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EXPERIMENTAL

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Orthogonal polarization spectroscopy to detect mesenteric hypoperfusion

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Abstract *Objective:* Orthogonal polarization spectral (OPS) imaging is used to assess mucosal microcirculation. We tested sensitivity and variability of OPS in the assessment of mesenteric blood flow (Q_{sma}) reduction. *Setting:* University Animal Laboratory. *Interventions:* In eight pigs, Q_{sma} was reduced in steps of 15% from baseline; five animals served as controls. Jejunal mucosal microcirculatory blood flow was recorded with OPS and laser Doppler flowmetry at each step. OPS data from each period were collected and randomly ordered. Samples from each period were individually chosen by two blinded investigators and quantified [capillary density (number of vessels crossing predefined lines), number of perfused villi] after agreement on the methodology. *Measurement and results:* Interobserver coefficient of variation (CV) for capillary density from samples representing the same flow condition was 0.34 (0.04–1.41) and intraobserver CV was 0.10 (0.02–0.61). Only one investigator observed a decrease in capillary density [to 62% (48–82%) of baseline values at 45% Q_{sma} reduction; $P = 0.011$], but comparisons with controls never revealed significant differences. In contrast, reduction in perfused villi was detected by both investigators at 75% of mesenteric blood flow reduction. Laser Doppler flow revealed heterogeneous microcirculatory perfusion. *Conclusions:* Assessment of capillary density did not reveal differences between animals with and without Q_{sma} reduction, and evaluation of perfused villi revealed blood flow reduction only when Q_{sma} was very low. Potential explanations are blood flow redistribution and heterogeneity, and suboptimal contrast of OPS images. Despite agreement on the method of analysis, interobserver differences in the quantification of vessel density on gut mucosa using OPS are high.

Keywords OPS · Splanchnic perfusion · Microcirculation · Laