpoint in interventional studies, is a comprehensive understanding of factors potentially affecting the reliability of the data obtained. Our results underline the importance of identifying and excluding video triplets of inadequate quality from the analysis of microvascular parameters, as their inclusion can distort our interpretation of the patient's microvascular state and lead to an overestimation of microcirculatory alterations.

Our study confirms the operator-dependence of sublingual microvascular monitoring. The involvement of multiple operators was necessary due to the demanding protocol of our study (data collection 7 days a week for 9 months). Notably, our comparison between PI and other investigators must not be merely read as "high versus low experience", as most of those who contributed to video acquisition had previously participated in microcirculatory studies [13, 21, 22] or had undergone an adequate training period. Our results suggest that the quality of microcirculatory data is enhanced if image acquisition is performed by one single and extensively-experienced investigator. Importantly, microvascular parameters may simply vary on the basis of the investigator responsible for video acquisition. The operator-dependence of SDF technique clearly hinders its widespread adoption in the clinical setting. Technological developments of video-microscope devices, such as Cytocam-IDF (Incident Dark Field Illumination) imaging, will hopefully increase their ease of use and limit the bias due to investigator's experience in the video acquisition and analysis [23]. The Cytocam system consists of a lightweight pen-like device with a field of view three times larger than previous devices and improved focus and contrast [24]. This system has also the capability of automatic video analysis, although it still needs validation.

Extensive experience may be not sufficient to ensure high-quality video recording, as indicated by the substantial amount of unacceptable videos among those recorded by the PI. The patient's cooperation during image acquisition is crucial for ensuring reliable microcirculatory data. We were able to demonstrate that the acquisition of reliable video triplets was easier in patients receiving sedation and/or mechanical ventilation. In addition, those among non-sedated patients who received higher-quality microcirculatory videos had significantly lower GCS score. Involuntary movements of the tongue or head can make it difficult to perform adequate image acquisition in fully conscious patients. As a result, microcirculatory assessment may be significantly biased in these patients, since unreliable microcirculatory images may produce an underestimation of microvascular perfusion. In our study, patients not requiring sedation or mechanical ventilation (who were therefore likely to be less severe) paradoxically showed a lower perfused vessel density, thus leading to a spurious dissociation between the microcirculation and the patient's clinical condition.

Our study may have important implications for future research. Image quality should be systematically assessed in studies evaluating the microcirculation. There is an urgent need to define a unified score to assess microcirculatory image quality. The score used in our study was adapted from the one proposed by Massey et al. [12] and takes into account the main domains of video quality. However, it may be limited in the criteria used to define adequateness of stability or duration. The strict criterion used to define stability (absolute pixel translation on X- or Y-axis <20) may explain the high percentage of videos scored as unacceptable for this domain and its relatively minor impact on microvascular parameters. In addition, priority was given to the avoidance of pressure artefacts during image acquisition rather than to stability or duration. An evolution of microcirculatory image quality scoring systems could include a differential weight of different domains in the overall score of quality, based on their impact on the reliability of the analysis. The importance of each component may also vary based on the particular aspect of interest in the study, e.g. more emphasis could be given to the avoidance of pressure artefacts in studies evaluating variations in microvascular convective flow, while aspects such as focus, brightness and content may have major relevance when focusing on vessel density. The exclusion of the confounding factor of video quality will possibly improve microcirculatory data consistency and allow a more reliable identification of even subtle variations in vessel density or perfusion following interventions.

The problem of quality should be taken in particular consideration when assessing patients with expected low cooperation (awake or not intubated). Video triplets of unacceptable quality should be identified and excluded from the analysis in order to avoid biases in the assessment of microvascular perfusion. Alternatively, the proportion of unreliable video sequences should be reported as a measure of possible methodological bias. In addition, it may be important that measurements are performed by one single investigator with extensive expertise in microcirculatory video acquisition, in order to enhance data quality and consistency.

CONCLUSION

Using a large database of SDF videos from a heterogeneous population of 100 critically ill patients monitored daily during their stay in the ICU, this study showed that microcirculatory image quality is an important problem when assessing the sublingual microcirculation. Recording high-quality images is a challenging task, depending not only on the operator's experience but also on the patient's cooperation. Low-quality videos produce spurious microcirculatory data, leading to an overestimation of microvascular alterations. It is thus important that microcirculatory video quality

is assessed systematically in studies evaluating the microcirculation and unreliable triplets must be excluded from the analysis.

Funding

None external.

Conflict of interest

CI is the inventor of sidestream dark field imaging technology and holds shares in MicroVision Medical and was a consultant for this company more than four years ago but has had no further contact with the company since then. He has no other competing interests in this field and there are no other relationships or activities that could appear to have influenced the submitted work. The other authors declare that they have no conflict of interest.

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Table 1 – Microcirculatory image quality score, as adapted from Massey et al. [12].

Category	Quality score		
	-12 (unacceptable)	**1 (acceptable)**	**2 (good)**
Brightness	The video is too bright/too dark to visualize all vessels	The video borders on being too bright/too dark but all vessels are still identifiable	Homogeneous illumination and contrast across the entire field of view
Focus	Totally out of focus, no small vessels can be seen	<1/2 field of view is out of focus or edges of vessels are slightly out of focus	Good focus for all vessels (erythrocytes/leukocytes are visible in most of the vessels)
Content	Prevalence of repeating capillary loop motif or saliva bubbles or blood occluding most of the field of view	<50% looped vessels; a few occluding artefacts (saliva, blood) that do not impede vessel or flow identification	Various size vessel architecture in the entire field of view, with prevalence of capillaries; no saliva bubbles or blood
Stability	Absolute pixel translation on X- or Y-axis >20	Absolute pixel translation on X- or Y-axis between 10 and 20	Absolute pixel translation on X- or Y-axis <10
Pressure	Obvious pressure artefacts associated with probe movements, flow that starts and stops, reversal of flow, obstructed flow in larger venules	Only localized pressure artefacts, flow is unobstructed in most vessels, good flow in larger venules	No signs of pressure artefacts
Duration	Analyzable video segment is <3s long	Analyzable video segment is 3-5s long	Analyzable video segment is >5s long

Table 2 – Distribution of microcirculatory videos based on the score assigned for each category of quality.

Category	Quality score		
	-12 (unacceptable)	**1 (suboptimal)**	**2 (optimal)**
Brightness	23 (1%)	548 (22%)	1884 (77%)
Focus	125 (5%)	1112 (45%)	1218 (50%)
Content	252 (10%)	565 (23%)	1638 (67%)
Stability	770 (31%)	759 (31%)	926 (38%)
Pressure	54 (2%)	444 (18%)	1957 (80%)
Duration	65 (3%)	131 (5%)	2259 (92%)

Fig. 1 Quality of all the microcirculatory videos analyzed

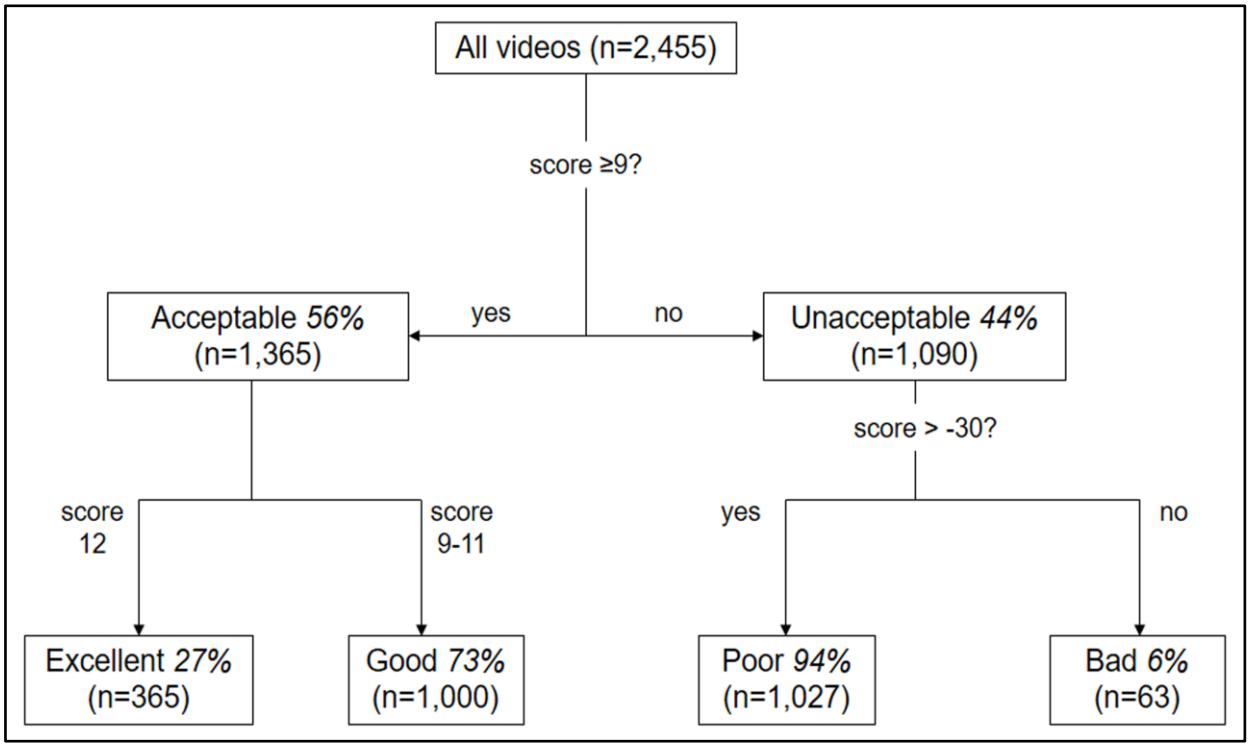


Fig. 2 Total vessel density, percentage of perfused vessels (PPV) and microvascular flow index (MFI) for small vessels in videos stratified based on their quality. ***p<0.001, Kruskal-Wallis test with Dunn's test for multiple comparisons. Data are expressed as median and interquartile range.

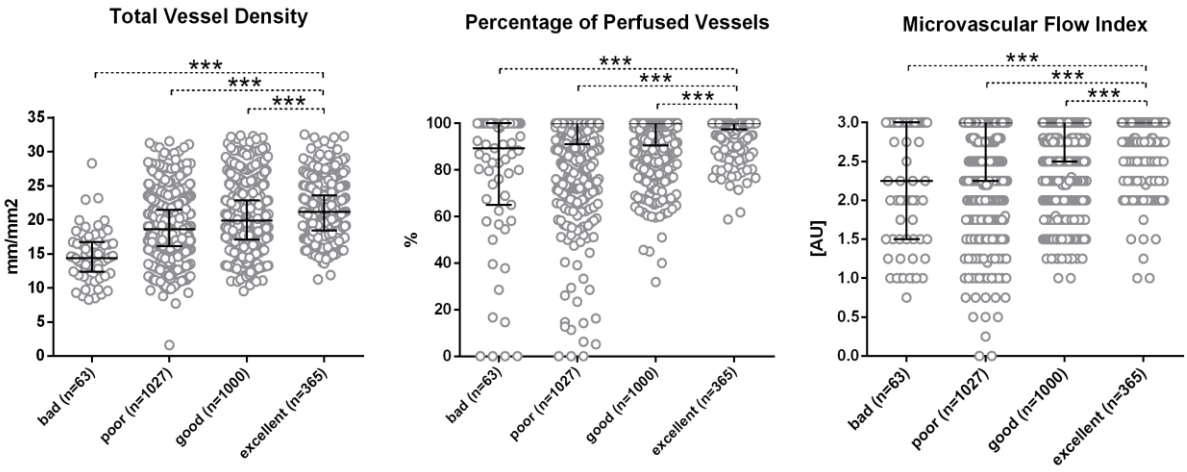
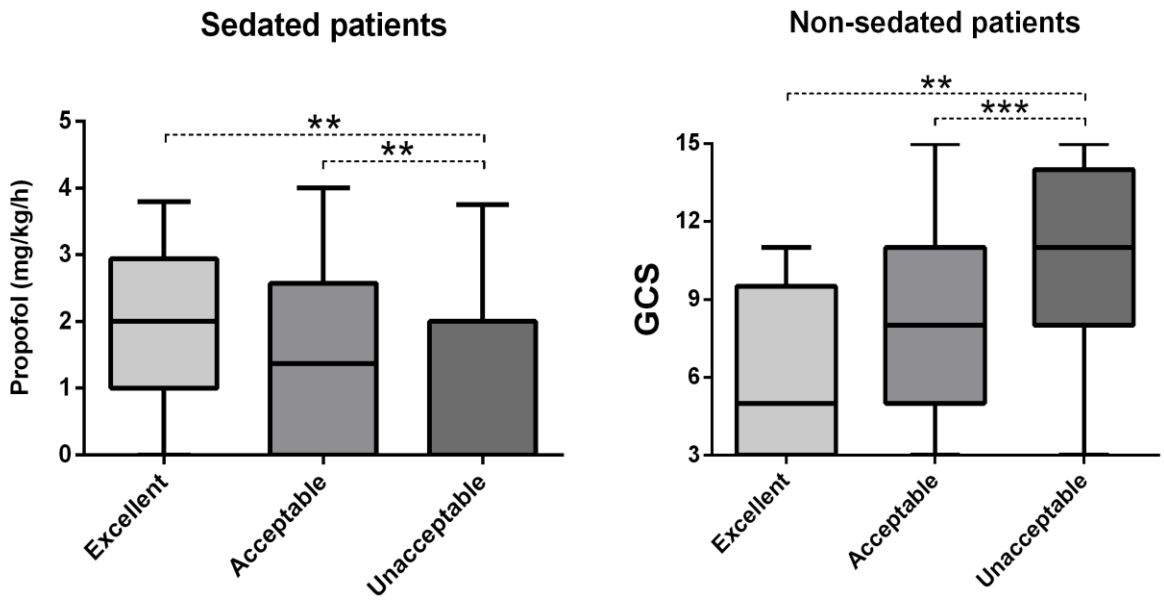


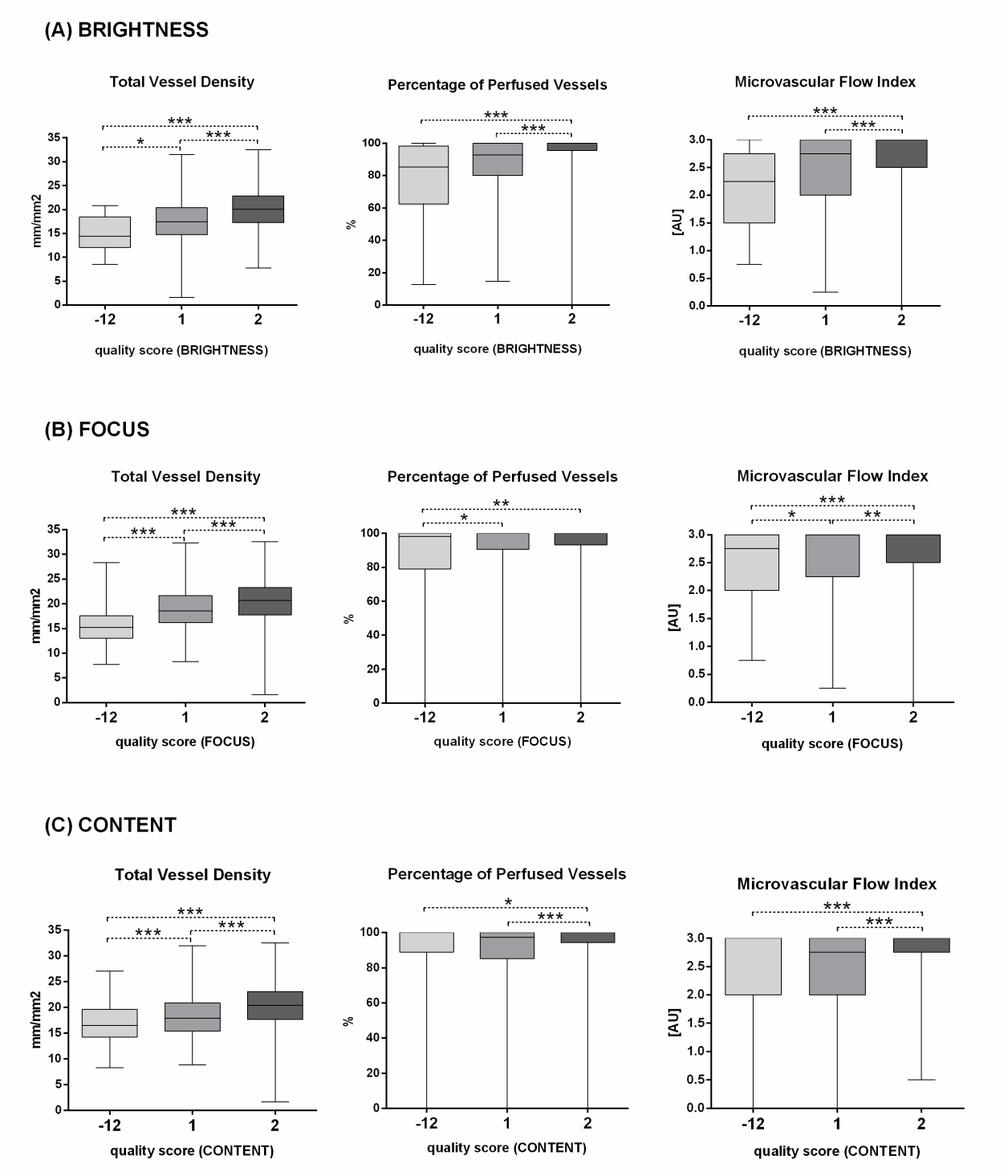
Fig. 3 Relationship between sedation (dose of propofol) or Glasgow Coma Scale and quality of microcirculatory video triplets. Sedation was defined based on the administration of any hypnotic and/or opioid agents at the time of microcirculatory assessment.



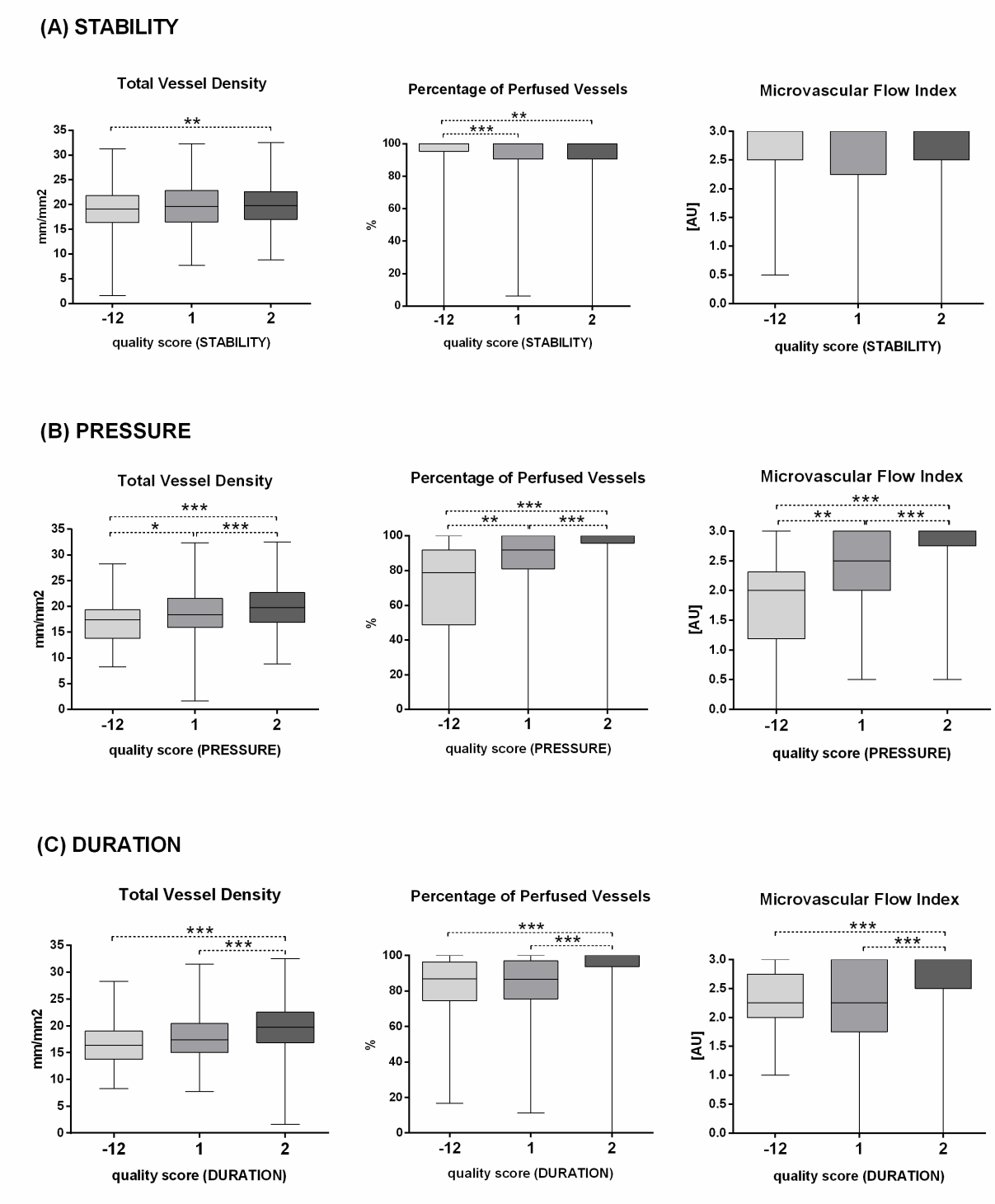
**p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's test for multiple comparisons. Data are expressed as median (Interquartile range). Error bars indicate maximum and minimum values

ELECTRONIC SUPPLEMENTARY MATERIAL

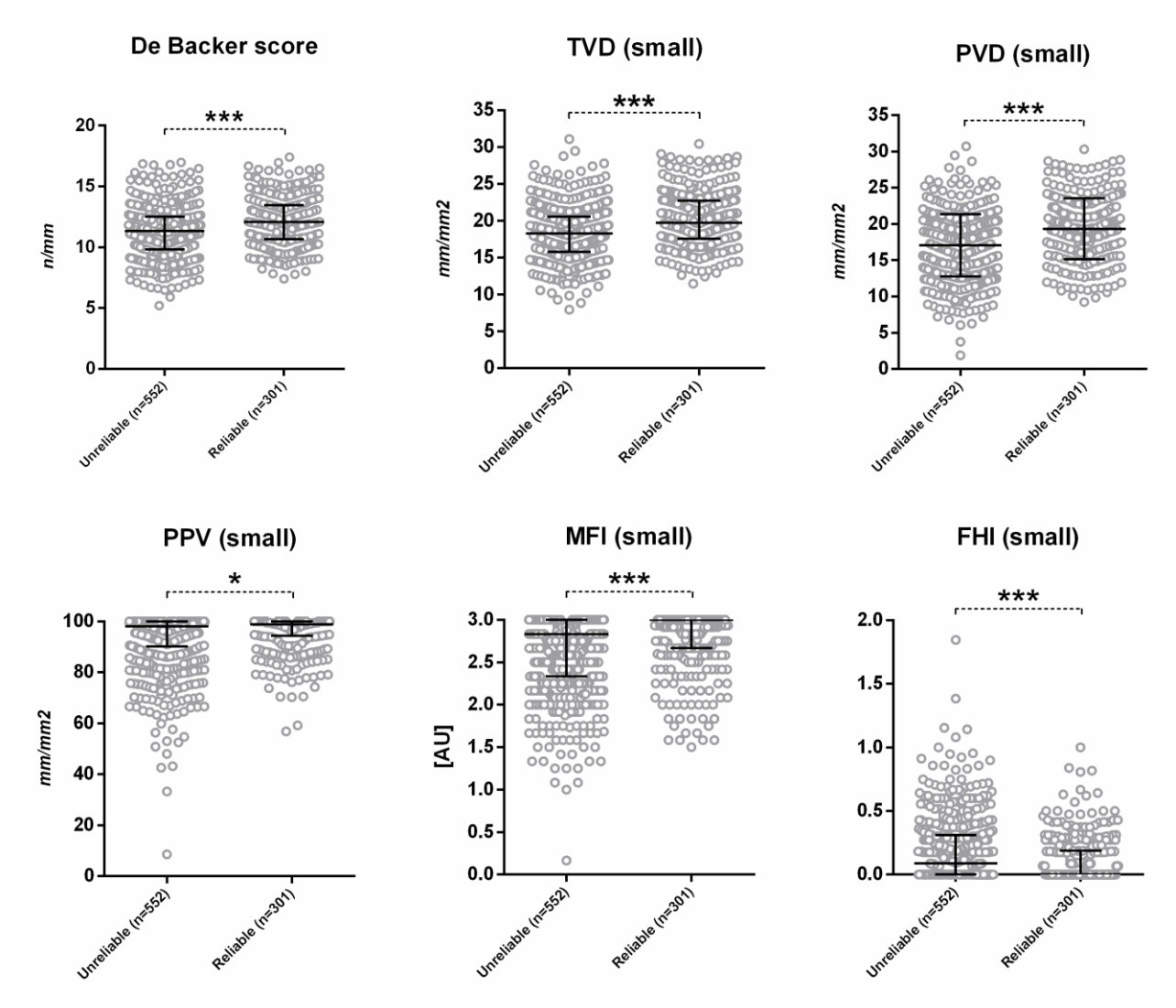
ESM 1 Vessel density, percentage of perfused vessels (PPV) and microvascular flow index (MFI) for small vessels in videos stratified based on their quality in the categories brightness (A), focus (B) and content (C). *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's test for multiple comparisons. Data are expressed as median (interquartile range), error bars indicate minimum and maximum values.



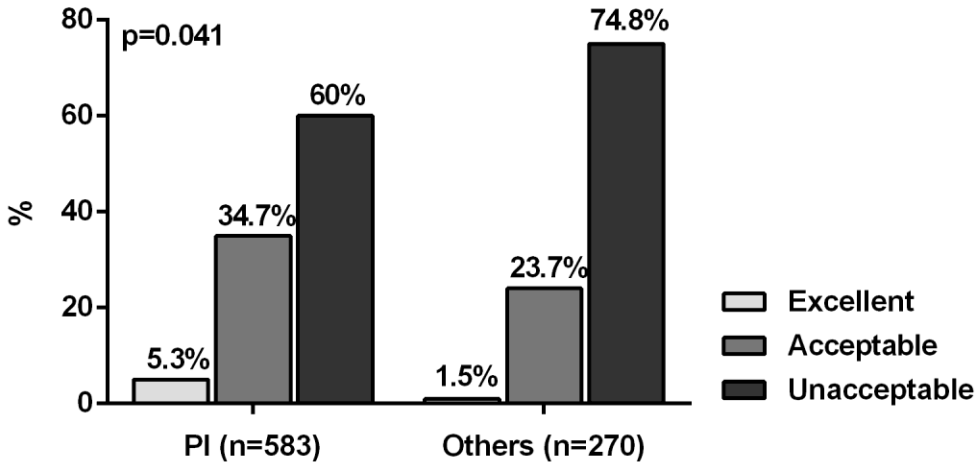
ESM 2 Vessel density, percentage of perfused vessels (PPV) and microvascular flow index (MFI) for small vessels in videos stratified based on their quality in the categories stability (A), pressure (B) and duration (C). *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's test for multiple comparisons. Data are expressed as median (interquartile range), error bars indicate minimum and maximum values.



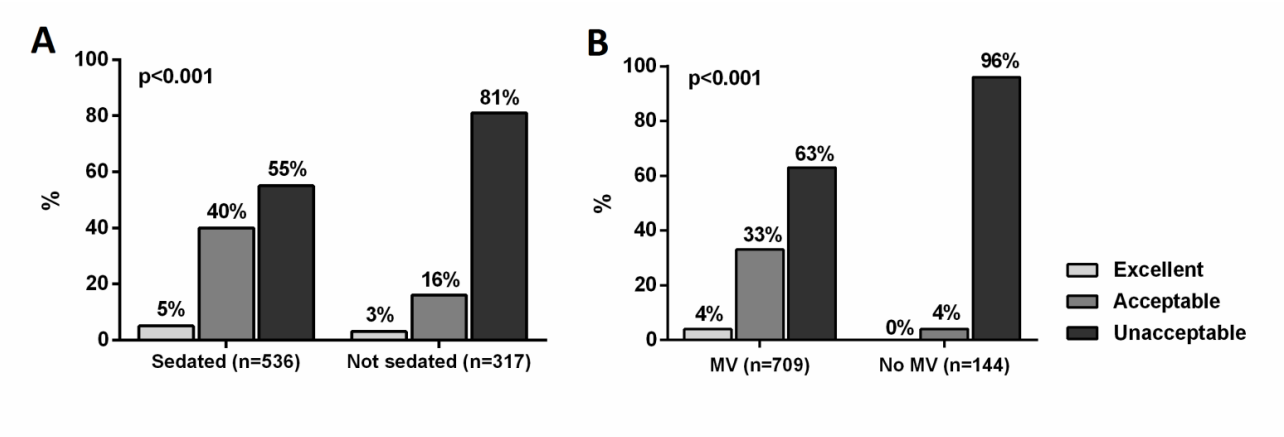
ESM 3 Comparison of microcirculatory parameters between reliable and unreliable video triplets. *TVD* total vessel density, *PVD* perfused vessel density, *PPV* percentage of perfused vessels, *MFI* microvascular flow index, *FHI* flow heterogeneity index. *p<0.05, ***p<0.001, unpaired t-test or Mann Whitney U-test. Data are expressed as mean (standard deviation) or median (interquartile range), as appropriate.



ESM 4 Percentage of excellent, acceptable or unacceptable video triplets among those collected by the principal investigator (PI) or other investigators



ESM 5 Percentage of excellent, acceptable or unacceptable video triplets in sedated versus non-sedated patients (A) and mechanically ventilated versus not mechanically ventilated patients (B). Sedation was defined based on the administration of any hypnotic and/or opioid agents at the time of microcirculatory assessment. *MV* mechanical ventilation.



Chapter 4

Near-Infrared Spectroscopy for assessing tissue oxygenation and microvascular reactivity in critically ill patients:

a prospective observational study

Near-Infrared Spectroscopy for assessing tissue oxygenation and microvascular reactivity in critically ill patients: a prospective observational study

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ABSTRACT

Background: *Impaired microcirculatory perfusion and tissue oxygenation during critical illness are associated with adverse outcome. The aim of this study was to evaluate the ability of thenar Near-Infrared Spectroscopy (NIRS) with a vascular occlusion test (VOT) to detect alterations in tissue oxygenation or microvascular reactivity and to predict outcome in critically ill patients.*

Methods: *Prospective observational study on adult critically ill patients admitted to a 12-bed Intensive Care Unit (ICU) of a University Hospital. NIRS with a VOT (using a 40% tissue oxygen saturation [StO_2] target) was applied daily until discharge from the ICU or death. A group of healthy volunteers was evaluated in a single session. During occlusion, StO_2 Downslope was measured for the first (Downslope 1) and the last part (Downslope 2) of the desaturation curve separately. The difference between Downslope 2 and 1 was calculated (Delta-Downslope). The upslope and the area of the hyperaemic phase (AUC StO_2) were calculated, reflecting microvascular reactivity. Outcomes of interest were ICU- and 90-day mortality.*

Results: *Patients (n=89) showed altered Downslopes and Upslopes as compared to healthy volunteers (n=27). Mean Delta-Downslope was higher in ICU Non-Survivors (2.8 [0.4-3.8] %/min versus 0.4 [-0.8, 1.8] in Survivors, p=0.004) and was able to discriminate 90-day mortality (area under the receiver operating characteristic [ROC] curve: 0.72 [95% confidence interval 0.59-0.84]). ICU Non-Survivors showed lower mean Upslope (141 [75-193] %/min versus 185 [143-217] in Survivors, p=0.016) and AUC StO_2 (7.9 [4.3-12.6] versus 14.5 [11.2-21.3], p=0.001). The Upslope and AUC StO_2 on admission were significant although weak predictors of 90-day mortality (area under the ROC curve = 0.68 [0.54-0.82] and 0.70 [0.58-0.82], respectively). An admission AUC StO_2 ≤6.65 (1^{st} quartile) was independently associated with higher 90-day mortality (hazard ratio 7.964 [95% CI 2.211-28.686]). The lowest Upslope in the ICU was independently associated with survival after ICU discharge (odds ratio: 0.970 [95% CI 0.945-0.996]).*

Conclusions: *In critically ill patients, NIRS with a VOT enables to identify alterations in tissue oxygen extraction capacity and microvascular reactivity that can predict mortality.*

Trial registration: *NCT02649088, www.clinicaltrials.gov, date of registration 23^{rd} December 2015, retrospectively registered.*

Keywords: tissue oxygenation; microcirculation; near-infrared spectroscopy; vascular occlusion test; critical illness

BACKGROUND

Imbalances between oxygen (O_2) delivery and demand during critical illness may result in tissue hypoxia and lead to organ dysfunction and death. The first goal of treatment is to optimize tissue perfusion and O_2 supply. Nonetheless, commonly used hemodynamic targets (mean arterial pressure [MAP], cardiac output) or markers of global oxygenation such as central venous O_2 saturation (SvO_2) or arterial lactate are not always specific and early predictors of organ perfusion [1]. Increasing evidence suggests a potential dissociation between microcirculation and macro-hemodynamic in the critically ill [2-4]: microcirculatory hypoperfusion may thus persist despite normalization of global hemodynamic parameters [5].

Near-Infrared Spectroscopy (NIRS) has been introduced some decades ago as a non-invasive tool for measuring oxygenation in the muscle and other tissues, although the thenar eminence is the most widely tested site [6]. Several studies reported an association between low thenar tissue O_2 saturation (StO_2) and poor outcome, especially during sepsis [7-10]. In conjunction with a vascular occlusion test (VOT), NIRS allows to analyse the changes in StO_2 during a brief ischemic challenge, providing dynamic parameters of tissue O_2 extraction and microvascular reactivity [10]. A slower StO_2 recovery during the reperfusion phase was an independent predictor of mortality in septic patients [9].

By using NIRS with a VOT daily in a cohort of patients admitted to our Intensive Care Unit (ICU), we aimed to confirm the relationship between static or dynamic NIRS-derived variables and outcome and further characterize their prognostic value during critical illness.

METHODS

This is a secondary analysis of the MICROcirculatory DAIly MONitoring in ICU (MICRODAIMON-ICU) study (NCT02649088, www.clinicaltrails.gov), a single-centre prospective observational study on a population of 100 critically ill patients with the primary goal of evaluating the relationship between changes in the sublingual microcirculation during the ICU stay and outcome. This study was performed between April and December 2013 in a 12-bed ICU of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona, Italy. At the time of enrolment, our ICU was divided in 3 sub-sections (General, Trauma and Respiratory ICU) of 4 beds each. All consecutive adult (≥18 year-old) patients admitted to each section during 3 trimesters (General: April-June 2013; Trauma: July-September 2013; Respiratory: October-December 2013) were included. Exclusion criteria were recent maxillo-facial surgery/trauma and

pregnancy. Every day until ICU-discharge/death, patients underwent microcirculatory assessment through sublingual videomicroscopy and NIRS monitoring. A group of healthy volunteers was studied as control in a single session. The study was approved by our local ethical committee of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona, Italy. Written informed consent was obtained from all patients or their next of kin.

NIRS monitoring

An InSpectra StO_2 Tissue Oxygenation Monitor (model 650; Hutchinson Technology, Hutchinson, MN, USA) was used to measure StO_2 at baseline and during a VOT with a 15mm-spaced probe applied on the thenar eminence, as described previously [11, 12]. After a 3-minute period of StO_2 signal stabilization, arterial inflow was arrested by inflation of a sphygmomanometer cuff to 50 mmHg above the systolic arterial pressure. The cuff was kept inflated until StO_2 decreased to 40% and then released [13]. StO_2 was continuously recorded during the reperfusion phase until stabilization. NIRS-derived parameters were calculated with a software package (version 3.03 InSpectra Analysis Program; Hutchinson Technology Inc.). StO_2 and tissue haemoglobin index (THI) [14] were calculated at baseline. The StO_2 downslope (%/minute) is generally calculated from the regression line of the first part of StO_2 decay after occlusion, providing an index of tissue O_2 extraction rate [15]. However, we noted that the desaturation slope may vary during the ischemic phase of the VOT, becoming more or less steep before reaching the 40% StO_2 threshold. In order to explore the meaning of this variation in the desaturation slope, the inflection point was visually identified and the Downslope was calculated for the first and the last part of the desaturation curve separately (Downslope 1 and Downslope 2, respectively). 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 then calculated as the difference between the last and the first part of the desaturation slope (Downslope 2 – Downslope 1), so that a positive value indicated a flattening in the second part of the slope (slower StO_2 decay). The StO_2 Upslope (%/min) and the area under the curve of the hyperaemic response (AUC StO_2) were calculated as indices of microvascular reactivity [13].

Clinical parameters and outcomes

For all patients we recorded age, gender, reason for ICU admission, comorbidities, Acute Physiology and Chronic Evaluation (APACHE) II score on admission. The Sequential Organ Failure Assessment (SOFA) score, presence of sepsis (as defined according to standard criteria [16]), main clinical and laboratory parameters, arterial and venous blood gas analyses were

recorded every day simultaneously with NIRS measurements. Outcomes of interest were ICU-mortality and 90-day mortality.

Statistical analysis

This was performed using IBM SPSS (version 19), GraphPad Prism version 5 (GraphPad software, La Jolla, CA, USA) and MedCalc version 12.5 (MedCalc software, Ostend, Belgium). Normality of distribution was checked through the Kolmogorov-Smirnov test. Continuous variables were expressed as mean ± standard deviation or median [25^{th}-75^{th} percentile], as appropriate. The Student's t test or the Mann Whitney U test were used to compare continuous variables between two groups. Nominal variables were compared across groups using the chi-square test. The Kruskal-Wallis test with Dunn's test for multiple comparisons was used to compare data between more than two groups. The area under the receiver operating characteristic (ROC) curve was calculated to evaluate the predictive value of the variables for 90-day mortality. The Logrank Mantel Cox test and multivariable Cox regression were used to evaluate differences in survival between patients stratified based on quartiles of NIRS variables. Multivariate binary logistic regression was performed to evaluate the independent association between NIRS-derived variables and outcome. A p value lower than 0.05 was used to indicate statistical significance.

RESULTS

A total of 27 healthy volunteers (30 [28-52] years old; 10 males, 17 females) and 89 patients (age 66 [45-74] years; 62 males, 27 females) was studied. NIRS monitoring was not performed in 11 patients out of the total cohort of 100 patients because of bilateral upper limb fractures, patient's refusal or unavailability of the NIRS-device at the time of enrolment. Mean length of stay in the ICU was 8 [4-15] days. Eighteen patients (20%) died in the ICU, while 90-day mortality was 31% (28 patients out of 89). On admission, ICU Non-Survivors showed higher APACHE II and SOFA score, higher heart rate (HR), lower MAP, higher lactate levels and had higher probability to receive vasopressors (Table 1). NIRS-derived variables for ICU Survivors and Non-Survivors are shown in Table 2.

StO_2, THI and outcome

StO_2 did not differ between healthy volunteers and patients on admission to the ICU (Figure 1), however ICU Non-Survivors showed significantly lower values on day 3 as compared to healthy volunteers or ICU Survivors. An StO_2 <70% on admission to the ICU was found in 16 patients

(18%), but was not associated with higher ICU-mortality (12.5% versus 23.5% among those with StO2 ≥70%, p=0.274) or 90-day mortality (31% versus 34%, p=0.498). Forty-four patients out of 89 (49%) showed an StO_2 <70% at least once during their stay in the ICU, but this was not associated with worse outcome. Similarly, an StO_2 >90% (observed at least once in 38 patients) was not associated with higher ICU- or 90-day mortality. THI was lower among patients as compared to healthy volunteers, with no difference between Survivors and Non-Survivors (Table 2 and Figure 1).

Tissue O_2 extraction rate and outcome

ICU Non-Survivors tended to show higher Delta-Downslope values (Figure 2 and Table 2). While Downslope 1 did not differ between Survivors and Non-Survivors, a slower desaturation in the second phase of the ischemic challenge (higher Downslope 2 and Delta-Dowslope) was observed among ICU Non-Survivors (Table 2). In pooled data, this was associated with the presence of sepsis, high lactate, hypotension or norepinephrine infusion (Additional File 1) and weak negative correlations were found between MAP and Downslope 2 (r=-0.12, p<0.001) or Delta-Downslope (r=-0.10, p=0.004). Mean Delta-Downslope during the ICU stay was able to discriminate 90-day Non-Survivors (area under the ROC curve = 0.72 [0.59-0.84], p = 0.001). Patients in the 4th quartile of mean Delta-Downslope (>2.19 %/min) showed higher 90-day mortality (59% in the 4th quartile versus 17%, 23% and 27% in the 1st, 2nd and 3rd quartiles, respectively, p=0.013). This association remained significant in Cox-regression analysis after adjustment for APACHE II score on admission, MAP on admission and diagnosis of sepsis on admission or during the ICU stay (Hazard Ratio = 3.414 [95% CI 1.084-10.756], versus 1st quartile of mean Delta-Downslope, p=0.036).

Microvascular reactivity and outcome

The Upslope was significantly lower in patients as compared to healthy volunteers, while AUC StO_2 was altered only in ICU Non-Survivors (Figure 3). More severe alterations in Upslope and AUC StO_2 were seen among ICU Non-Survivors (Figure 3 and Table 2). The presence of sepsis, high lactate, hypotension or norepinephrine infusion was associated with altered microvascular reactivity (Additional File 1). In pooled data, MAP was weakly correlated with the Upslope (r=0.18, p<0.001) but not with the AUC StO_2. The upslope on admission and its mean value during the ICU stay were able to predict 90-day survival (area under the ROC curve = 0.68 [0.54-0.82] and 0.69 [0.57-0.82] respectively, p<0.01 in both cases). Similarly, the AUC StO_2 on admission and its mean value were able to predict 90-day survival (area under the ROC curve = 0.70 [0.58-

0.82] and 0.68 [0.56-0.80] respectively, p<0.01 in both cases). Patients in the lowest quartile of Upslope on admission (Upslope ≤88 %/min) showed higher risk for 90-day mortality (67% versus 28% for the 4th quartile, p=0.004) independently of MAP and the presence of sepsis on admission or during the ICU stay, however this association became non-significant when the APACHE II score was included in the model (hazard ratio = 2.607 [0.982-6.919], p = 0.054). An AUC StO_2 ≤6.65 (1st quartile) on admission to the ICU was associated with higher risk of 90-day mortality, independently from the APACHE II score, MAP and the presence of sepsis on admission or during the ICU stay (Hazard Ratio 7.964 [2.211-28.686] versus 4th quartile, p = 0.002).

Prognostic value of NIRS-derived variables for mortality after ICU discharge

Among the 71 ICU-Survivors, 10 patients died within the first 90 days from ICU-admission. During their stay in the ICU, these patients reached higher SOFA scores and lactate levels and lower Upslope and THI values as compared to 90-day Survivors, while no significant differences were found in worst values of MAP, heart rate (HR), haemoglobin (Hb), PaO_2, $ScvO_2$ or the other NIRS-derived parameters (Additional File 2). The lowest Upslope, lowest THI and lowest StO_2 during the ICU stay were fair or good predictors of 90-day mortality similarly to the highest SOFA score or lactate levels (Additional File 3). A lowest Upslope <68.8 %/min was able to predict mortality after ICU discharge with a sensitivity of 90% and a specificity of 72%. In a multivariate logistic regression model including highest SOFA, highest lactate, lowest MAP, highest HR, lowest $ScvO_2$, lowest StO_2, lowest Upslope and lowest THI, only the lowest Upslope was independently associated with survival after ICU discharge (odds ratio: 0.970 [95% CI 0.945-0.996], p = 0.025).

DISCUSSION

Through a daily use of NIRS monitoring on the skeletal muscle in conjunction with a VOT, we showed significant alterations in tissue Hb content, O_2 extraction and microvascular reactivity in a heterogeneous population of critically ill patients in comparison to healthy volunteers. The introduction of an ischemic challenge, as opposed to a static StO_2 assessment, allowed to evaluate the response of the tissue to a physiologic perturbation and its reserve capacity. While we did not find a clear and consistent relationship between StO_2 or THI and outcome, an altered desaturation slope, a slower re-oxygenation during reperfusion and a less pronounced reactive hyperaemia were associated with mortality.

A number of studies indicated a prognostic value of skeletal muscle StO_2 in several patient populations [7, 17-22]. In our study, the initial StO_2 was similar to that observed among healthy volunteers in both ICU -Survivors and Non-Survivors, while a significant decrease was seen in ICU Non-Survivors only on day 3. We could not find any association between ICU-mortality and mean StO_2 during the ICU stay. Similarly to the SvO_2, StO_2 reflects the balance between regional O_2 delivery and consumption [6]. If on one hand a lower StO_2 may result from a reduced O_2 supply, on the other hand an apparently stable or even higher StO_2 may depend on a reduction in O_2 extraction and consumption, which may be associated with worse outcome [23]. However, those patients who exhibited at least one StO_2 <70% or >90% during their ICU stay did not show a worse outcome. The muscle StO_2 at rest could not have been accurate enough to predict mortality in this population, where a lot of factors (including global haemodynamics and oxygenation, use of vasopressors or sedation) may have influenced regional O_2 levels and blood flow. A lower StO_2 was however associated with the presence of sepsis, increased lactate levels or norepinephrine infusion, i.e. with conditions of likely hemodynamic instability, inadequate tissue oxygenation and activation of anaerobic metabolism.

The tissue O_2 extraction rate was significantly reduced in critically ill patients as compared to healthy volunteers. More interestingly, while the first part of the Downslope did not differ between Survivors and Non-Survivors, the desaturation rate tended to be slower in the late ischemic phase in Non-Survivors, as well as in presence of sepsis, hypotension, high lactate levels or patients receiving norepinephrine. A higher mean Delta-Downslope (i.e. a flattening in the second part of the desaturation curve) was independently associated with higher 90-day mortality, although a larger population would be needed to enhance the strength of this association. To our knowledge, no other study has previously explored the potential relevance of variations in the desaturation slope during the ischemic phase of the VOT. By using microelectrodes to measure muscle and subcutaneous oxygenation, Sair *et al.* showed that the decline in tissue O_2 tension during ischemia was initially more rapid in patients with sepsis as compared to controls, although the overall rate of decline was similar: this would suggest a reduction in the desaturation rate in the final part of the ischemic challenge in these patients, however the authors did not discuss of a possible meaning [24]. During ischemia, the progressive decrease in local O_2 levels triggers vasodilatory mechanisms including the release of adenosine triphosphate (ATP) from red blood cells or nitric oxide (NO) from S-nitrosylated-Hb [25]. In a model of cecal ligation and puncture in rats, Bateman et al. demonstrated a delayed capillary response time within hypoxic capillaries and an impaired release of ATP from erythrocytes in response to hypoxia, suggesting a loss of microvascular autoregulation [26]. We speculate that a constant tissue O_2 extraction rate during ischemia may

reflect a more effective redistribution of blood flow to more hypoxic regions, allowed by a local microvascular vasodilation. A flattening in the final part of the desaturation curve may thus reflect an altered microvascular autoregulation and limited tissue O_2 extraction capacity in more severe patients.

The StO_2 Upslope and the AUC StO_2 in the post-ischemic hyperaemic phase are considered as reflections of microvascular reactivity and endothelial integrity [6]. Previous studies showed an association between slower re-oxygenation rate and worse outcome [27-29]. In this study the Upslope did not significantly differ between ICU Survivors and Non-survivors during the first 3 days in the ICU, unlike the AUC StO_2 that was higher in Survivors on admission. Both the Upslope and the AUC StO_2 on admission, as well as their mean values during the ICU stay, were found to be weak predictors of 90-day mortality. Importantly however, among patients who were discharged alive from the ICU, the lowest Upslope during the ICU stay was a fairly good predictor of 90-day mortality. This result remained robust in multivariate regression analysis, and would suggest that those patients who experienced a more severe impairment in microvascular function can remain at higher risk for adverse outcome even after stabilization and normalization of clinical parameters.

Our study has several limitations. Firstly, we could not evaluate the relationship between NIRS-derived variables and outcome in different disease categories (e.g. sepsis) due to the low number of patients and/or deaths in each subgroup. Secondly, our study cannot demonstrate a direct causal relationship between altered tissue oxygenation or microvascular dysfunction and outcome. Several studies showed a correlation between macro-haemodynamic parameters and NIRS-derived variables [30-31]. In accordance with previous findings, our study showed more severe alterations in NIRS-derived variables in presence of hypotension. Even if we cannot exclude that a reduction in perfusion pressure was the primary responsible for the impairment in tissue O_2 extraction and microvascular reactivity, it is noteworthy that the association between Delta-Downslope, Upslope, AUC StO_2 and survival was independent of MAP values. Interventional studies incorporating NIRS parameters among resuscitation targets are needed to demonstrate a causal relationship between improved tissue O_2 extraction or microvascular reactivity and better outcome. By evaluating 89 patients daily for 8 [4-15] days per patient, we performed more than 800 NIRS sessions, thus creating probably one of the largest existing databases. Nonetheless, the sample size could have been small to evaluate differences in mortality, thus some analyses may have been underpowered. Moreover, results from the analyses of mean values during the ICU stay may be partly biased by the lower number of measurements available for ICU Non-Survivors as compared to Survivors due to a shorter ICU length of stay, possibly leading to an overestimation

of the statistical significance for these comparisons. Lastly, we did not assess other parameters that can be derived as part of the VOT, such as the nirVO_2 (an index of local O_2 consumption) [32], which may have proved of important physiologic and prognostic value.

CONCLUSIONS

Our study confirms the association between altered NIRS-derived measurements and mortality in critically ill patients and supports the usefulness of NIRS monitoring in conjunction with a VOT for risk stratification of ICU patients. Moreover, this is the first study to explore the potential relevance of variations in the desaturation slope during ischemia. A decrease in the desaturation rate in the last part of the ischemic phase of the VOT was associated with worse outcome, and could suggest an impaired microcirculatory autoregulation which limits the tissue O_2 extraction capacity.

LIST OF ABBREVIATIONS

O_2 oxygen, *MAP* mean arterial pressure, *SvO_2* central venous oxygen saturation, *NIRS* near infrared spectroscopy, *StO_2* tissue oxygen saturation, *VOT* vascular occlusion test, *ICU* Intensive Care Unit, *THI* tissue haemoglobin index, *AUC StO_2* area under curve of the hyperaemic response, *APACHE II* Acute Physiology and Chronic Evaluation score, *SOFA* Sequential Organ Failure Assessment, *ROC* receiver operating characteristics, *HR* heart rate, *Hb* haemoglobin, *ATP* adenosine triphosphate, *NO* nitric oxide

DECLARATIONS

- **Ethics approval and consent to participate**

The study protocol was approved by our local ethical committee of Azienda Ospedaliera Universitaria "Ospedali Riuniti" of Ancona, Italy (protocol number 212639). Written informed consent was obtained from all patients or their next of kin.

- **Consent for publication**

Not applicable.

- **Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

- **Competing interests**

The authors declare that they have no competing interests.

- **Funding**

Local departmental funding.

- **Authors' contributions**

AD, EA, RR, PP, MS designed the study, contributed to the interpretation of the data and revised the manuscript critically for important intellectual content. ED, RD, CS, AC, ST and VM contributed to the acquisition and analysis of the data and drafted the manuscript. All authors gave final approval of the version to be published. All authors agreed 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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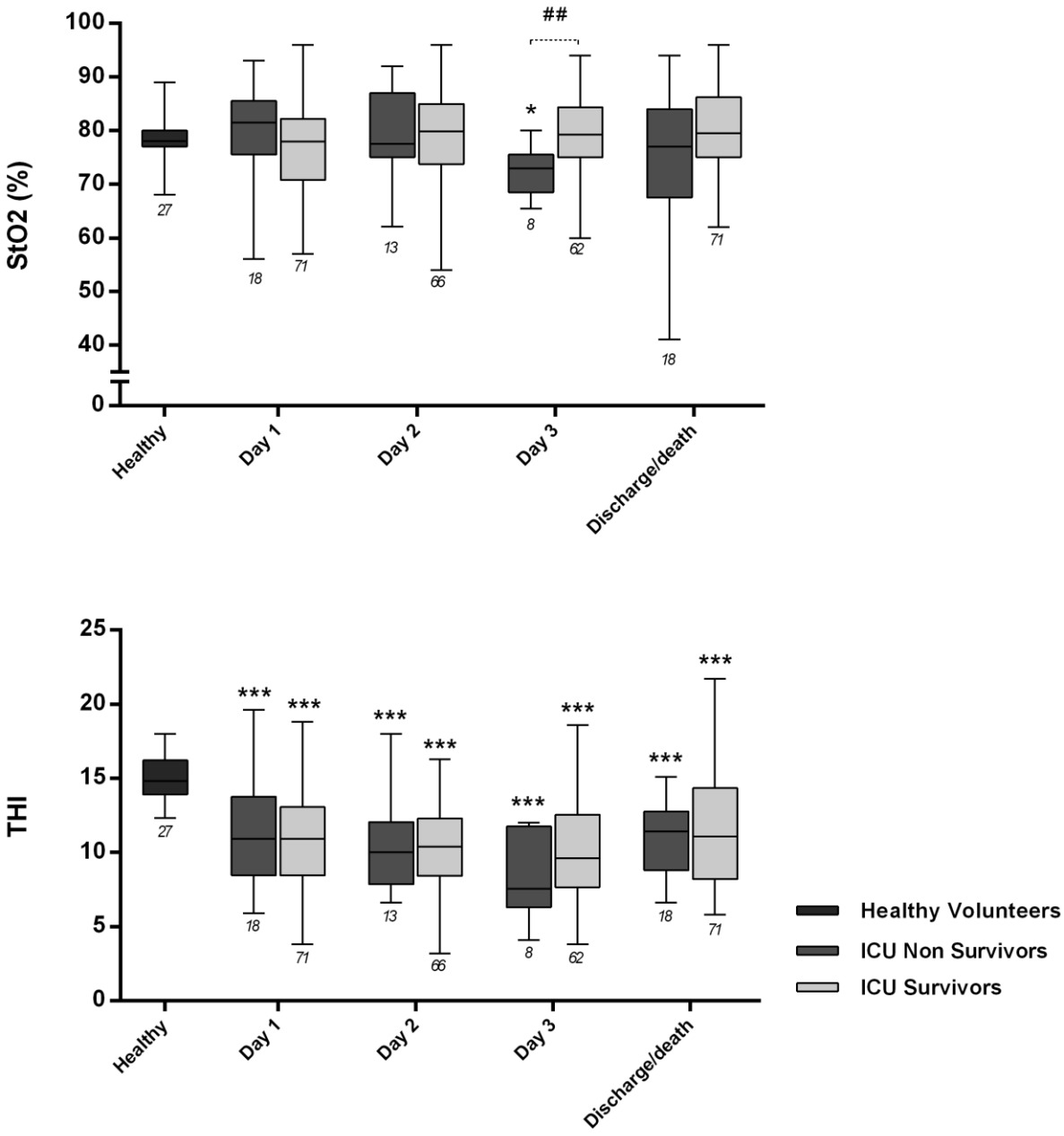
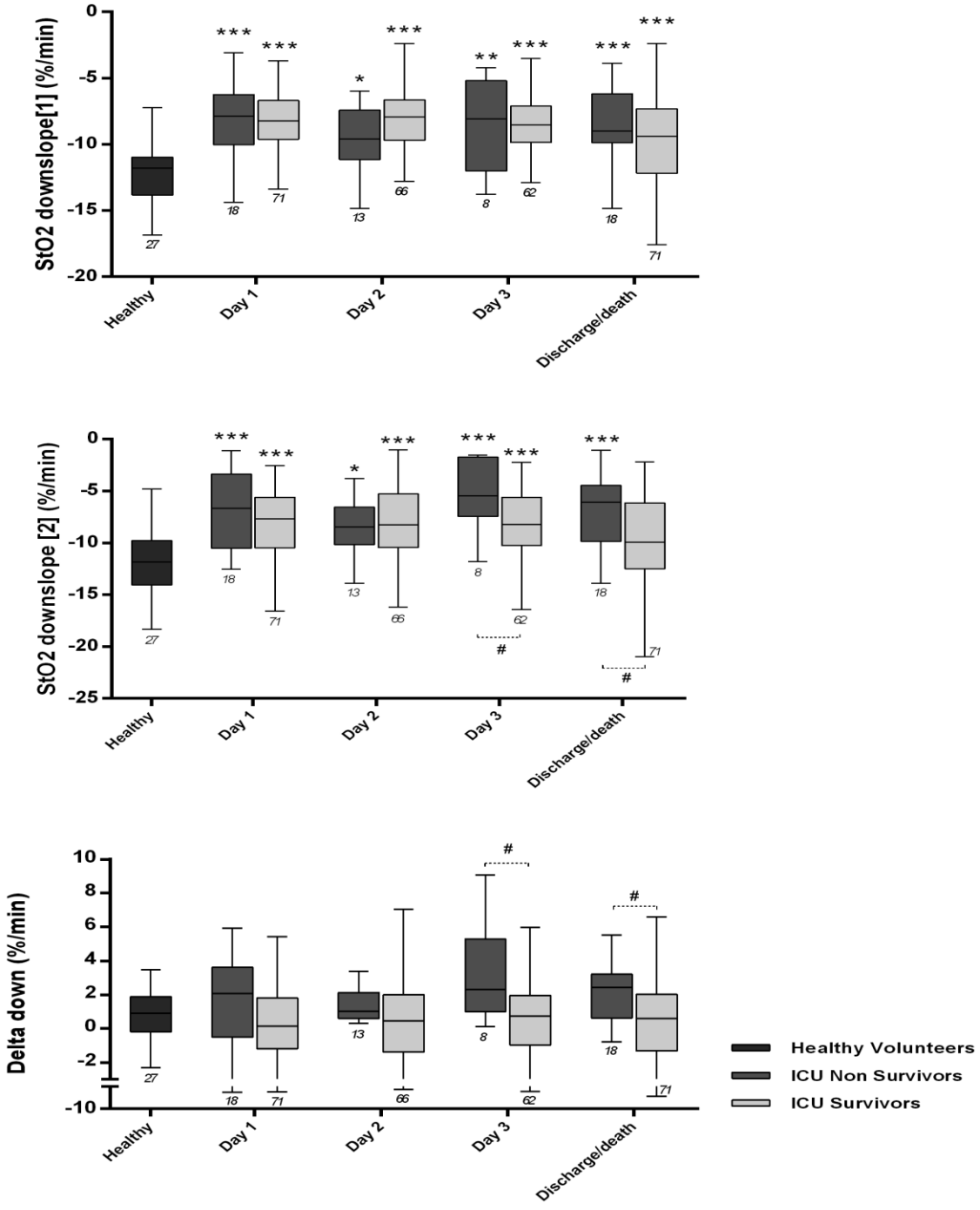
Figure 1 – Tissue O_2 saturation (StO_2) and Tissue Hemoglobin Index (THI) in healthy volunteers, ICU-Survivors and ICU Non-Survivors (on the first three days and on the day of death/discharge).



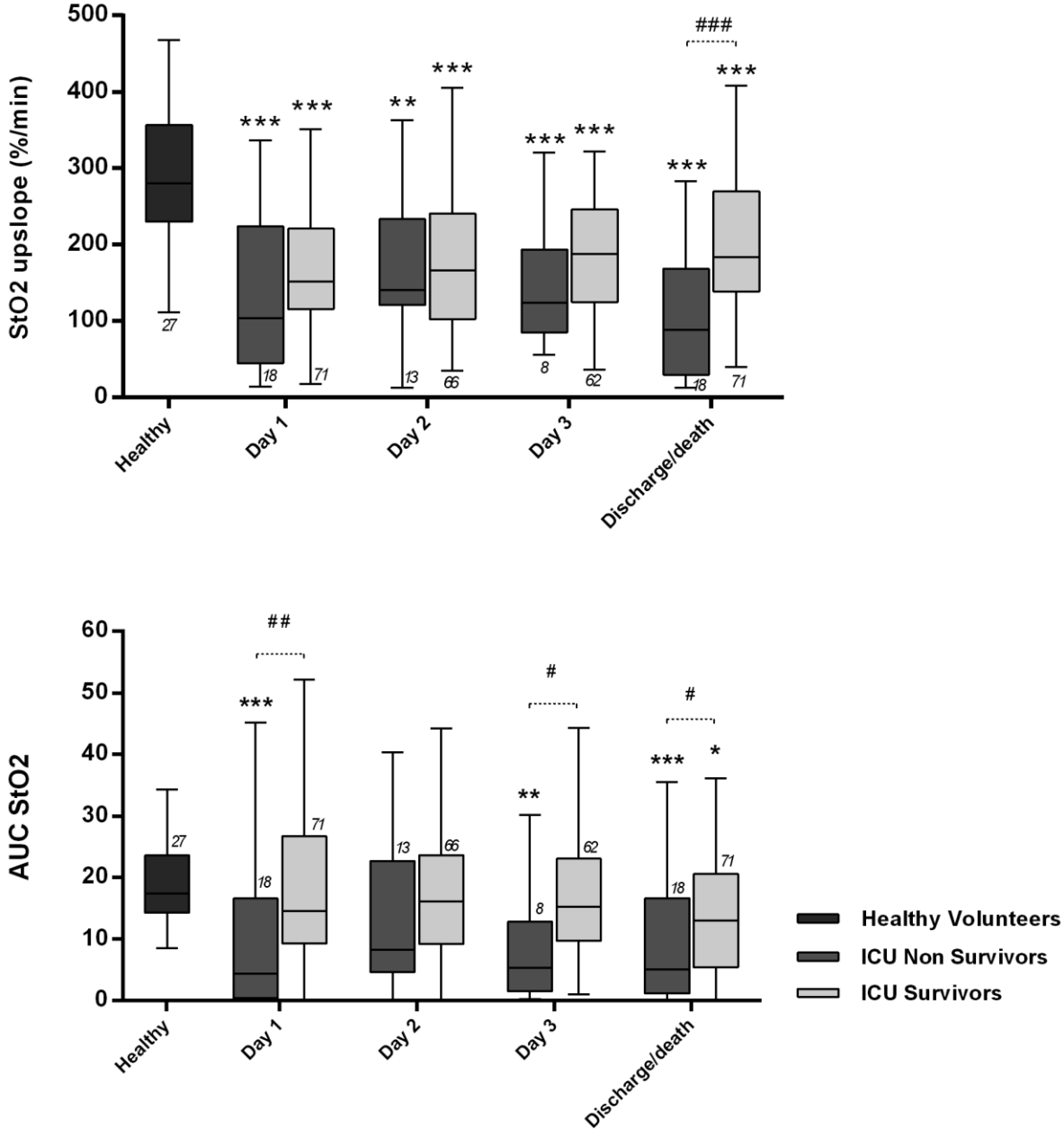
*p<0.05, **p<0.01, ***p<0.001, versus Healthy volunteers, Kruskal Wallis test with Dunn's test for multiple comparisons ##p<0.01, Mann-Whitney U test. Number of patients is indicated near the error bars.

Figure 2 – Downslope 1, Downslope 2 and Delta-Downslope in healthy volunteers, ICU-Survivors and ICU Non-Survivors (on the first three days and on the day of death/discharge).



*p<0.05, **p<0.01, ***p<0.001, versus Healthy volunteers, Kruskal Wallis test with Dunn's test for multiple comparisons, #p<0.05, ##p<0.01, Mann-Whitney U test. Number of patients is indicated near the error bars.

Figure 3 – Upslope and AUC StO_2 in healthy volunteers, ICU-Survivors and ICU Non-Survivors (on the first three days and on the day of death/discharge).



*p<0.05, **p<0.01, ***p<0.001, versus Healthy volunteers, Kruskal Wallis test with Dunn's test for multiple comparisons, #p<0.05, ##p<0.01, Mann-Whitney U test. Number of patients is indicated near the error bars.

Table 1 – Description of the patients on admission to the ICU: comparison between ICU Survivors and Non-Survivors.

	ICU Survivors (n=71)	**ICU Non-Survivors (n=18)**	**p**
Age (years)	59 ± 18	67 ± 18	0.080
Gender (M;F)	49; 22	13; 5	0.518
APACHE II score	15 ± 7	23 ± 5	<0.001
SOFA score	6 ± 3	11 ± 5	<0.001
Admission diagnosis (n)			0.131
Trauma (36)	33	3	
Neurologic (20)	17	3	
Respiratory (8)	6	2	
Sepsis (8)	5	3	
Surgery (7)*	4	3	
Cardiac (3)	2	1	
Other (7)	4	3	
Comorbidities (n)			0.362
None (34)	30	4	
Arterial hypertension (27)	20	7	
Chronic vascular disease (17)	11	6	
Cardiac disease (24)	16	8	
Chronic respiratory disease (15)	8	7	
Diabetes mellitus (14)	12	2	
Obesity (13)	9	4	
Cancer (8)	5	3	
Hypercholesterolemia (6)	4	2	
Chronic renal failure (6)	4	2	
ICU length of stay	9 [5-15]	3 [2-11]	0.006
Heart rate (bpm)	80 ± 22	93 ± 28	0.036
Mean Arterial Pressure (mmHg)	87 [74-95]	74 [56-87]	0.046
Hb (g/dL)	11 ± 1.5	11 ± 2.3	0.959
Arterial lactate (mmol/L)	1.3 [0.9-1.8]	3.1 [1.5-5.6]	<0.001
Any vasopressor			0.006
yes	34	15	
no	37	3	

APACHE Acute Physiology and Chronic Evaluation, *SOFA* Sequential Organ Failure Assessment
*3 high-risk scheduled surgery (2 pulmonary lobectomies, 1 partial pancreatectomy complicated by intra-operative bleeding), 4 emergency interventions (3 ruptured abdominal aortic aneurysm, 1 fasciotomy for soft tissue infection)

Table 2 – NIRS-derived parameters in ICU Survivors and Non-Survivors.

	ICU Survivors (n=71)	ICU Non-Survivors (n=18)	p
First measurement			
StO_2 (%)	78 [70-82]	81 [75-85]	0.176
Downslope 1 (%/min)	-8.1 [-9.6, -6.7]	-7.9 [-10, -6.2]	0.764
Downslope 2 (%/min)	-7.7 [-10.5, -5.6]	-6.7 [-10.5, -3.4]	0.200
Delta-Downslope (%/min)	0.1 [-1.2, 1.7]	2.1 [-0.5, 3.6]	0.050
Upslope (%/min)	150 [114-206]	103 [44-224]	0.202
AUC StO_2	14.6 [9.2-26.8]	4.3 [0.5-16.6]	0.002
THI	10.9 [8.4-12.9]	10.9 [8.4-13.7]	0.612
Mean values			
StO_2 (%)	80 [76-84]	79 [73-85]	0.567
Downslope 1 (%/min)	-8.8 [-10.3, -7.6]	-8.7 [-10.5, -7.9]	0.927
Downslope 2 (%/min)	-9.0 [-10, -7.2]	-6.5 [-8.6, -5.4]	0.010
Delta-Downslope (%/min)	0.4 [-0.8, 1.8]	2.8 [0.4-3.8]	0.004
Upslope (%/min)	185 [143-217]	141 [75-183]	0.016
AUC StO_2	14.5 [11.2-21.3]	7.9 [4.3-12.6]	0.001
THI	11.0 [9.1-12.8]	11.4 [9.5-13.7]	0.432

StO_2 tissue O_2 saturation, *AUC StO_2* area under the curve of reactive hyperaemia

Additional File 1 – NIRS-derived variables stratified based on the presence of sepsis, hypotension, tachycardia, high lactate levels and norepinephrine administration.

	StO_2 (%)	Downslope 1 (%/min)	Downslope 2 (%/min)	Delta Downslope (%/min)	Upslope (%/min)	AUC StO_2	THI
Sepsis							
no (725)	81 [75-86]	-8.9 [-11.2, -7.2]	-8.5 [-11.1, -6.1]	0.5 [-1, 2.5]	183 [121-248]	13 [6-21]	11 [8-13]
yes (98)	79 [73-83]*	-8.7 [-10.8, -7.0]	-7.2 [-9.1, -4.3]***	2.1 [0.3-3.9]***	145 [95-227]**	11 [5-19]	10 [7-12]
MAP							
≥65 mmHg (770)	80 [75-86]	-8.9 [-11, -7.1]	-8.4 [-10.9, -6]	0.5 [-0.9, 2.6]	185 [122-249]	13 [6-21]	10 [8-13]
<65 mmHg (53)	81 [73-85]	-8.6 [-11.8, -7.1]	-7.3 [-9.9, -5]***	1.4 [-0.2, 3.3]***	120 [78-179]***	11 [5-20]	10 [8-12]
Heart rate							
≤90 bpm (605)	81 [75-86]	-8.6 [-10.8, -7.1]	-8.3 [-10.8, -6]	0.4 [-1.1, 2.4]	183 [124-241]	13 [7-21]	11 [8-13]
>90 bpm (218)	80 [74-86]	-9.4 [-12.6, -7.6]	-8.4 [-10.9, -5.6]	1.3 [-0.6, 3.8]	169 [103-255]	11 [5-19]*	10 [8-13]
Arterial lactate							
≤1.5 mmol/L (680)	81 [76-86]	-8.7 [-10.8, -7.1]	-8.5 [-11, -6.2]	0.4 [-1.1, 2.3]	186 [127-248]	13 [7-22]	11 [8-13]
>1.5 mmol/L (144)	79 [72-85]**	-9.2 [-12.4, -6.8]	-7.3 [-9.7, -4.3]***	2.3 [0.1-4.2]***	125 [85-222]***	11 [5-19]*	10 [8-12]*
Norepinephrine							
no (587)	81 [76-87]	-8.8 [-12.3, -7.2]	-8.5 [-11, -6.2]	0.4 [-1.1, 2.4]	187 [131-253]	13 [7-22]	11 [9-13]
yes (236)	79 [73-84]***	-8.9 [-10.7, -7.1]	-7.3 [-10.2, -5]***	1.4 [-0.1, 3.4]***	150 [97-223]***	12 [6-20]	10 [7-12]***

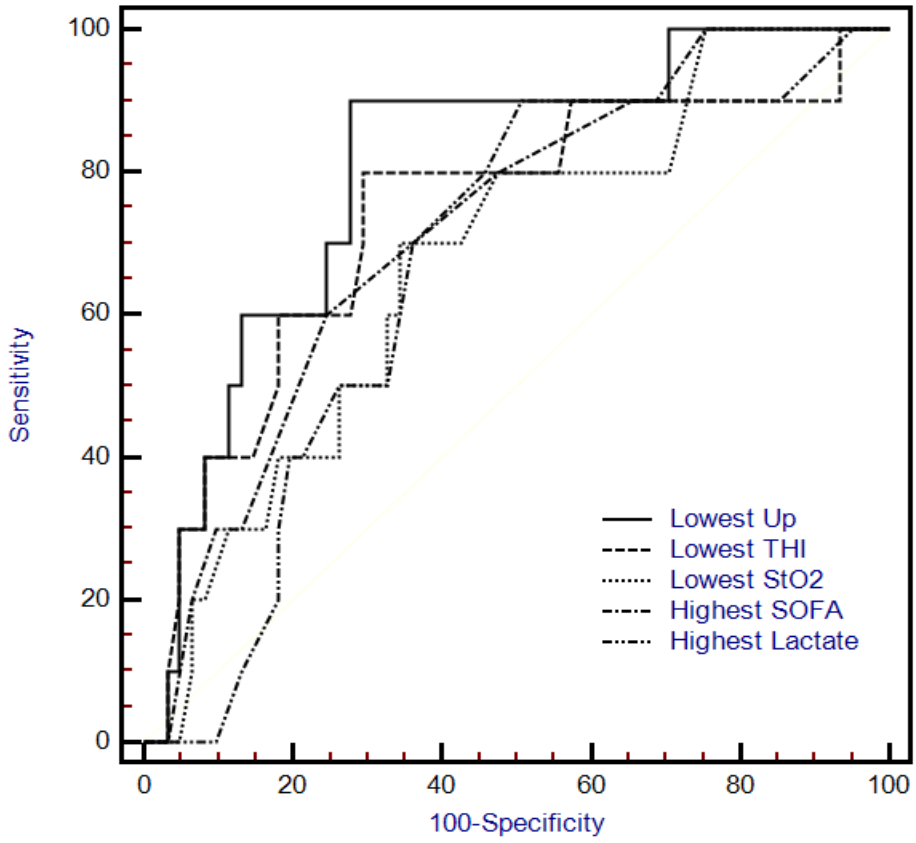
Numbers of measurements are indicated in parenthesis. *StO_2* tissue O_2 saturation, *AUC StO_2* area under the curve of reactive hyperaemia, *MAP* mean arterial pressure. *p<0.05, **p<0.01, ***p<0.001, Mann Whitney U test.

Additional File 2 – Worst values of clinical and NIRS-derived parameters during the ICU stay: comparison between 90-day Survivors and 90-day Non-Survivors. ICU Non-Survivors were excluded from this analysis.

	90-day Survivors (n=61)	**90-day Non-survivors (n=10)**	**p**
Highest SOFA score	7 [4-9]	10 [8-12]	0.021
Highest HR (bpm)	101 [87-112]	104 [86-127]	0.435
Lowest MAP (mmHg)	72 [64-79]	71 [56-83]	0.675
Lowest Hb (g/dL)	9.0 [8.3-10.1]	9.7 [7.8-10.3]	0.785
Lowest PaO_2 (mmHg)	82 [70-109]	84 [79-92]	0.984
Highest Lactate (mmol/L)	1.4 [1.0-1.9]	1.7 [1.5-2.2]	0.099
Lowest $ScvO_2$ (%)	69 [64-73]	67 [61-71]	0.159
Lowest StO_2 (%)	70 [65-75]	66 [58-70]	0.076
Highest Downslope 1 (%/min)	-5.5 [-6.7, -4.7]	-6.3 [-7, -4.4]	0.693
Highest Downslope 2 (%/min)	-3.4 [-6.1, -2.6]	-2.9 [-4.9, -2.5]	0.486
Highest Delta-Downslope (%/min)	3.7 [1.7-5.3]	4.5 [2.3-16]	0.312
Lowest Upslope (%/min)	90 [66-145]	46 [31-68]	0.001
Lowest AUC StO_2	3 [0-10]	5 [1-6]	0.697
Lowest THI	7 [6-10]	5 [4-6]	0.015

SOFA Sequential Organ Failure Assessment, *HR* heart rate, *MAP* mean arterial pressure, *Hb* haemoglobin, *PaO_2* arterial O_2 tension, *$ScvO_2$* central venous O_2 saturation, *StO_2* tissue O_2 saturation, *AUC StO_2* area under the curve of reactive hyperaemia, *THI* tissue haemoglobin index.

Additional File 3 – Receiver operating characteristics (ROC) curve analysis for 90-day mortality after ICU discharge.



Lowest Upslope: area under the curve (AUC) 0.80 [95% confidence interval 0.69-0.89]; Lowest THI: AUC 0.74 [95% confidence interval 0.62-0.83]; Lowest StO_2: AUC 0.68 [95% confidence interval 0.55-0.78]; Highest SOFA score: AUC 0.73 [95% confidence interval 0.61-0.82]; Highest Lactate: AUC 0.66 [95% confidence interval 0.54-0.77].

Chapter 5

MicroDAIMON study:

Microcirculatory DAIly MONitoring in critically ill patients. A prospective observational study

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ABSTRACT

Background: *until now, the prognostic value of microcirculatory alterations in critically ill patients has been mainly evaluated in highly selected subgroups. Aim of this study is to monitor the microcirculation daily in mixed group of Intensive Care Unit (ICU)-patients and to establish the association between (the evolution of) microcirculatory alterations and outcome.*

Methods: *Prospective longitudinal observational single-centre study in adult patients admitted to a 12-bed ICU in an Italian teaching hospital. Sublingual microcirculation was evaluated daily, from admission to discharge/death, using Sidestream Dark Field imaging. Videos were analysed offline to assess flow and density variables. Laboratory and clinical data were recorded simultaneously. A priori, a microvascular flow index (MFI) <2.6 was defined as abnormal. A binary logistic regression analysis was performed to evaluate the association between microcirculatory variables and outcomes; a Kaplan-Meier survival curve was built. Outcomes were ICU and 90-day mortality.*

Results: *Trial registration: NCT 02649088, www.clinicaltrials.gov. Date of registration: 23rd December 2015, retrospectively registered.*

97 patients were included. An abnormal MFI was present on day 1 in 20.6%, and in 55.7% of cases during ICU-admission. Patients with a baseline MFI<2.6 had higher ICU-, in-hospital and 90-day mortality (45% vs 15.6%, p=0.012; 55% vs 28.6%, p=0.035; 55% vs 26%, p=0.017 respectively). An independent association between baseline MFI <2.6 and outcome was confirmed in a binary logistic analysis (Odds Ratio 4.594 [1.340-15.754], p=0.015). A heart rate (HR) ≥90 bpm was an adjunctive predictor of mortality. However, a model with stepwise inclusion of mean arterial pressure<65mmHg, HR≥90 bpm, lactate>2mmol/L and MFI<2.6 did not detect significant differences in ICU-mortality. In case an abnormal MFI was present on day 1, ICU-mortality was significantly higher in comparison to patients with an abnormal MFI after day 1 (38 vs. 6%, p=0.001), indicating a time-dependent significant difference in prognostic value.

Conclusions: *in a general ICU-population an abnormal microcirculation at baseline is an independent predictor for mortality. In this setting, additional routine daily microcirculatory monitoring did not reveal extra prognostic information. Further research is needed to integrate microcirculatory monitoring in a set of commonly available hemodynamic variables.*

Keywords: microcirculation, physiologic monitoring, critical illness, tachycardia, video microscopy, capillaries.

BACKGROUND

The microcirculation is a vast network of small vessels (terminal arterioles, capillaries and venules < 100μm diameter) in which the exchange of oxygen and nutrients with tissues takes place. [1] Its derangement, defined as "microcirculatory shock" [2], is recognized as an important cause of organ dysfunction in critically ill patients, affected by various disease states, such as sepsis, severe trauma, hemorrhagic shock and post-cardiac arrest. [2-5] Furthermore, microcirculatory abnormalities and its persistence despite adequate macro-hemodynamic resuscitation were independently associated with morbidity and mortality in many critical conditions. [6-12]

Today, the development of new technologies of in-vivo video microscopy, and its integration in easy-to-handle microscopes as in Sidestream Dark Field (SDF) imaging, allow us to assess the (sublingual) microcirculation at the bedside, in a non-invasive way. [13] However, until 2015, data on microcirculatory alterations in the Intensive Care Unit (ICU) were restricted to small sample-sized studies in high-risk patients. [7,8,14]

The MicroSOAP study by Vellinga and colleagues [15] gave a first insight in the *prevalence* of microcirculatory alterations in a large number of ICU patients. However, due to its design with a single time-point observation, the *incidence* in a time-dependent manner remains to be elucidated. Primary aim of the study was to detect a difference in the incidence of microvascular flow abnormalities between ICU survivors and non-survivors. Secondary outcomes were long-term mortality (in-hospital mortality and 90-day mortality) and development of organ dysfunction (described by Sequential Organ Failure Assessment, SOFA).

METHODS

PATIENTS ENROLLMENT AND DATA COLLECTION

The MicroDAIMON (Microcirculation DAIly MONitoring in critically ill patients) is a single-centre prospective observational study (clinicaltrials.gov, NCT 02649088 registered on 23rd December; retrospectively registered). The recruiting phase was performed in a 9 months period in 2013 (from 1st April to 31st December) in a 12-bed mixed ICU of an Italian teaching hospital with a mean number of yearly-admitted patients of 400. The ICU was structured in 3 subunits of 4 beds each, caring for respiratory, traumatology and medical critically ill patients respectively. For the study purpose, each subunit was subsequently included and monitored during a 3 months period for patients' screening and the recruitment: from 1st April to 30th June 2013 the medical

subunit, from 1st July to 30th September 2013 the traumatology subunit and from 1st October to 31st December 2013 the respiratory subunit.

Patients were screened and included in the study within the first 12 hours from ICU admission. Exclusion criteria were age<18 years, lack of informed consent and pathophysiological conditions that may interfere with the sublingual microcirculation videos acquisition (maxillofacial traumas/surgery, oral bleeding, mucositis etc.). In context to the microcirculatory assessments, demographic, laboratory, microbiologic, hemodynamic and other clinical data were recorded. All patients were followed up for 90 days after the ICU admission.

The study protocol was approved by the Local Ethics Committee and conducted in respect of the principles of Helsinki declaration (last revision, Edinburgh 2000). A written informed consent was obtained from all the included subjects or their next of kin in compliance with national applicable laws.

MICROCIRCULATION ASSESSMENT:

The sublingual microcirculation was evaluated at the moment of the inclusion and every 24 hours until discharge/death with SDF-imaging (Microscan®, Microvision Medical, Amsterdam, The Netherlands). [13]

The video acquisition technique is extensively described in previous papers. [16] For every session, videos from at least 5 different sites were registered trying to obtain a good video quality and to avoid artefacts that may affect flow or vessels density variables. [16]

The three best videos were chosen from each session, in compliance with recommendations from Massey et al. [17] and blindly analysed offline with a dedicated software (Automated Vascular Analysis, AVA Software 3.0, MicroVision Medical, Amsterdam, The Netherlands) by a restricted group of four experienced investigators. Inter-observer variability was calculated, based on the simultaneous analysis of ten randomly selected SDF videos by all the investigators. Variables of flow (Microvascular Flow Index, MFI and Proportion of Perfused Vessels, PPV), as well as capillary density (Total Vessel Density, TVD, Perfused Vessel Density, PVD) and flow distribution (Heterogeneity Index, HI) were calculated according to international criteria [18,19]. Flow was scored per quadrant as 0 (no flow), 1 (intermittent flow), 2 (sluggish flow) and 3 (continuous flow). The MFI is the average over 4 quadrants x 3 areas of interest. Total vessel density (TVD, mm/mm^2) was calculated as the total length of vessels divided by the total area of the image. The percentage of perfused vessels (PPV) was estimated as follows: 100 x [(total number of grid crossings - [no flow + intermittent flow])/total number of grid crossings] and expressed as percentage. The perfused vessel density (PVD, mm/mm^2) was estimated by

multiplying TVD by PPV as estimated with the De Backer method. The flow heterogeneity index (FHI, arbitrary units) was calculated as the highest MFI minus the lowest MFI, divided by the mean MFI of all sublingual sites. [18]
Analogous to previous data, a threshold for the MFI < 2.6 was a priori established to define an abnormal microcirculation. [3,8,15,20]

STATISTICAL ANALYSIS:
Data analysis was conducted with SPSS Software 17.0 (IBM, New York, NY) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA). All data are presented as mean ± standard deviation (SD) or median [interquartile range, IQR].
Descriptive statistics were performed to obtain patients' baseline characteristics. Quantitative variables distribution was tested with Kolmogorov-Smirnov normality test. Parametric (Student's t test with Welch's correction) and non-parametric tests (Mann-Whitney U test) were applied to describe the differences between groups for the variables of interest as appropriate. Fisher's Exact test was performed for comparisons between categorical variables and the results are presented as percentage, Odds Ratio (OR) and 95% confidence interval (CI). Kaplan-Meier 90-day survival curves with Tarone-Ware test for the comparison of the Hazard Ratio between groups were built for the survival analysis.
Binary logistic regression analysis was performed with a forward stepwise entry method. A p value of less than 0.05 was considered statistically significant.

RESULTS

Population characteristics:

During the study period, 40, 37 and 38 patients were admitted respectively in the medical, traumatology and respiratory ICU subunits, for a total amount of 115 patients. 100 patients met the inclusion criteria. All the patients were included in the study within 12 hours from ICU admission, with no exceptions due to timing or organizational issues. Three patients were a posteriori excluded because no SDF videos were available for the baseline assessment. Therefore, 97 patients were included in the final analysis. The flow-chart for the patients' inclusion process is illustrated in Additional file 1.
Baseline characteristics of the patients are illustrated in Table 1. Patients were predominantly male (66%) with a median age of 67 years [46-75], a mean Acute Physiology And Chronic Health

Evaluation (APACHE) II score of 16±7 and a median SOFA score of 7[4-10]; the most frequent cause of ICU admission was trauma (38.1%). Patients admitted for sepsis represented the 9.3% of the sample. During the ICU stay, 10 more patients developed sepsis: 2 trauma patients (5.4%), 1 neurologic patient (4.8%), 2 respiratory patients (18.2%) and 5 other patients (26.3%).

Median ICU length of stay was 7 [4-15] days; ICU mortality was 21.6%, in-hospital mortality 34%, 90-day mortality 31.9% (two patients died in the hospital after 90 days from ICU admission).

Microcirculatory abnormalities at baseline and outcome:

2455 videos were collected and analyzed off-line to obtain microcirculatory variables. The coefficient of variation (inter-observer variability) for MFI was 1.4%±3% for small vessels. Baseline microcirculatory variables are described in Table 1. The incidence of MFI abnormality at the day of ICU admission was 20.6%.

ICU non-survivors showed a higher baseline APACHE II score and SOFA score, higher age, heart rate (HR), Cumulative Vasopressor Index [21], arterial lactate level, serum creatinine and lower platelets count. (Table 1)

Subsequently, patients were divided in two groups based on normal (≥2.6) or abnormal (<2.6) baseline MFI. In comparison to patients with a normal MFI at baseline, patients with an abnormal MFI showed a higher ICU-mortality (45% vs 15.6%, p=0.012) (Table 1), in-hospital mortality (55% vs 28.6%, p=0.035) and 90-day mortality (55% vs 26%, p=0.017). (Additional files 2-3)

Survival analysis, by Kaplan-Meier method, confirmed a significant difference between the two groups for 90-day mortality (Tarone-Ware χ^2=6.15, p=0.003). (Figure 1, panel a). In the binary logistic regression analysis, the presence of an abnormal MFI at baseline was associated with ICU-mortality (OR 4.594 [95% CI 1.340-15.754], p=0.015) independently of the APACHE II score (Table 2).

The role of tachycardia in combination with an abnormal microcirculation was additionally tested. Patients were divided in four groups based on the presence of tachycardia (defined as the presence of an HR≥90 beats per minute, bpm) [22-25] and/or MFI abnormality at the baseline. ICU-mortality was significantly different between the four groups (overall χ^2=12.76, p=0.002). Survival analysis confirmed a significant difference between the groups in terms of 90-day mortality (Tarone-Ware χ^2= 24.98, p<0.0001) with a survival rate as low as 12.5% among patients with tachycardia plus abnormal MFI (Figure 1, panel b). The combination of tachycardia and an abnormal MFI on day 1 was associated with an increased risk for ICU-mortality (OR 10.732 [95% CI 1.685-68.354], p=0.012) independently of the APACHE II score (Table 2).

Integration of an abnormal microcirculation in a set of common hemodynamic variables

In order to clarify the additional prognostic value of an abnormal microcirculation (MFI <2.6) at baseline in a set of commonly available hemodynamic variables, i.e. mean arterial blood pressure (MAP), HR and lactate, we divided these variables into normal and abnormal: MAP ≥65mmHg = normal, <65 mmHg = abnormal; HR < 90 bpm=normal, ≥90 bpm=abnormal; arterial lactate ≤2 mmol/L= normal, >2 mmol/L=abnormal. In the first model, all variables were normal. A stepwise addition of each variable was associated with a non-significant reduction in ICU mortality. (Figure 2). In the second model, all variables were abnormal. A stepwise addition of each variable was associated with a non-significant increment in ICU mortality. (Figure 2). The comparison between the two models, revealed in each step a significantly higher ICU mortality in the 'abnormal' model. (Figure 2).

Microcirculatory longitudinal monitoring and outcome:

The median duration of follow-up for each patient category was: 6 [3-12] days for trauma, 8 [3-14] days for neurologic, 5 [1-6] days for respiratory, 8 [3-11] days for septic and 4 [2-8] days for other patients.

The total *incidence* of an abnormal microcirculation during the entire ICU stay was 55.7% (20.6% on day 1, 35.1% after day 1). Microcirculatory imaging was restricted to day 1 in ten patients (6 died, 4 were discharged); missing data (SOFA and/or MFI) prevented further analysis in 19 patients. MFI and SOFA score over time are depicted in figure 3. Twenty-two patients showed an increment in MFI between day 1 and 2 (ΔMFI (+)), 21 patients showed a reduction in MFI between day 1 and 2 (ΔMFI (-)), and 25 remained indifferent. ΔMFI (+) was not associated with a significant reduction in SOFA score between day 2 and 3 (corresponding with the same timeframe of MFI day 1 and 2) or mortality, as compared to patients with a ΔMFI (-). Any increase/decrease in MFI was considered relevant for this analysis.

Post-hoc, patients were divided into four groups according to the timing of the presence of an abnormal MFI. Group 1: patients with a normal MFI on day 1 AND later on (n=36). Group 2: patients with a normal MFI on day 1 but with one or more episodes of an abnormal MFI later on (n=34). Group 3: patients with an abnormal MFI on day 1 AND a normal MFI later on (n=6). Group 4: patients with an abnormal MFI on day 1 AND one or more episodes of an abnormal MFI later on (n=10). Mortality was significantly different across groups (p<0.001). If an abnormal MFI was present on day one (group 3 and 4) mortality was 6/16 (38%), whereas in patients with an abnormal MFI only after day 1 (group 2) ICU mortality was 2/34 (6%, p=0.001), indicating a

significant difference in prognostic value of an abnormal MFI on day 1 in comparison to an abnormal MFI after day 1.

DISCUSSION

This MicroDAIMON study is currently the largest prospective longitudinal observational study to describe the incidence of microcirculatory derangements among a mixed group of critically ill patients, offering a day-by-day follow-up. The incidence of baseline microcirculatory flow abnormalities was 20.6% and more than half (55.7%) of the patients displayed an abnormal MFI in at least one observation during ICU stay. The main finding of this study is that in this mixed ICU population, an abnormal baseline MFI is independently associated with unfavorable outcome in terms of ICU, in-hospital and 90-day mortality. In addition, the contemporary presence of tachycardia showed an additive predictive power towards mortality in the survival analysis. However, the change of MFI over time was not associated with outcome, both in terms of organ failure (SOFA) and mortality. In contrast to an abnormal MFI on day 1, we could not associate an abnormal MFI after day 1 with unfavorable outcome. No associations were found between the other microvascular variables and outcome.

In 2014, the MicroSOAP study provided the first and largest database on the prevalence and the significance of the microcirculatory alterations in a heterogeneous ICU population, with a time point-observation across 36 ICUs worldwide. [15] The authors reported a prevalence of MFI abnormalities of 17%, using the same predefined cut-off value [15,26]. This difference in reported percentage of MFI abnormalities can be explained by the difference in study design (longitudinal vs point-prevalence). Our data confirm previous observations, showing an important prognostic role of the microcirculation in various subsets of critically ill patients. [4-9,14] In contrast to the existing literature, these findings extend the predictive value of early microcirculatory alterations towards 90-day mortality. Patients with an abnormal MFI at baseline showed an absolute risk of non-survival almost three times higher in comparison to patients with a normal MFI.

However, in the present study routine day-by-day microcirculatory monitoring does not confirm previous observations. In 2004 Sakr et al. introduced for the first time the concept of serial observations of microcirculation in a cohort of 49 patients with septic shock.[11] In this highly selected group of patients, the persistence of microcirculatory alterations was associated with persistence of shock, development of multiple organ failure and mortality. Conversely, ICU-survivors showed early improvement of microcirculation. These data were confirmed by

others. [21] Duranteau and colleagues observed in another selected cohort of 18 patients with traumatic hemorrhagic shock, early derangements of microcirculatory flow and vessel density, as well as its persistence, were able to predict a worse SOFA score after 96 hours from ICU admission.[5] A possible explanation for this discrepancy may lie in the heterogeneous composition of our study population and in considerable differences in microcirculatory baseline abnormalities. Alternatively, microvascular alterations represent differences in underlying pathology between study populations. Careful selection of patients at risk may contribute to the prognostic power of microcirculatory observation. As of now, our data indicate that routine daily monitoring of the microcirculation in an unselected group of ICU patients is of limited prognostic value.

This study has several limitations. Although this study contains the largest reported database on day-by-day monitoring of the microcirculation in critically ill, it appears to have insufficient sample size to correlate differences in the evolution of microcirculatory conditions over time with clinically relevant endpoints (SOFA, mortality) also due to the considerable number of patients lost to follow up, due to death/discharge. And although the independent predictive value of an abnormal MFI on day 1 was established, the integration of such variable in a model with more commonly used hemodynamic variables was clearly limited by the sample size as well. Further research is need to establish the additive value of microcirculatory imaging on top of the existing hemodynamic variables. In addition, it is conceivable that other microvascular variables and different cut-off yields different results. We did not find any significant association between the other microcirculatory variables (TVD, PVD, PPV) and the outcome either on day 1 or in the following days. This could be explained by the fact that the MFI, especially if used as a dichotomous variable based on an a priori cut off of 2,6, could have been the most sensitive variable to detect an association with the outcome in a such heterogeneous population which is expected to cause a “dilution effect” on the microcirculatory alterations. It is also possible that a vessel by vessel MFI calculation could have been more precise and provide different results depending on a more accurate evaluation of the capillary blood flow especially in presence of marked heterogeneity. In this respect, the burden of time-consuming off-line analysis remains a major practical limitation for the study population sample size until the time of the development and full validation of automated analysis software. Real-time “eyeballing” the microcirculation by bedside assessment of MFI is a major advantage in the development of a bedside tool, and showed good agreement with the gold standard off-line analysis. [27] Post-hoc analysis of our data confirmed 2.6 as the optimal cut-off for the discrimination between survivors and non-survivors. Finally, this was a pure observational study: patients were treated following the international

guidelines and principles of good clinical practice and clinicians had no information about the microcirculation during the study. Therefore, our study design is insufficient to draw conclusions on the applicability of microcirculatory monitoring as a tool to guide resuscitation. Even in the setting where there is an absence of additional prognostic information, derived from microcirculatory monitoring, the observation itself may contain valuable information about the underlying pathophysiologic mechanisms. For example, an increased lactate may adequately predict outcome, but does not reveal its underlying mechanism. Under these conditions additional assessment of microvascular blood flow may not be useful to predict outcome, but may be helpful for the clinician to select the appropriate resuscitation strategy. Further research is needed to address this topic. Careful selection of subgroups and adequate timing remain of the essence in this process.

CONCLUSIONS

This MicroDAIMON study provides data about incidence of microcirculatory alterations in a heterogeneous group of critically ill patients. Microcirculatory flow abnormalities at the baseline were independently associated with an increased risk of unfavorable outcome. Simultaneous presence of tachycardia enhanced this predictive value. However, neither the evolution of MFI over time, nor the development or new abnormalities after day 1 was associated with organ function or mortality in our population with a sample size limitation. Further studies are needed to incorporate microcirculatory monitoring into a set of currently available hemodynamic variables and to establish its value as a tool to guide specific resuscitation strategies.

List of abbreviations:

SDF, Sidestream Dark Field; ICU, Intensive Care Unit; SOFA, Sequential Organ Failure Assessment; MFI, Microvascular Flow Index; PPV, Proportion of Perfused Vessels; TVD, Total Vessel Density; PVD, Perfused Vessel Density; SD, Standard Deviation; IQR, InterQuartile Range; OR, Odds Ratio; CI, Confidence Interval; APACHE II, Acute Physiology And Chronic Evaluation score; HR, Heart Rate; MAP, Mean Arterial Pressure.

Declarations

Ethics approval and consent to participate: The study protocol was approved by the Local Ethics Committee of Azienda Ospedaliera Universitaria “Ospedali Riuniti” of Ancona, Italy (protocol number 212639) and conducted in respect of the principles of Helsinki declaration (last revision,

Edinburgh 2000). A written informed consent was obtained from all the included subjects in compliance with national applicable laws.

Consent for publication: not applicable.

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

Competing interests: CI is the inventor of Sidestream Dark Field imaging technology. He has been a consultant for MicroVision Medical in the past but he has actually no contact with this company for more than 5 years, except that he still holds shares. He has no other competing interests in this field other than his commitment to promoting the importance of the microcirculation during patient care; and there are no other relationships or activities that could appear to have influenced the submitted work.

The other authors have no competing interests to declare.

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Author's contributions: CS, AD, ED, RR, EA designed the study, contributed to the interpretation of the results and critically revised the manuscript. CS and ED performed the statistical analysis, drafted the manuscript, and interpreted the data. ED, CS, RD, AC, SP, ST, VM, SC, MR, BM made a substantial contributions to the acquisition of the data and the analysis of SDF videos and revised the manuscript for important intellectual content. CB made a substantial contribution in drafting the manuscript and interpreting the results. CI made a substantial contribution in the study design, critically revised the manuscript for important intellectual content. 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.

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Table 1 Baseline characteristics and comparison between ICU survivors vs non survivors.

PATIENTS CHARACTERISTICS	*n*	All (97)	*ICU survivors (76)*	*ICU non survivors (21)*	*p*
Male gender (n, %)	*97*	64 (66)	50 (65.8)	14 (66.7)	1
Age (years, n)	*97*	67 [46-75]	64[44-73]	71[56-81]	**0.034**
APACHE II (pts)	*97*	16 ± 7	14±7	22±6	**<0.001**
SOFA (pts)	*97*	7 [4-10]	6[4-9]	12[8-15]	**<0.001**
ICU admission diagnosis, n (%)	*97*				**0.046**
		Trauma 37 (38.1)	34	3(8.1)	
		Neurologic 21 (21.6)	17	4(19)	
		Respiratory 11 (11.3)	8	3(27.3)	
		Sepsis 9 (9.3)	6	3 (33.3)	
		Other 19 (19.7)	11	8(42.1)	
Heart rate (bpm)	97	79 [61-102]	77[61-95]	96[69-107]	**0.045**
Mean arterial pressure (mmHg)	97	84 ± 19	86±17	79±27	0.255
Vasoactive drugs (treated)	54		38(50)	16(76.2)	**0.046**
Noradrenaline (mcg/kg/min)	52	0.28 [0.14-0.61]			
Dopamine (mcg/kg/min)	5	6.1 [4.8-7.4]			
Dobutamine (mcg/kg/min)	8	2.54 [2-4.5]			
Cumulative Vasopressor Index	54	4 [4-4]	1[0-4]	4[2-4]	**0.008**
Glasgow Coma Scale (pts)	97	10 [3-15]	10[4-15]	4[3-14]	0.074
Mechanical ventilation (n, %)	97	91 (93.8)	71(93.4)	21(100)	0.581
Peep (cmH_2O)	91	7 [6-9]	7[6-9]	8[7-10]	0.095
Haemoglobin (g/dL)	97	11 ± 1.78	11.1±1.7	10.8±2.2	0.58
White Blood Cells ($nx10^3$/mmc)	97	12.1 [8.83-14.81]	11.3[8.8-15.7]	12.7[9.5-14.6]	0.518
Platelets ($nx10^3$/mmc)	97	150 [102-199]	165[110-201]	115[56-173]	**0.02**
Creatinine (mg/dL)	97	1.0 [0.8-1.45]	1[0.8-1.2]	1.4[1.1-1.8]	**<0.001**
Bilirubine (mg/dL)	97	0.8 [0.5-1.2]	0.75[0.5-1.1]	0.9[0.4-1.8]	0.264
PaO_2 (mmHg)	97	146 [104-175]	147[106-176]	140[93-172]	0.63
Arterial lactates (mmol/L)	97	1.4 [1.0-2.15]	1.3[0.9-1.67]	3.1[1.4-5.6]	**<0.001**
$ScvO_2$ (%)	60	77.2 [71-82.3]	77.7[72.3-82.5]	75.6[62.7-80.8]	0.24
MICROCIRCULATORY VARIABLES:					
TVD (small) (mm/mm^2)	97	20.4 ± 3.7	20.5[17.2-22.7]	20.9[17.8-22.7]	0.817
PVD (small) (mm/mm^2)	97	19.3 ± 4.4	19.3±4	19.3±6	0.951
De Backer score (small) (n/mm)	97	11.9 ± 2	11.8±2	12.2±2	0.489
PPV (small) (%)	97	98.3 [95.4-100]	98.2[94.8-100]	98.3[97-100]	0.688
MFI (small) (AU)	97	3 [2.7-3.0]	3[2.75-3]	2.93[2.3-3]	0.155
HI (small)	97	0 [0.0-0.2]	0[0-0.2]	0[0-0.3]	0.417
Abnormal MFI (n,%)	97	20 (20.6)	11(14.5)	9(42.9)	**0.012**

Data are presented as mean ± or as median [IQR] unless stated otherwise. APACHE Acute Physiologic And Chronic Health Evaluation II, calculated over the first 24 hours from ICU admission;
SOFA Sequential Organ Failure Assessment, calculated over the first 24 hours from ICU admission. CVI Cumulative Vasopressor Index; ICU Intensive Care Unit; COPD, Chronic Obstructive Pulmonary Disease. TVD Total Vessel Density; PVD Perfused Vessel Density; PPV Proportion of Perfused Vessel; HI Heterogeneity Index;. MFI Microvascular Flow Index. Abnormal MFI is defined as MFI < 2,6. Cut off value for small vessels diameter < 20 μm.

Table 2 Binary logistic regression analysis for ICU mortality

Variables	Odds Ratio (95% CI)	p value
ICU MORTALITY (abnormal MFI)		
APACHE II Score	1.204 (1.089-1.331)	<0.001
MFI < 2.6	4.594 (1.340-15.754)	0.015
ICU MORTALITY (abnormal MFI + tachycardia)		
APACHE II Score	1.191 (1.077-1.316)	0.001
MFI < 2.6 + tachycardia	10.732 (1.685-68.354)	0.012

APACHE Acute Physiologic And Chronic Health Evaluation II, calculated in the first 24 hours from ICU admission; ICU Intensive Care Unit; MFI Microvascular Flow Index. Abnormal MFI is defined as MFI < 2,6 for small vessels (diameter <20 μm). Tachycardia is defined as a heartrate ≥90 bpm.

In the upper model baseline MFI abnormality was the independent variable. Model AUC 0.836 [0.747-0.904], Nagelkerke R^2 0.359, Hosmer and Lemeshow χ^2 4.733, p=0.822. In the lower model the presence of abnormal MFI plus tachycardia was the independent variable. Model AUC 0.836 [0.747-0.903], Nagelkerke R^2 0.374, Hosmer and Lemeshow χ^2 2.670, p=0.914.

FIGURES

Figure 1. Kaplan Meier survival analysis. Panel a represents 2 subgroups, separated by microvascular blood flow (MFI) < 2.6 versus MFI ≥ 2.6. Panel b represents 4 subgroups, separated by MFI with identical cut-off value and heart rate (HR) ≥ 90 versus < 90 bpm.

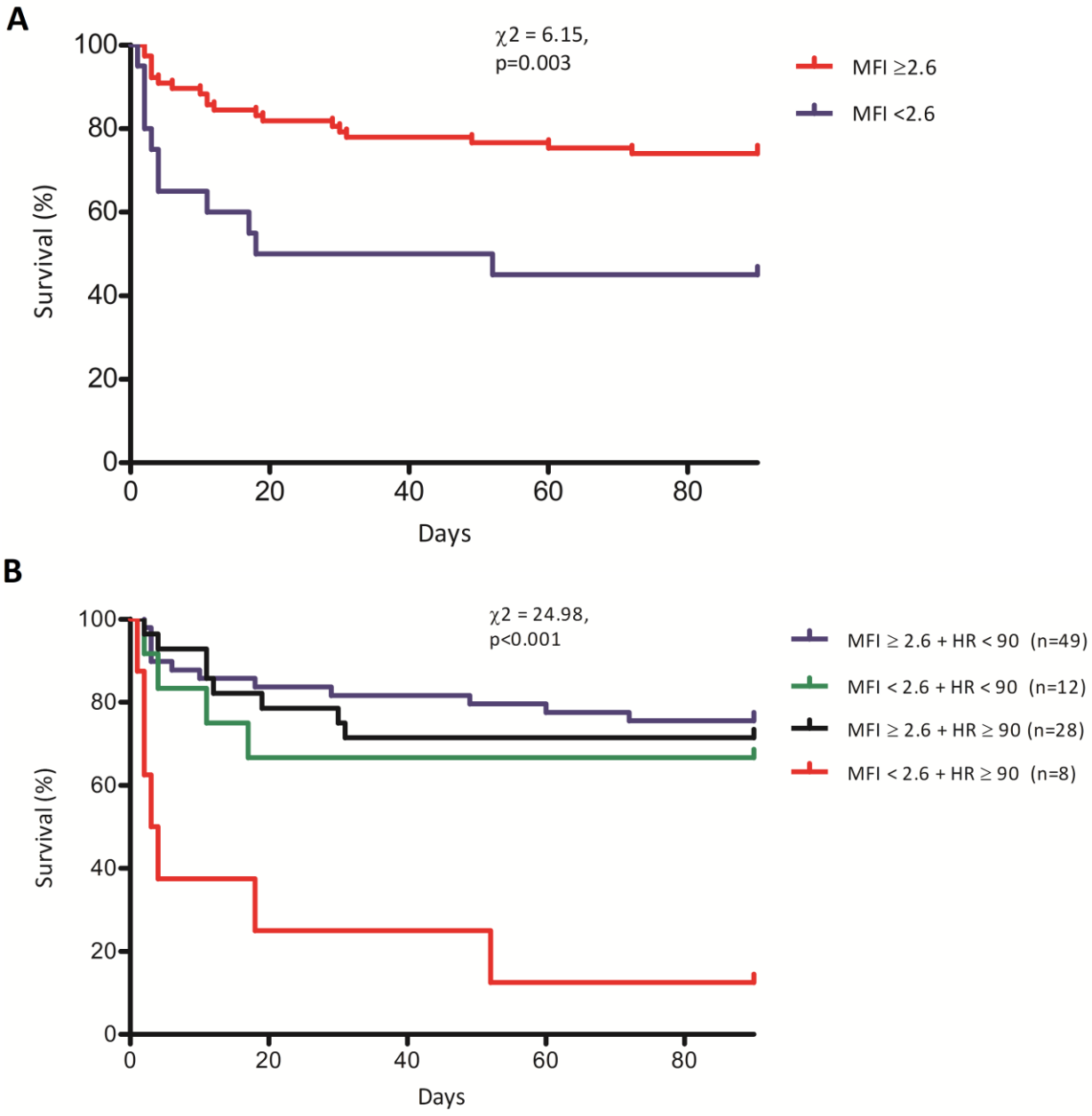


Figure 2. Prognostic model with stepwise inclusion of consecutive hemodynamic variables: mean arterial pressure (MAP) in mmHg, heart rate (HR) in bpm, (arterial) lactate in mmol/L and microvascular flow index (MFI) in AU.

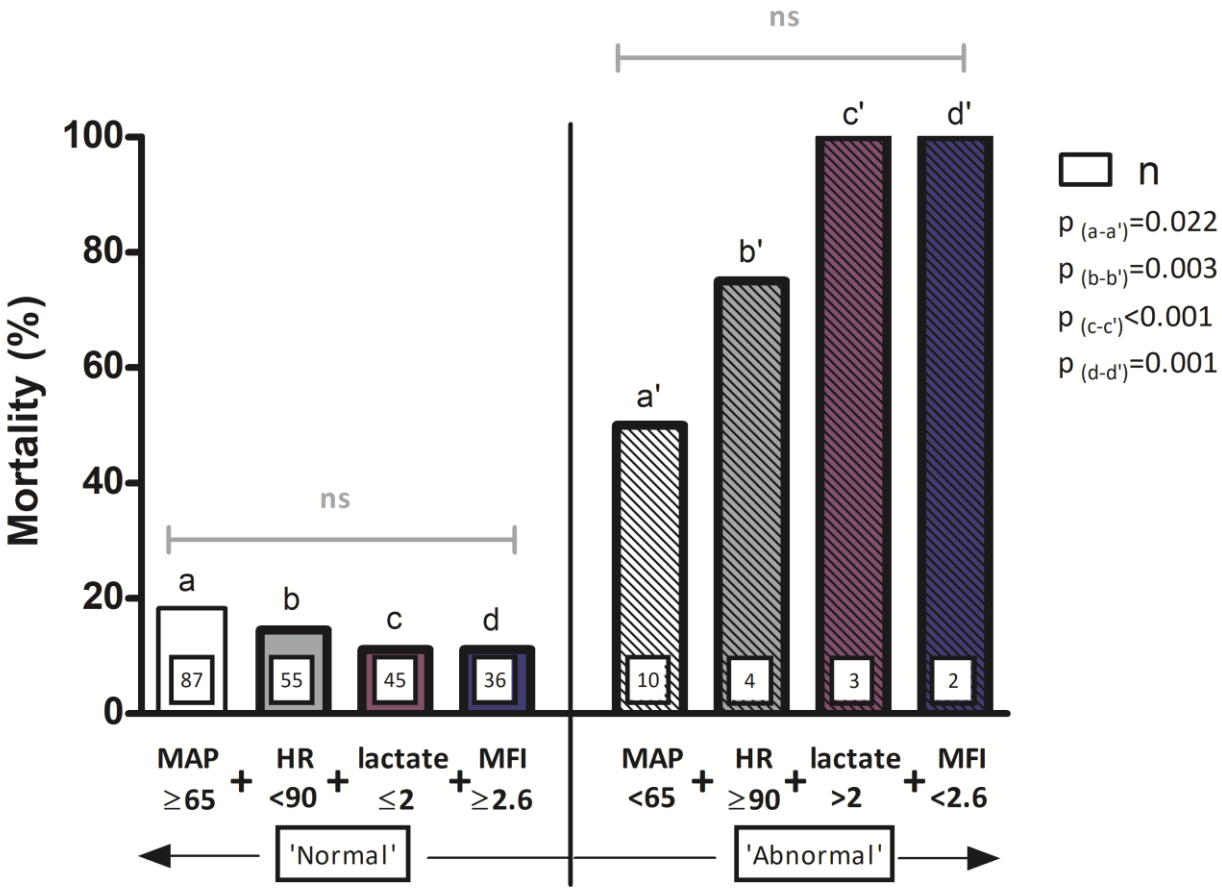
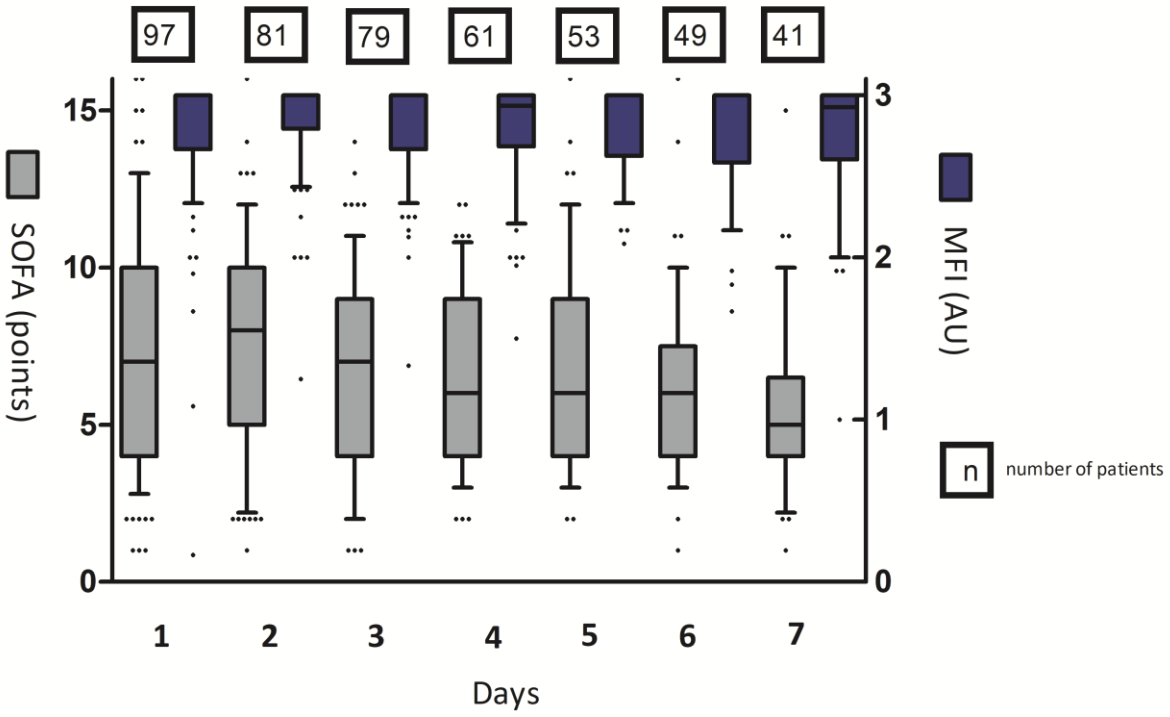
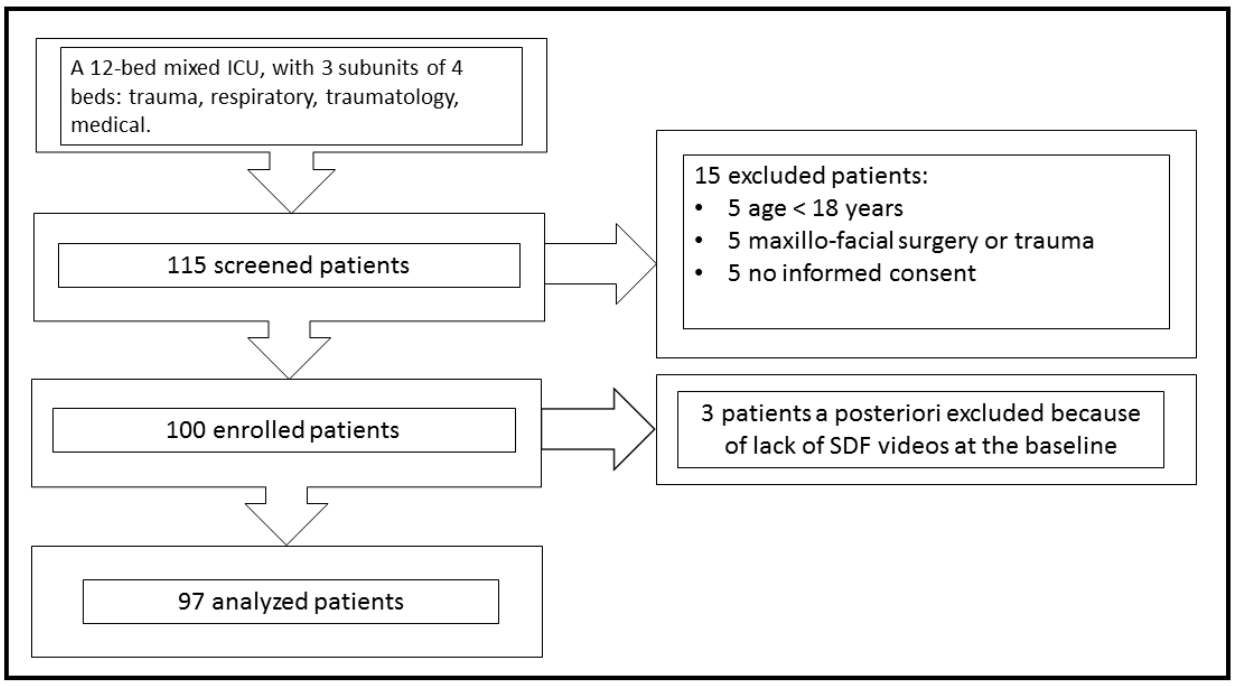


Figure 3. Evolvement over time of sequential organ failure assessment (SOFA) score and microvascular flow index (MFI) in the first 7 days of ICU admission. Box and 10-90th percentile whisker plots with individual outliers.



ADDITIONAL FILES

Additional file 1 - Flow chart for patients' recruitment. A schema to clarify the procedures for the patients' recruitment for the study.



Additional file 2 - Comparison between in-hospital survivors and non survivors. The table illustrates the results of the univariable analysis for baseline clinical and microcirculatory variables between in-hospital survivors and non survivors.

PATIENTS CHARACTERISTICS	*n*	*In-hospital survivors (64)*	*In-hospital non survivors (33)*	*p*
Male gender (n, %)	97	43 (67.2)	21 (63.6)	0.821
Age (years, n)	97	58[43-71]	73[63-82]	**<0.001**
APACHE II (pts)	97	14±7	21±6	**<0.001**
SOFA (pts)	97	5[3-9]	10[8-13]	**<0.001**
ICU admission diagnosis, n (%)	97			0.173
Trauma	37	29	8(21.6)	
Neurologic	21	14	7(33.3)	
Respiratory	11	7	4(36.4)	
Sepsis	9	5	4 (44.4)	
Other	19	9	10(52.6)	
Heart rate (bpm)	97	74[61-98]	88[65-107]	0.11
Mean arterial pressure (mmHg)	97	86±17	80±23	0.161
Vasoactive drugs (treated)	54	31(48.4)	23(69.7)	0.054
Cumulative Vasopressor Index	54	1[0-4]	4[0-4]	**0.044**
Glasgow Coma Scale (pts)	97	13[5-15]	6[3-11]	**0.007**
Mechanical ventilation (n, %)	97	71(93.7)	33(100)	0.163
Peep (cmH_2O)	91*	7[5-9]	8[6-10]	0.088
Haemoglobin (g/dL)	97	11.1±1.7	11±2	0.763
White Blood Cells ($nx10^3/mmc$)	97	12[9-15.7]	12.2[8.1-14.6]	0.899
Platelets ($nx10^3/mmc$)	97	169[111-201]	126[93-182]	**0.048**
Creatinine (mg/dL)	97	1[0.8-1.4]	1.2[0.9-1.5]	0.052
Bilirubine (mg/dL)	97	0.7[0.5-1]	1[0.5-1.8]	**0.037**
PaO_2 (mmHg)	97	149[114-178]	134[91-158]	0.103
Arterial lactates (mmol/L)	97	1.2[0.9-1.67]	1.6[1.4-3.5]	**<0.001**
$ScvO_2$ (%)	59**	78.8[71.7-83.8]	75.4[66.1-80.5]	0.164
Comorbidities, n(%) - **Obesity** - **Hypertension** - **Diabetes mellitus** - **Hypercholesterolemia** - **Malignancy** - **Vascular disease** - **Cardiomiopathy** - **COPD** - **Renal insufficiency**				ns
MICROCIRCULATORY VARIABLES:				
TVD (small) (mm/mm^2)	97	20.1[16.9-22.6]	20.9[17.4-22.8]	0.817
PVD (small) (mm/mm^2)	97	19.1±4	19.8±5	0.52
De Backer score (n/mm)	97	11.7±2	12.3±2	0.152
PPV (small) (%)	97	98[94.2-99.6]	98.6[97-100]	0.199
MFI (small) (AU)	97	3[2.8-3]	3[2.5-3]	0.624
HI (small)	97	0[0-0.2]	0[0-0.3]	0.982
Abnormal MFI (n,%)	97	9(16.4)	11(50)	**0.035**

Data are presented as mean ± SD or as median [IQR] unless stated otherwise. APACHE Acute Physiologic And Chronic Health Evaluation II, calculated over the first 24 hours from ICU admission;
SOFA Sequential Organ Failure Assessment, calculated over the first 24 hours from ICU admission. CVI Cumulative Vasopressor Index; ICU Intensive Care Unit; COPD, Chronic Obstructive Pulmonary Disease. TVD Total Vessel Density; PVD Perfused Vessel Density; PPV Proportion of Perfused Vessel; HI Heterogeneity Index; MFI Microvascular Flow Index. Abnormal MFI is defined as MFI < 2,6. Cut off value for small vessels diameter < 20 μm.

Additional file 3 - Comparison between 90-day survivors and non survivors. The table illustrates the results of the univariable analysis for baseline clinical and microcirculatory variables between 90-day survivors and non survivors.

PATIENTS CHARACTERISTICS	***n***	***90-day survivors (66)***	***90-day non survivors (31)***	***p***
Male gender (n, %)	97	45 (68.2)	19 (63.6)	0.502
Age (years, n)	97	58[43-71]	73[63-81]	**<0.001**
APACHE II (pts)	97	14±7	21±6	**<0.001**
SOFA (pts)	97	5[4-9]	10[8-13]	**<0.001**
ICU admission diagnosis, n (%)	97			0.112
Trauma	37	30	7(18.9)	
Neurologic	21	15	6(28.6)	
Respiratory	11	7	4(36.4)	
Sepsis	9	5	4 (44.4)	
Other	19	9	10(52.6)	
Heart rate (bpm)	97	74[61-95]	88[69-107]	0.086
Mean arterial pressure (mmHg)	97	86±17	81±23	0.264
Vasoactive drugs (treated)	54	31(46.9)	23(74.2)	**0.016**
Cumulative Vasopressor Index	54	0[0-4]	4[0-4]	**0.011**
Glasgow Coma Scale (pts)	97	13[5-15]	6[3-11]	**0.011**
Mechanical ventilation (n, %)	97	61(92.4)	31(100)	0.174
Peep (cmH_2O)	91*	7[6-8]	8[7-10]	**0.037**
Haemoglobin (g/dL)	97	11.1±1.7	10.9±2.1	0.724
White Blood Cells ($nx10^3$/mmc)	97	11.5[8.9-15.2]	12.5[8.4-14.6]	0.76
Platelets ($nx10^3$/mmc)	97	168[116-200]	122[92-187]	**0.049**
Creatinine (mg/dL)	97	1[0.8-1.4]	1.2[1-1.5]	**0.023**
Bilirubin (mg/dL)	97	0.7[0.5-1]	1[0.5-1.7]	**0.041**
PaO_2 (mmHg)	97	149[115-177]	122[90-158]	0.081
Arterial lactates (mmol/L)	97	1.2[0.9-1.73]	1.6[1.4-3.6]	**<0.001**
$ScvO_2$ (%)	59**	79[71.9-83.5]	75.2[65.2-80.8]	0.127
Comorbidities, n(%) - **Obesity** - **Hypertension** - **Diabetes mellitus** - **Hypercholesterolemia** - **Malignancy** - **Vascular disease** - **Cardiomiopathy** - **COPD** - **Renal insufficiency**				ns
MICROCIRCULATORY VARIABLES:				
TVD (small) (mm/mm^2)	97	20.1[17.1-22.6]	20.9[17.2-22.7]	0.817
PVD (small) (mm/mm^2)	97	19.1±4	19.7±5	0.595
De Backer score (n/mm)	97	11.7±2	12.3±2	0.184
PPV (small) (%)	97	98[94.5-99.7]	98.6[96.7-100]	0.28
MFI (small) (AU)	97	3[2.8-3]	3[2.4-3]	0.378
HI (small)	97	0[0-0.2]	0[0-0.3]	0.731
Abnormal MFI (n,%)	97	9(13.6)	11(35.5)	**0.017**

Chapter 6

Sublingual microcirculation and tissue perfusion as predictors of organ failure in major trauma: a subgroup analysis of a prospective observational study

Sublingual microcirculation and tissue perfusion as predictors of 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 relation 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 important predictors of 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 hemostatic, 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 edema, which may lead to microcirculatory alterations similar to those observed during sepsis [2, 7]. An impairment in microvascular perfusion and tissue oxygenation has been described after traumatic hemorrhagic 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].

MATHERIALS 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 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]. (Supplementary Figure 1)

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 (Supplementary Table 1). 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 hemodynamically 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 $\leq$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$\leq$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), hemoglobin and RBC transfusion requirements (Table 1).

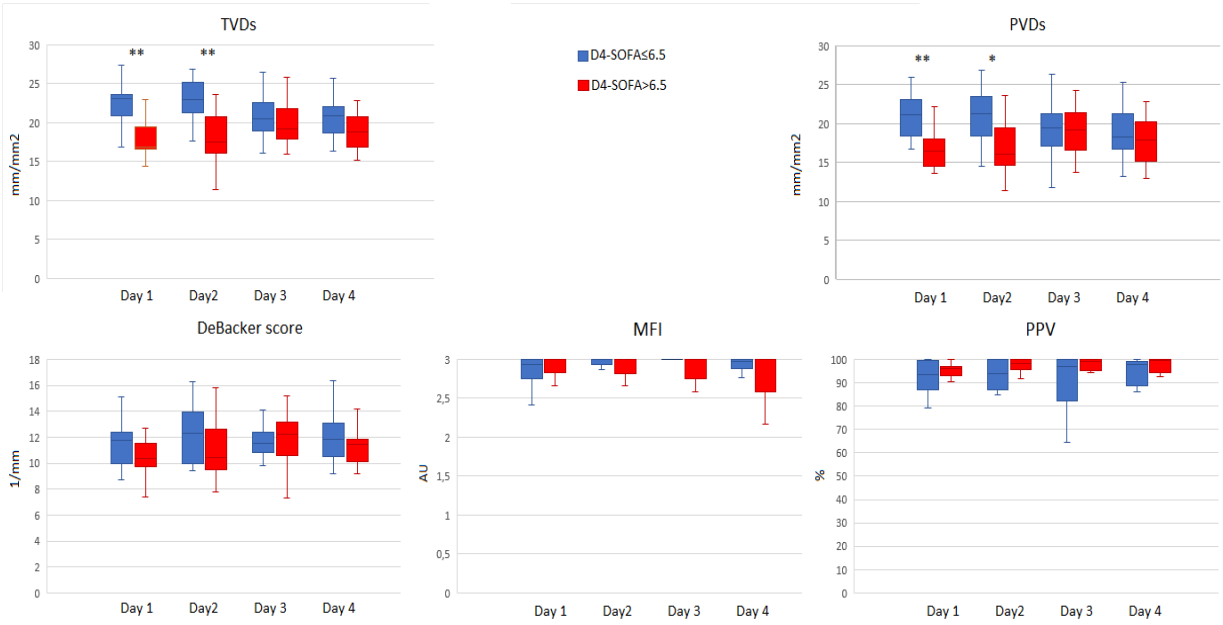
		SOFA≤6.5 at D4	***SOFA>6.5 at D4***	***p value***
		15 (53.6%)	13 (46.4%)	
MAP, mmHg				
	D1	86 [75-99]	85 [77-95]	0.964
	D2	82 [71-99]	89 [71-95]	0.648
	D3	89 [84-102]	88 [79-97]	0.413
	D4	91 [74-106]	88 [84-89]	0.932
HR, bpm				
	D1	63 [57-83]	83 [74-102]	0.142
	D2	77 [64-100]	84 [60-101]	0.717
	D3	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, 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, %				
	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, 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, %				
	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

Table 1: *Hemodynamic variables in the first four days of ICU admission for the two groups of patients (SOFA score at D4≤6.5 and SOFA score at D4>6.5). Hemodynamic data were collected daily, at the same time of microcirculatory assessment. SOFA = Sequential Organ Failure Assessment; MAP= Mean Arterial Pressure; HR= Heart Rate; Hb= Haemoglobin; Hct= Haematocrit, BE= Base Excess. Data presented as median [IQR] or number [%].*

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).

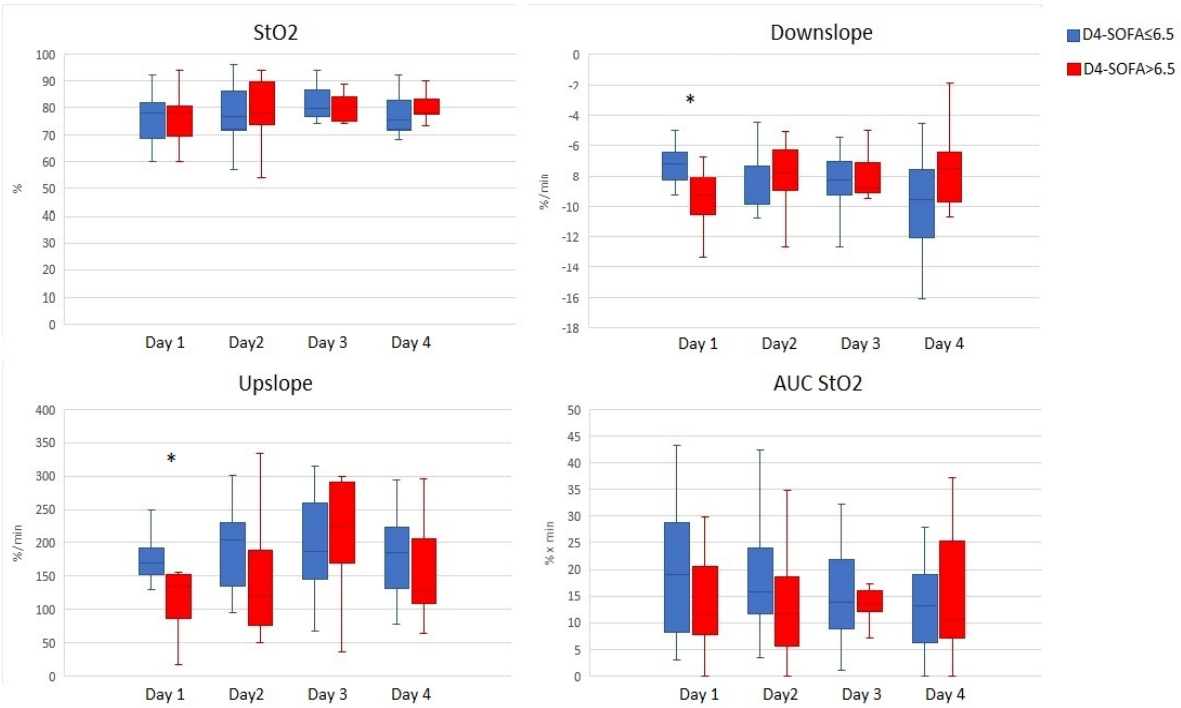
Fig 1: Changes in the sublingual microvascular parameters in the first 4 days of admission in ICU for the two groups of patients (SOFA score at D4≤6.5 and SOFA score at D4>6.5). * p<0.05; ** p<0.01. *TVDs: total small vessel density; PVDs: perfused small vessel density; MFI: microvascular flow index;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



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.

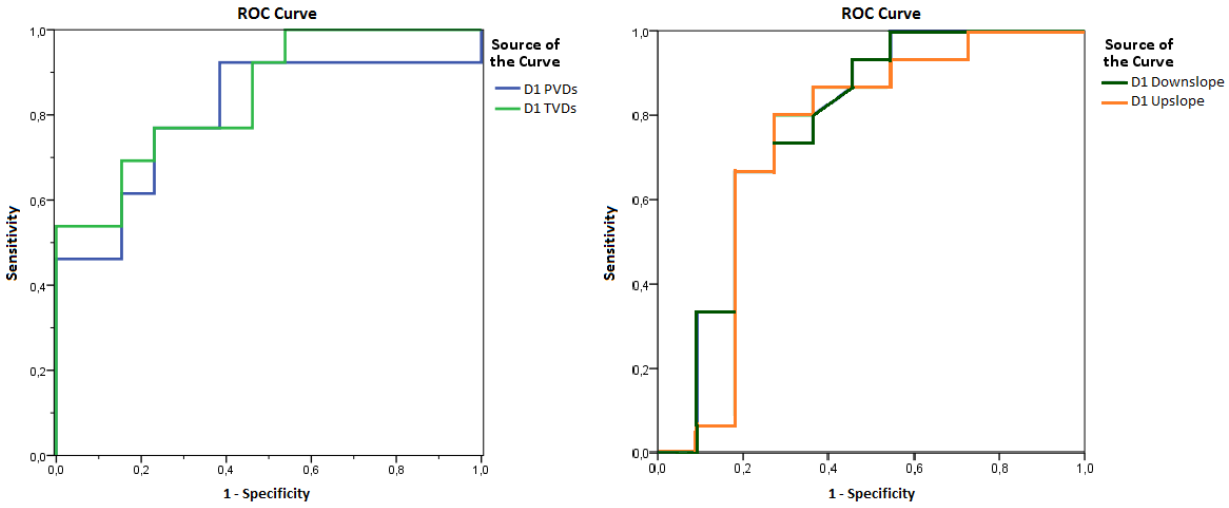
Fig 2: Changes in NIRS-derived parameters in the first 4 days of admission in ICU for the two groups of patients (SOFA score at D4≤6.5 and SOFA score at D4>6.5). * p<0.05. *StO2: tissue oxygen saturation; AUC StO2: area under the curve of tissue oxygen saturation.*



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).

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.*



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 hemodynamic 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 hemorrhagic 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 ischemic 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 hypovolemia: microvascular disturbances have been previously showed in hypovolemic 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 hypovolemic and hemorrhagic 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 edema. 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. [28-30] 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, it suggests that early identification of microvascular and tissue hypoperfusion in trauma patients may be relevant to detect patients at higher risk of multiple organ failure and to prevent it with tailored intensive care treatment and acute resuscitation.

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 hemodynamic 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 developed more severe organ dysfunction. Our data suggest that early impairment in microvascular perfusion after severe trauma may be an important predictor of organ failure. Our study would support the hypothesis that restoration of macrocirculation can be dissociated from restoration of peripheral and tissue perfusion. Evaluating the microcirculation 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.

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Chapter 7

Effects of ketanserin on microcirculatory alterations in septic shock: an open-label pilot study

Effects of ketanserin on microcirculatory alterations in septic shock: an open-label pilot study

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ABSTRACT

Introduction

Microcirculatory alterations in sepsis are associated with increased morbidity and mortality. These alterations occur despite macrohemodynamic resuscitation. Alternative pro-microcirculatory strategies, including vasodilatory drugs, have been suggested to improve capillary blood flow. Ketanserin, a serotonin receptor antagonist, is an attractive candidate, because of its vasodilatory, antithrombotic and anti-inflammatory effects.

Methods

This is an open-label pilot study on the effect of ketanserin administration on microcirculatory alterations in septic shock, defined as microvascular flow index (MFI) ≤ 2.5 after a strict macrohemodynamic resuscitation protocol. Sidestream dark field imaging (SDF) was applied to assess the microcirculation. A stepwise incremental dose regimen was applied until an MFI>2.9, the primary endpoint, was reached.

Results

Ten patients (Acute Physiology and Chronic Health Evaluation IV scores of 115 [100-136]) were included. Baseline MFI was 1.71 [1.31-2.32] and was significantly increasing to 2.96 ([2.54-3.00]; P 0.021) during the ketanserin infusion. The total ketanserin dose was 0.09 [0.08-0.13] mg/kg per patient in 60 [30-60] minutes. In 3 patients (30%) the ketanserin infusion was discontinued due to refractory hypotension.

Conclusion

An improvement in microcirculatory perfusion was observed during ketanserin administration in patients with septic shock after macrohemodynamic resuscitation. This finding needs further exploration in a placebo-controlled setting.

INTRODUCTION

The microcirculation in sepsis is characterized by heterogeneous alterations in both flow and vessel density [1]. A consistent observation is the absence of a clear correlation between microcirculatory alterations and systemic hemodynamic parameters despite macrohemodynamic resuscitation [2]. Because these microcirculatory alterations are associated with increased morbidity and mortality, the microcirculation appears to be an appealing target in sepsis resuscitation [3,4]. Main aim of microcirculatory resuscitation is capillary recruitment in order to improve oxygen transport to the cells [1]. The classical paradigm is to increase perfusion pressure in order to enhance organ perfusion. However, since the introduction of in vivo microscopy at the bedside, it has become clear that augmenting blood pressure will not have beneficial effects on microcirculatory perfusion per se [5-9]. Alternatively, treatment could be directed at opening up the microcirculation by recruitment of weak microcirculatory units [10,11]. Nevertheless, studies with different types of vasodilators have not yielded unequivocal results either [12-18]. Therefore, the search for a pro-microcirculatory resuscitation strategy continues. Ketanserin could be an attractive candidate for recruitment of the microcirculation: in addition to vasodilatory properties via serotonin (5HT) receptor $5HT_{2a}$ and the adrenergic α_1-receptor, ketanserin also attenuates thrombocyte aggregation and could therefore improve the microcirculation by preventing formation of occlusive microthrombi [19-22]. Furthermore, serotonin receptor antagonists are reported to have beneficial effects on cytokine profiles and leukocyte-endothelium interactions in animal sepsis models [23-25]. In cardiac surgery, ketanserin treatment lowered the incidence of endotoxemia [26]. Data on the direct effect of ketanserin on the human microcirculation are limited: in post cardiac surgery hypertension ketanserin lowered blood pressure, without impairment of sublingual microcirculatory flow or vessel density. An improvement of digital capillary blood flow was observed in patients with Raynaud's phenomenon after ketanserin treatment [27-29]. The aim of the present pilot study is to investigate the effect of a stepwise dose finding schedule of intravenous ketanserin on compromised sublingual microcirculation in patients with severe sepsis and septic shock after initial macrohemodynamic resuscitation.

METHODS

Study design

This study is an open label dose finding pilot study in 10 patients on the effect of ketanserin on sublingual microcirculatory alterations in patients 18 years or older with severe sepsis and septic shock (NCT01329887, clinicaltrials.gov). Inclusion was characterized by 2 main criteria: 1. Fulfillment of international criteria for severe sepsis or septic shock [30]; 2. Persistence of microcirculatory alterations after standardized resuscitation (see below), defined as a microvascular flow index (MFI) ≤ 2.5 in small vessels (<20 □m). Exclusion criteria were age less than 18 years, prolonged corrected QT-interval or recent oropharyngeal surgery interfering with Sidestream Dark Field (SDF) measurements. The study was approved by the local ethics committee (Medical Research Ethics Committee, Medical Center Leeuwarden, the Netherlands). Written informed consent was obtained from all included patients or their legal representative in accordance with local legislation.

Sidestream Dark Field (SDF) imaging and analysis

Sidestream dark field imaging and subsequent image analysis were performed in line with international consensus [31,32]. An SDF camera (MicroScan, MicroVision Medical, Amsterdam, the Netherlands) is a form of handheld intravital microscopy enabling direct visualization of the microcirculation [33]. In short, the SDF camera emits stroboscopic green light with a wavelength (530 nm) within the absorption spectrum of hemoglobin, thereby depicting erythrocytes as black cells on the screen. The area of visualization is 1 mm^2.

Offline software-assisted analysis (AVA 3.0, MicroVision Medical, Amsterdam, the Netherlands) yields the semi-quantitative MFI, ranging from 0 (no flow) to 3 (continuous flow), and percentage of perfused vessels (PPV), providing information on convection, whereas total vessel density (TVD) and perfused vessel density (PVD) provide information on diffusion [34]. Heterogeneity index was calculated as the difference between the highest and the lowest quadrant MFI divided by the average MFI of all sublingual sites [35]. Vessels were separated into large (mostly venules) and small (mostly capillaries) using a diameter cutoff value of 20 μm. Initial analysis of images during the screening process of patients was performed at the bedside by eyeballing MFI. Measurements were performed by a small group of dedicated researchers. The MFI was calculated as the mean over 3 different sublingual regions. Bedside assessment of MFI has good agreement with offline analysis [36]. Subsequent detailed offline analysis was performed blinded and in a randomized order to prevent coupling between images.

Resuscitation

Resuscitation was performed in line with a strict protocol aiming for the following resuscitation goals: a mean arterial pressure (MAP) of 60 mmHg or greater, a mixed or central venous oxygen saturation ($S(c)vO_2$) at least 70% and a cardiac index (CI) at least 2.5 L/min/m^2. This protocol reflects standard practice in our ICU [13,37]. Systemic hemodynamic monitoring consisted of continuous invasive monitoring of arterial blood pressure and continuous cardiac output and SvO_2 measurements using a pulmonary artery catheter (Vigilance, Edwards Lifesciences, Saint-Prex, Switzerland) or a pulse contour analysis system (PiCCO, Pulsion Medical Systems AG, Munich, Germany) in combination with repeated $S(c)vO_2$ measurements via a central venous line in the jugular or subclavian vein. Stepwise goal-directed protocolized resuscitation consisted of 1) repeated infusions of at least 250 mL of crystalloids, colloids, or blood products (no albumin because of high protein binding of ketanserin), until the increase in stroke volume was < 10%. Crystalloids were the resuscitation fluids of choice; colloids and blood products were administered at the discretion of the attending physician, with the threshold for red blood cell transfusion being a hematocrit <25%; 2) treatment of inadequate systemic oxygen supply (defined as CI < 2.5 L/min/m^2 or $S(c)vO_2$ < 70%) with dopamine or dobutamine administered at up to 10 □g/kg per minute and additional enoximone in case of an inadequate response to dopamine or dobutamine; and 3) treatment with norepinephrine in case of MAP < 60 mmHg despite the aforementioned steps. In case of hypotension despite vasopressor support, hydrocortisone at a maximum dose of 300 mg per day was administered. During the study period, therapeutic goals and resuscitation protocol remained unchanged.

Screening of eligible patients

After fulfillment of the resuscitation end points, eligible patients were screened for inclusion. In case bedside SDF-assessment revealed a small vessel MFI ≤ 2.5, patients were included for this study after obtaining informed consent of the patient or legal representative.

Ketanserin administration and data collection

In case of a small vessel MFI ≤ 2.5 after fulfillment of the above-mentioned resuscitation goals, baseline measurements (SDF, hemodynamics, blood samples) were made. After the baseline measurements, intravenous ketanserin infusion was started following the scheme in figure 1. Each step consisted of a ketanserin bolus, followed by a 30-minute continuous infusion, the first step being a bolus of 0.015 mg/kg and a 30 minute continuous infusion of 0.03 mg/kg/hour. In case of hypotension (MAP < 60 mmHg for > 1 minute) during ketanserin administration, patients were resuscitated following the standard resuscitation protocol. In case of MAP < 60 mmHg for more

than 10 minutes or < 50 mmHg for more than 5 minutes despite resuscitation, the ketanserin infusion was discontinued (refractory hypotension). After each step, data on hemodynamics, applied therapy and arterial blood gas analysis were collected. Furthermore, SDF measurements of the sublingual microcirculation were acquired. In case of a capillary MFI <2.9, the ketanserin dose was increased following the scheme in figure 1, with a maximum study period of 2 hours (4 steps). The ketanserin infusion was stopped in case of a MFI≥2.9, refractory hypotension (see above) or after completion of all 4 steps.

Statistical analysis

Since this is a pilot study, a true sample size calculation was not applicable. The sample size was in accordance with comparable previous studies [12, 18]. Primary aim was to describe the changes in microcirculation observed during ketanserin administration. Secondary end points were the administered ketanserin dose and incidence of (refractory) hypotension. Whenever appropriate, a non-parametric test (Wilcoxon signed-rank test) was used to test for differences between time points. Data are presented as median [interquartile range].

RESULTS

Patients

Out of 60 screened patients 15 patients fulfilled the entry criteria of an MFI ≤ 2.5. Five denied informed consent. Ten patients with APACHE IV scores of 115 [100-136], SOFA scores of 11 [8-14] and highest lactate levels before inclusion of 5.5 [4.3-7.9] mmol/L, were included after bedside confirmation of a small vessel MFI ≤ 2.5 (table 1). The majority of patients (70%) were admitted because of abdominal sepsis. All patients but one fulfilled the criteria of septic shock and in 80% of patients positive cultures confirmed the presence of an infection. The ICU mortality was 50% for an APACHE IV predicted mortality of 65 [40-85] %. The average total ketanserin dose during the study period was 7.5 [6.2-9.8] mg per patient (0.09 [0.08-0.13] mg/kg) in 60 [30-60] minutes. Five patients fulfilled stop criteria within two doses and two patients within four doses of ketanserin.

Microcirculation

Before start of the ketanserin infusion, small vessel MFI was 1.71 [1.13-2.32]. During the ketanserin infusion, MFI increased to 2.96 [2.54-3.00] (P = 0.021). In 6 out of 7 patients without refractory hypotension, MFI improved to MFI ≥2.9 between T0 and the end of the ketanserin infusion. MFI also increased in 2 out of 3 patients with refractory hypotension (figure 2). Heterogeneity index (HI) over the course of the study showed a significant decrease compared to baseline measurements (1.34 [0.57-2.35) vs. 0.17 [0.00-0.49], P=0.021). Small vessel PPV increased (0.78 [0.55-0.90] vs. 0.92 [0.78-0.93], P=0.037). Vessel densities for both small and large vessels and large vessel MFI did not show any significant changes (table 2, figure 3 and 4).

Macrohemodynamics

Over the course of the ketanserin infusion, MAP was lowered by 6 [1-11] mmHg leading to increases in vasopressor use in 6 patients. Average norepinephrine dosage increased from 0.19 [0.11-0.34] to 0.21 [0.19-0.36] (P=0.026). In 3 patients (30%), the study was discontinued due to refractory hypotension. One of these patients had a MAP of 44 mmHg at baseline, but remained unresponsive to further therapy. Inotropic therapy was restricted to dobutamine in one patient and enoximone in two patients. Average fluid administration during the study period was 550 [190-1275] mL. No significant changes in heart rate, CI, lactate levels and CVP were observed, whereas $S(c)vO_2$ significantly increased from 71 [60-78] to 75 [69-81] % (P=0.024). Central-to-toe temperature gradient decreased from 8.6 [5.1-10.2] to 7.4 [4.6-9.0] ºC (P=0.012) (see table 3 and figure 5 for an overview of macrohemodynamic data).

DISCUSSION

Main finding of the present study is that microcirculatory blood flow but not vessel density increases during ketanserin administration in septic patients with a small vessel MFI ≤2.5 at baseline. The presence of profound microvascular alterations despite macrohemodynamic resuscitation is in line with previous reports [3,13,15]. Albeit slightly lower as predicted based on APACHE IV scores, the considerable mortality underlines the severity of disease.

At the level of the macrocirculation, ketanserin administration was accompanied by a decrease in blood pressure necessitating increases in vasopressor dose in 60% and dictating discontinuation of the study drug in 3 (30%) patients. It is difficult to determine whether this could be due to a direct (vasodilatory) effect of ketanserin or that other factors might have played a role. Ketanserin might have unmasked hypovolemia despite fulfillment of resuscitation endpoints in 2 out of 3 patients, as illustrated by the considerable amount of fluids that was administered during the study

period in most patients. Interestingly, the one patient in whom MAP was < 60 mmHg before the start of the ketanserin infusion, managed to increase MFI to 3 despite persistence of refractory hypotension. Taken together with the significant decreases in blood pressure during the study period, this demonstrates the absence of a linear association between hypotension and microcirculatory alterations.

Ketanserin may influence microcirculatory perfusion in several ways. Ketanserin is a selective $5HT_{2a}$-receptor antagonist as well as an α_1-receptor antagonist. Besides direct vasodilation by antagonism of α_1-receptors, ketanserin can also induce effects on the microcirculation via the $5HT_{2a}$-receptor, one of fourteen serotonin receptor subtypes [19,38]. Serotonin in the peripheral circulation is released by activated platelets [39]. Platelet activation appears to play a pivotal role in impairment of microcirculatory perfusion in sepsis [40,41]. Inflammatory stimuli lead to secretion of P-selectin, Von Willebrand factor and serotonin, causing the platelets to adhere to the endothelium [42,43]. Ketanserin is known for attenuating $5HT_{2a}$-mediated platelet aggregation and might therefore prevent the formation of occlusive microthrombi [44,45].

Serotonin has a complex variety of cardiovascular effects, mediated by several subtypes of the 5HT-receptor. It is a potent vasoconstrictor by stimulating $5HT_{2a}$-mediated vasoconstriction of vascular smooth muscle cells, whereas stimulation of the $5HT_{2b}$-receptor results in endothelium dependent vasodilation by increased nitric oxide (NO) release [46]. Therefore, ketanserin can induce vasodilation by blockage of $5HT_{2a}$-receptors, whilst $5HT_{2b}$-mediated vasodilation due to endothelial NO release is preserved. The resulting action of serotonin depends on the receptor subtype involved as well as the local conditions such as endothelial damage or hypoxia [46]. Under conditions of hypoxia, NO scavenging by erythrocytes can lead to profound serotonin induced vasoconstriction [47].

Data on the effects of ketanserin in human sepsis are limited. In cardiac surgery, treatment with ketanserin attenuated endotoxemia [26]. The only study applying direct in vivo microscopy of the microcirculation during ketanserin administration was performed in post-cardiac surgery hypertension. Ketanserin lowered blood pressure with a concomitant increase in large vessel PVD, whereas small PVD and small vessel MFI remained unchanged. This was interpreted as shunting as a result of ketanserin administration [27]. Although we did not observe signs of shunting at the level of the microcirculation in our patients, the significant increase in $S(c)vO_2$ together with stabile CI and lactate levels and a lower central-to-toe temperature gradient could also indicate a shunting effect without an increase in oxygen consumption at the level of the microcirculation.

Being the first study on the effect of ketanserin on the sublingual microcirculation in septic patients, no data on potentially effective doses are available. Therefore, we decided to titrate the

ketanserin dose based on microcirculatory response. Inclusion of patients with a compromised microcirculatory flow allows for a better evaluation of the effect of ketanserin: a MFI of 3 cannot be improved further. Although MFI has been validated for bedside assessment, one of the patients had a MFI of 3 at inclusion [36].

Moreover, a stepwise increase in the dose allowed for more safety with respect to inducing hypotension. Although hypotension can result from numerous causes during septic shock, administration of a vasodilator should of course be done with caution. Indeed, in 3 out of 10 patients refractory hypotension occurred. In these cases, ketanserin might have unmasked hypovolemia despite resuscitation. However, in 2 out of 3 patients, hypotension persisted despite discontinuation of the ketanserin infusion.

The major limitation of this pilot study is its open label design without a placebo group and a limited number of patients. It is difficult to determine whether the observed increase in MFI is due to the ketanserin infusion or other factors, such as timing. Our findings of improvement of microcirculatory perfusion fit in with other open label studies on the effects of nitroglycerin and dobutamine [12,18]. However, no significant effects could be observed when these drugs were tested in a randomized, placebo-controlled setting [13,16]. As the endothelium in sepsis remains responsive to vasodilating stimuli, maximal endogenous precapillary smooth muscle relaxation could be a factor to take into account when applying vasodilators with both non-endothelium as well as endothelium mediated properties [15,18]. This is illustrated by the blunting of any microcirculatory effects of vasodilation in the context of thoracic epidural analgesia after hypervolemic hemodilution [48]. Further experimental research is needed to elucidate the position of vasodilators in microcirculatory recruitment.

Being a pilot study, we decided to include patients with impaired microvascular flow after fulfillment of macrohemodynamic endpoints. Therefore, it is difficult to extrapolate our findings to patients with less severe disease. On top of that, little is known about pharmacokinetics and pharmacodynamics of ketanserin in sepsis. In non-septic patients, ketanserin displays a non-linear elimination with sequential half-lives of 8 minutes, 2 hours and 14 hours, because of the partly reoxidation of one of the non-pharmacologically active metabolites to ketanserin. Reduced hepatic clearance, but not renal impairment, can influence ketanserin bioavailability, which can be of importance in our study group. Doses in our study were below the doses reported to be safe in patients with non-septic organ impairment [19]. Spronk et al. reported the use of 2 mg/hour of ketanserin in sepsis before administering nitroglycerin, but no information on the exact indications for starting the drug was provided [12].

The considerable incidence of hypotension raises questions about the safety of using this vasodilator in severely ill patients. By using a stepwise dosing protocol, we aimed to find the lowest possible dose for inducing capillary recruitment in order to minimize chances of refractory hypotension. Of course, optimal conventional macrohemodynamic resuscitation remains the cornerstone of patient care in sepsis and therefore, every effort should be made to avoid suboptimal macrohemodynamic resuscitation during administration of a vasodilator.

In conclusion, the observed improvement in microcirculatory perfusion during short-term ketanserin administration could fit in with the observed effects in (animal) experiments on platelet aggregation, vasodilation, inducible NO synthase, baroreceptor reflex and cytokine profiles. Furthermore, this study provides a framework for the ketanserin dose that might lead to capillary recruitment. Although the open label design, the small number of patients as well as the considerable incidence of refractory hypotension do not allow strong conclusions, we believe that further elaboration of ketanserin -induced promotion of microcirculatory blood flow deserves exploration in a randomized placebo-controlled setting.

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TABLES

Age (years)	63 [54-72]
Male - n (%)	5 (50)
APACHE II	30 [25-34]
Predicted mortality (APACHE II)(%)	75 [65-87]
APACHE IV	115 [100-136]
Predicted mortality (APACHE IV) (%)	65 [40-85]
SOFA	11 [8-14]
Sepsis type- n (%)	
Abdominal sepsis	7 (70)
Pneumosepsis	1 (10)
Necrotizing fasciitis	1 (10)
Meningitis	1 (10)
Confirmed infection- n (%)	8 (80)
LOS ICU (days)	8 [3-26]
LOS hospital (days)	24 [23-50]
ICU mortality- n (%)	5 (50)
Hospital mortality- n (%)	5 (50)
Highest arterial lactate before inclusion (mmol/L)	5.5 [4.3-7.6]
Fluid balance (0-24 hours after ICU admission) (mL)	5450 [3658-10450]
Steroids- n (%)	7 (70)
Mechanical ventilation - n, %	10 (100)
PEEP (cm H2O)	12 [10-14]
FiO_2 (%)	50 [40-75]
Renal replacement therapy - n (%)	4 (40)

Table 1. Baseline patient characteristics. Values are median [interquartile range], range unless stated otherwise. APACHE indicates Acute Physiology and Chronic Health Evaluation score. SOFA, Sequential Organ Failure Assessment. LOS, length of stay. ICU, intensive care unit. PEEP, positive end expiratory pressure. FiO_2, fraction of inspired oxygen. N, number.

	Baseline	*After ketanserin*	*P*
MFI (small vessels) (AU)	1.71 [1.13-2.32]	2.96 [2.54-3.00]	0.021
MFI (large vessels) (AU)	2.66 [2.23-2.87]	3.00 [2.79-3.00]	0.080
TVD (small vessels) (mm/mm^2)	23.52 [19.78-25.70]	22.31 [19.76-25.42]	0.646
TVD (large vessels) (mm/mm^2)	3.57 [2.52-4.95]	4.76 [4.35-5.24]	0.139
PVD (small vessels) (mm/mm^2)	18.64 [11.14-21.62]	20.49 [17.06-23.44]	0.241
PVD (large vessels) (mm/mm^2)	3.49 [3.70-5.75]	4.76 [4.34-5.24]	0.445
PPV (small vessels)	0.78 [0.55-0.90]	0.92 [0.78-0.93]	0.037
Heterogeneity index (small vessels)	1.34 [0.57-2.35]	0.17 [0.00-0.49]	0.021

Table 2. Microcirculatory parameters before and after ketanserin administration. Small vessels; vessels < 20 μm. MFI, microvascular flow index. AU, arbitrary units. TVD, total vessel density. PVD, perfused vessel density. PPV, proportion of perfused vessels. Data are expressed as median [interquartile range] unless stated otherwise.

	Baseline	*After ketanserin*	*P*
Heart rate (beats per minute)	110 [99-130]	109 [103-131]	0.683
Mean arterial pressure (mmHg)	66 [60-77]	60 [53-64]	0.028
Central venous pressure (mmHg)	13 [8-15]	10 [5-16]	0.160
S(c)vO_2 (%)	71 [60-78]	75 [69-81]	0.024
Cardiac index (L/min/m^2)	3.0 [2.3-3.9]	2.9 [2.3-3.6]	0.474
Central-to-toe temperature gradient (^oC)	8.6 [5.1-10.2]	7.4 [4.6-9.0]	0.012
Arterial lactate (mmol/L)	4.7 [2.7-6.5]	4.1 [1.8-6.5]	0.609
Dopamine dose (n, μg/kg/min)	N=4	N=5	
	5.7 [2.0-9.1]	3.3 [2.1-8.8]	0.317
Norepinephrine dose (n, μg/kg/min)	N=9	N =9	
	0.19 [0.11-0.34]	0.21 [0.19-0.36]	0.027

Table 3. Macrohemodynamic parameters, dopamine and norepinephrine dose and lactate levels before and after ketanserin administration. S(c)vO_2, central/mixed venous oxygen saturation. N, number of patients. Data are expressed as median [interquartile range] unless stated otherwise.

FIGURES

Figure 1. Ketanserin trial scheme. Each step consists of an intravenous bolus followed by a continuous intravenous infusion during 30 minutes. T indicates time, T = 0 minutes denotes the start of the study. Before step 1 and after each step, SDF measurements are repeated. In case of a capillary MFI ≤2.5 after a step, the ketanserin dose is increased according to this scheme.

	Bolus	*Continuous infusion*
Step 1 (T=0 minutes)	0,015 mg/kg	0,03 mg/kg/hour
Step 2 (T=30 minutes)	0,03 mg/kg	0,06 mg/kg/hour
Step 3 (T=60 minutes)	0,03 mg/kg	0,09 mg/kg/hour
Step 4 (T=90 minutes)	0,03 mg/kg	0,12 mg/kg/hour
Maximum total dose	**0,24 mg/kg in 2 hours**	

Figure 2. Small vessel (<20 μm) microvascular flow index (MFI) at baseline and after ketanserin administration for all patients (boxplots) and for individual patients without refractory hypotension (n=7) and with refractory hypotension (n=3). AU, arbitrary units.

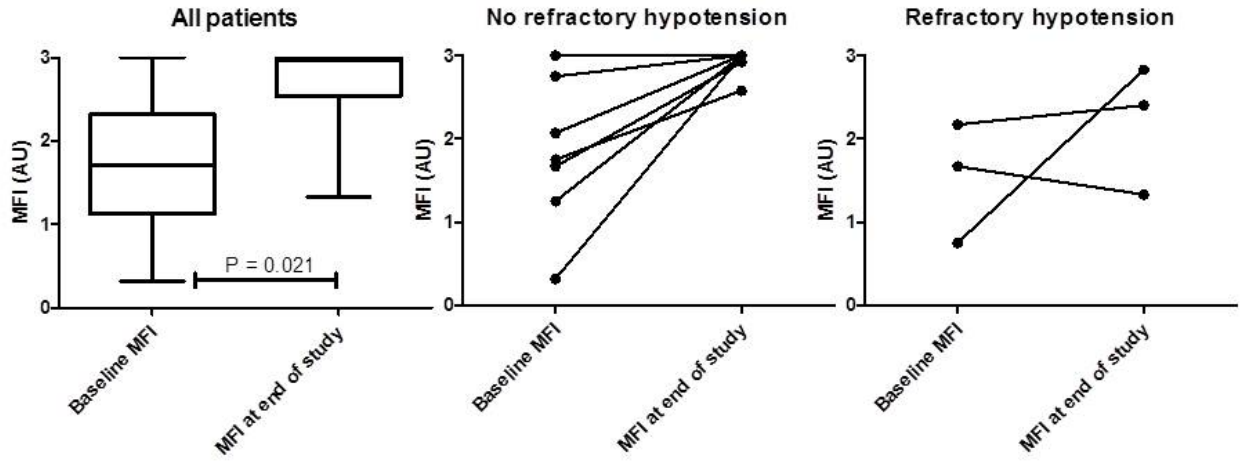


Figure 3. Heterogeneity index (HI), proportion of perfused vessel (PPV), total vessel density (TVD) and perfused vessel density (PVD) for small (s) vessels (<20 μm) before and after ketanserin administration.

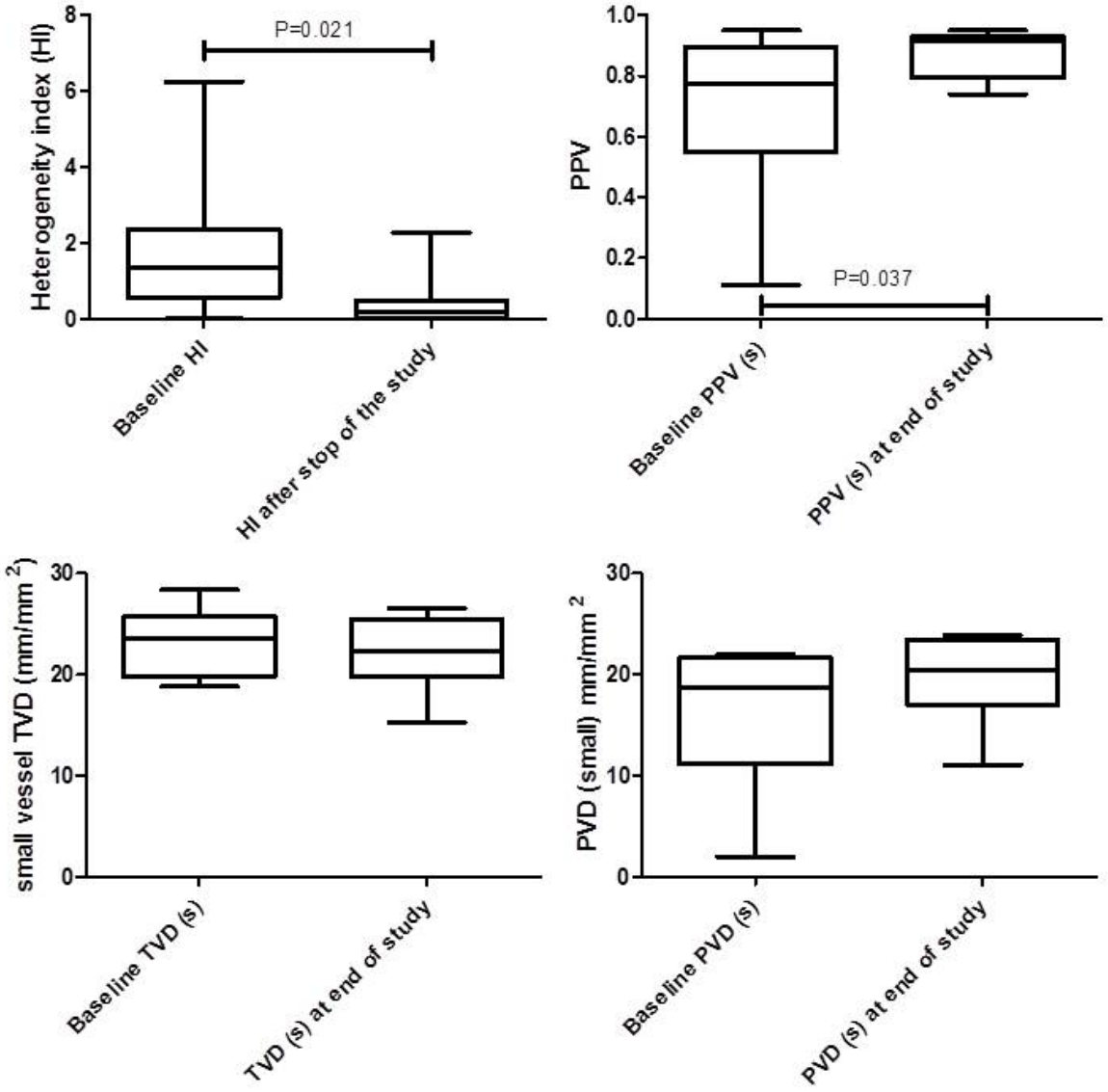


Figure 4. Microvascular flow index (MFI), total and perfused vessel density (TVD, PVD) for large (L) vessels (> 20 μm) before and after ketanserin administration.

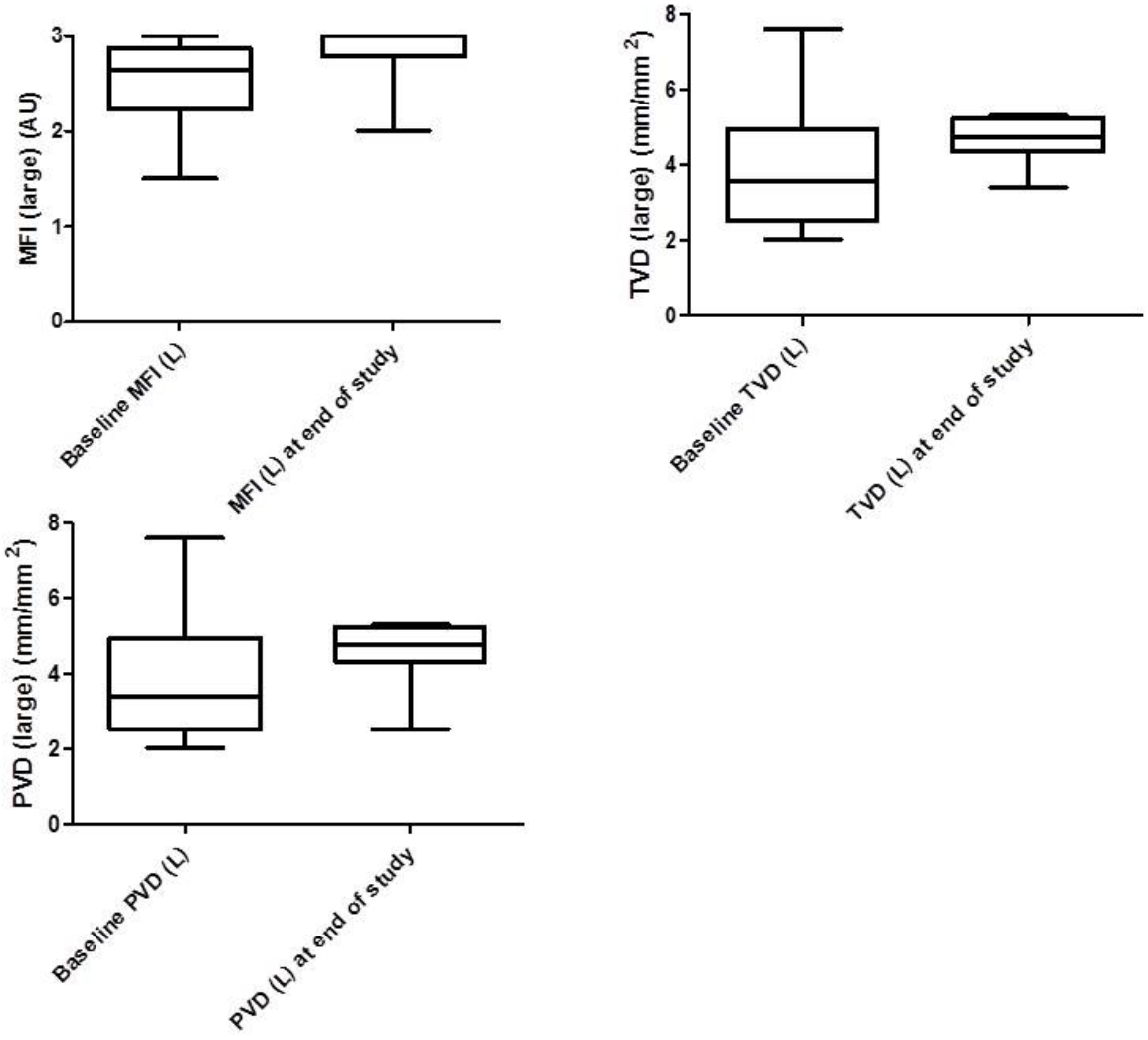
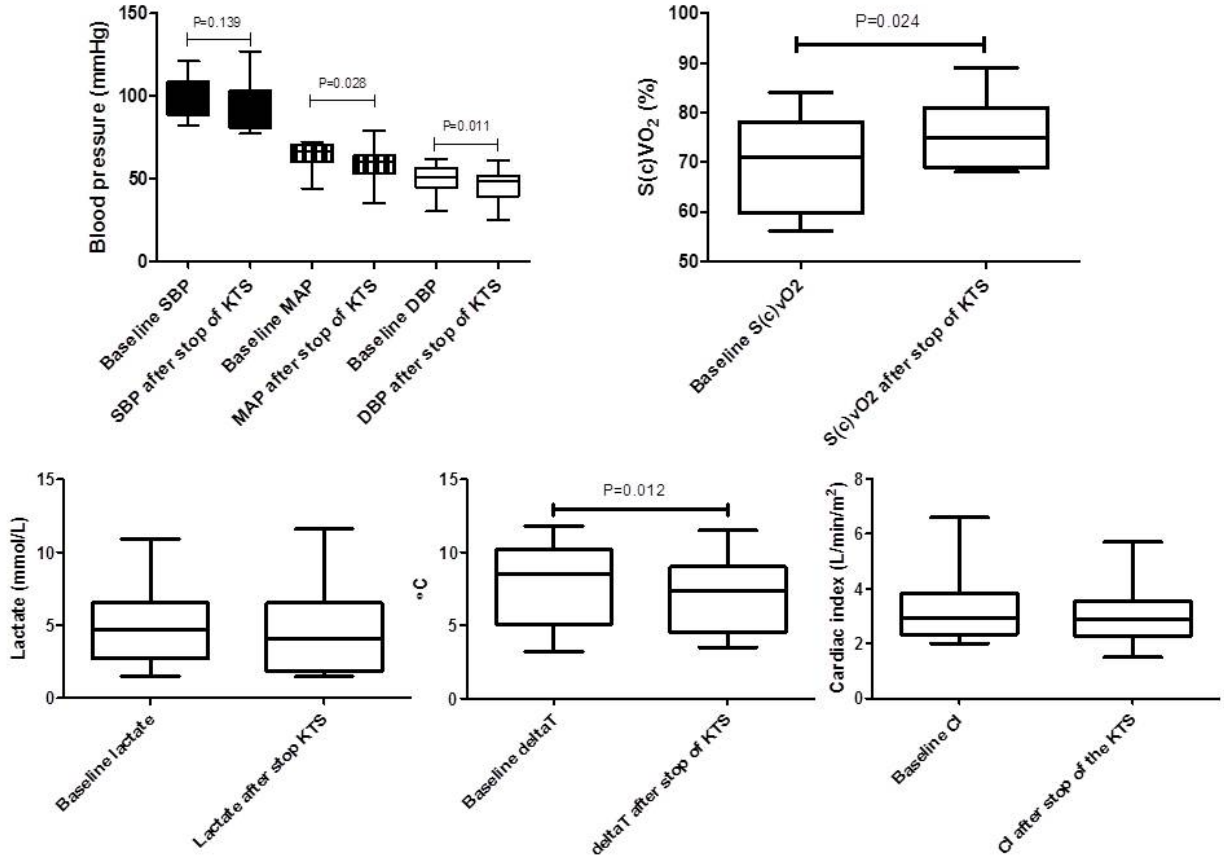


Figure 5. Systolic (SBP), mean arterial (MAP) and diastolic blood pressure (DBP), central/mixed venous oxygen saturation ($S(c)vO_2$), arterial lactate levels, central-to-toe temperature (deltaT) and cardiac index (CI) before and after ketanserin administration.



Chapter 8

Conclusion and future perspectives

Conclusion and future perspectives

Keeping an eye on microcirculation in critically ill patients, offers a more physiologic approach to their management: an impaired microcirculation, as the "motor" of tissues hypoperfusion and organ dysfunction, should theoretically represent the primary target of medical interventions, especially when the restoring of the standard macrohemodynamic variables appears to be insufficient to ameliorate the patient clinical status and outcome.

Despite this huge potential in clinical practice, microcirculatory monitoring still remains a fascinating and intriguing research tool.

The results presented in this thesis showed only a small part of the efforts of the past 4 years trying to cut the distances between pure pathophysiology research and clinical practice, bringing the microcirculatory monitoring to the bedside in critical care. During the development of the present research project, many other studies have been planned, designed and are still in the recruiting or data analysis phases (see PhD Portfolio).

Monitoring microcirculation as a routine clinical procedure: is the current technology sufficient to support the diffusion of this practice at the bedside?

In the past 20 years, great progresses are made in imaging human in vivo microcirculation. Starting with OPS and continuing with SDF, in vivo microscopy has become reality and widely applicated in many research settings.

The third-generation microscopes, based on the IDF, offers a great boost to the features of the available technology. The integration of the incident dark field illumination system and the optical system into a pen-like probe weighting only 120 grams, results in a highly handy hardware. The camera is fully digital, both in video acquisition system (highly resolution sensor offering sharper imaging with a 3,5 megapixel frame size) and focus depth/brightness adjustment, facilitating the acquisition process at the beside. Furthermore, the device is connected with a computer allowing the operator to directly register the videos without any supplemental hardware.

Chapter 2 has described the validation process of this technology on 25 healthy volunteers comparing it to the second generation SDF videomicroscope. IDF microscopes detected 30% more microvessels than SDF, as quantified by an off-line calculation of total vessel density, showing improved video quality in terms of both contrast and sharpness. Conversely, variables of blood

flow, such as MFI and PPV, did not differ between the two techniques. This should be accounted in comparing future studies performed with the new camera with the past studies, majorly regarding vessel density variables. Moreover, the quantitative focus mechanism, enabling the operator to obtain patient-specific focus depth [1] could results in a deeper analysis in mucosal interstitial fluid accumulation and tissue edema, possible signs of fluid overload.

Today, the only variable that could be potentially used at the bedside is qualitative flow evaluation with the MFI, which showed a good agreement with the gold standard off-line analysis. [2] In the MicroDAIMON study, MFI, especially if used as a dichotomous variable based on an a priori cut off of 2,6, is confirmed as the most sensitive variable to detect an association with the outcome in a such heterogeneous population which is expected to cause a "dilution effect" on the microcirculatory alterations. Post-hoc analysis of our data confirmed 2.6 as the optimal cut-off for the discrimination between survivors and non-survivors. Advances in the analysis software and, hopefully, the future validation of a system of fully automated analysis of microcirculatory videos could cut enormously the issue of time-consuming off-line analysis. In association with this new generation hardware, this could represent a further boost to bring a research tool to the bedside, giving the possibility to have a point-of-care tool to detect both flow and vessel density variables in real time.

Understanding physiology at the bedside: what is going wrong with my patient? Can improvement of the microcirculation be a hemodynamic endpoint?

In 2014, the MicroSOAP study provided the first and largest database on the prevalence and the significance of the microcirculatory alterations in a heterogeneous ICU population, with a time point-observation across 36 ICUs worldwide. [3] The authors reported a prevalence of MFI abnormalities of 17%, using the same predefined cut-off value [3-4]. In Chapter 5, the described MicroDAIMON study is currently the largest prospective longitudinal observational study to describe the incidence of microcirculatory derangements among a mixed group of critically ill patients, offering a day-by-day follow-up during the ICU admission. The incidence of baseline microcirculatory flow abnormalities was 20.6% and more than half (55.7%) of the patients displayed an abnormal MFI in at least one observation during ICU stay, showing an unexpected widespread phenomenon. This difference in reported percentage of MFI abnormalities can be explained by the difference in study design (longitudinal vs point-prevalence).

In this mixed ICU population, an abnormal baseline MFI is independently associated with unfavorable outcome in terms of ICU, in-hospital and 90-day mortality. In addition, the

contemporary presence of tachycardia showed an additive predictive power towards mortality in the survival analysis (reinforcing the findings of the microSOAP study results in high risk population). However, the change of MFI over time was not associated with outcome, both in terms of organ failure (SOFA) and mortality. In contrast to an abnormal MFI on day 1, we could not associate the development of an abnormal MFI after day 1 with unfavorable outcome.

Our data confirm previous observations, showing an important prognostic role of the microcirculation in various subsets of critically ill patients. [5-11] Moreover, these findings extend the predictive value of early microcirculatory alterations towards 90-day mortality. Patients with an abnormal MFI at baseline showed an absolute risk of non-survival almost three times higher in comparison to patients with a normal MFI.

However, routine day-by-day microcirculatory monitoring does not confirm previous observations. [6, 12-13]. A possible explanation for this discrepancy may lie in the heterogeneous composition of our study population and in considerable differences in microcirculatory baseline abnormalities. Alternatively, microvascular alterations represent differences in underlying pathology between study populations. Careful selection of patients at risk may contribute to the prognostic power of microcirculatory observation. As of now, our data indicate that routine daily monitoring of the microcirculation in an unselected group of ICU patients is of limited prognostic value.

Despite the findings in the general population, in a post-hoc subgroup analysis on 28 traumatic patients (Chapter 6) a persistent microcirculatory under-resuscitation in presence of normalized systemic hemodynamic variables was associated with more severe organ dysfunction and adverse clinical outcome. This could suggest that early identification of microvascular and tissue hypoperfusion in trauma patients may be relevant to detect patients at higher risk of multiple organ failure and to prevent it with tailored intensive care treatment and acute resuscitation.

Similarly to sepsis, severe traumatism may lead to the activation of multiple complex mechanisms of microvascular derangement: the tissue damage and hypoperfusion may induce release of inflammatory mediators, cytokines and reactive oxygen species producing a Systemic Inflammatory Response Syndrome with impairment of the endothelial and glycocalyx function, alterations in red blood cell deformability and increase in leukocyte adhesion. Anyone 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 edema. 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. [14]

Finally, the MicroDAIMON was a pure observational study: patients were treated following the international guidelines and principles of good clinical practice and clinicians had no information about the microcirculation during the study. Therefore, the study design is insufficient to draw conclusions on the applicability of microcirculatory monitoring as a tool to guide resuscitation. Under these conditions, additional assessment of microvascular blood flow may not be useful to predict outcome, but may be helpful for the clinician to select the appropriate resuscitation strategy or high-risk patients who needs to be more promptly or aggressively treated. Further research is needed to address this topic: careful selection of subgroups and adequate timing of microcirculatory reassessment during resuscitation and further treatments remain essential in this process. Studies to test the direct effect on prognosis and organ function of microcirculation-directed treatments are needed as following steps in this field.

Evaluate the microcirculatory response to therapy: most therapies which aim to improve hemodynamic failure should finally improve the microcirculation. Does it really happen?

As extensively discussed by many researchers, studies on microcirculatory resuscitation do not show uniform and univocal results. Furthermore, a wide variability in studies settings, patient stratification and selection by severity and underlying disease could have affected the conclusions. The physiopathology of microvascular dysfunction is probably variable in different clinical conditions and the underlying mechanisms are not completely understood yet. Another crucial point in targeting microcirculation as resuscitation endpoint is the timing of the treatment, being prompt treatment of main importance in obtain a possibility to interrupt the way to irreversible organ dysfunction and death. In this point of view, microcirculatory monitoring could be inserted in a wider concept of patient-tailored therapy, consenting to individualize treatments to the specificity of the single subject.

Many treatments have been proposed for improving microcirculation perfusion. In Chapter 7, the effect of intravenous ketanserin has been explored. This study has been conducted following a strict protocol of patients' selection and dosing titration on response: only patients with a persistent impaired MFI after an appropriate resuscitation were included, in order to be able to detect improvement of microcirculatory perfusion at the bedside.

The pleiotropic effects of ketanserine, both on vasodilation and positive modification of platelets' aggregation and cytokines dynamics, showed the capacity of ameliorate the microcirculatory dysfunction in patients with persistent abnormal MFI after resuscitation. A major limitation of this pilot study was the open label design and the lack of control group, creating possible confusion in the interpretation of the results. In fact, the presence of a placebo control group allows to reduce at minimum the effect of the regression to the mean, excluding that the detected response is due to confounding factors and not to the tested treatment. According to this observation many placebo-controlled trials, designed to demonstrate a positive effect of drugs on recruiting microcirculation, failed to detect any beneficial effect, despite positive and promising results of pilot uncontrolled studies. [15-18]

As part of this research project, diverse other clinical studies are already planned, designed and already started, trying to investigate the effects of some microcirculatory-targeted therapeutic strategies:

- Effects of the infusion of IgM-enriched immunoglobulins on sublingual microcirculation in patients with severe sepsis and septic shock. A randomized controlled study to investigate whether the administration of IgM-enriched immunoglobulins could improve microcirculation derangements in septic patients by inducing a modulation in the immune response to sepsis. The enrollment phase is concluded; data analysis is ongoing.
- Changes in cytokines, hemodynamic and microcirculation in patients with sepsis/septic shock undergoing renal replacement therapy and blood purification with Cytosorb. An observational study to explore the microcirculatory response to the blood purification from cytokines by using a dedicated sorbent cartridge. Original manuscript containing the main results is already submitted for publication.
- Circulating free hemoglobin and microcirculatory response after administration of paracetamol in febrile septic patient. An observational study primarily designed to evaluate the potential scavenging effect of paracetamol against free hemoglobin, know as potential oxidative agent and recognize to be detrimental for glycocalyx integrity and microvascular perfusion. Enrollment phase is ongoing.

Are clinicians ready to apply such a perfusion-based approach to their daily clinical practice?

Beyond all the technical issues, the common practice and the clinical habits are often the hardest obstacle to fight when trying to introduce a new tool, treatment or protocol in the clinical practice even if clearly beneficial for the patients. An example of this aspects of clinicians' mindset was elegantly shown by Rameau et al [19]: the introduction of a fluid resuscitation protocol based on fluid responsiveness tested by means of a passive leg raising test, showed a compliance of only 56%, despite an adequate staff training. Patients resulting to be not fluid responsive after the performance of the test, but still judged under-resuscitated by the attending physician (based on other clinical variables), received fluids despite the protocol. This attitude is common also with other interventions and practices.

The introduction of microcirculatory monitoring in daily practice has several implications for the clinicians: new equipment to deal with, new procedures to learn, accurate quality check of videos with potential pitfalls which could affects measurements results and lead to consistent inter-individual variability if not adequately detected, and, last but not least, a completely different approach to the patient, targeting treatments on different endpoints. This complexity can potentially discourage who tries to get into this world causing an exacerbation of the wary attitude of the so called "human factor".

In Chapter 3, the major issues in obtaining good quality microcirculatory imaging were investigated, searching the potential avoiding errors and defining precise criteria to select optimal and acceptable videos, suitable for analysis or clinical evaluation. Videos of unacceptable quality, conducted to a false increasing in microcirculatory alterations: this can cause important bias in the interpretation. Pressure artifacts are the most important errors to avoid when collecting microcirculatory videos, leading to the most consistent alterations of microvascular flow and vessel density. Interestingly, not only the investigator's experience in microcirculatory imaging, but also patient-related factors (mainly the neurological state, cooperation and capacity to maintain a stable position of the tongue) played a preeminent role in determining a good-quality video recording.

A good training with experts and a sufficient learning curve is thus desirable, such as with many other imaging tools, in order to minimize the intra and inter-observer variability, to correctly use microcirculatory imaging in clinical practice avoiding misinterpretation of the results and incorrect conclusions. Consequently to this study, the second consensus conference on the assessment of microcirculation in critically ill patients recommends to include specific scoring systems (included a cut off value for acceptability) that address all the aspects of video quality (content, illumination, focus, pressure artifacts and duration). [20] This aspect acquires even more relevance in clinical settings.

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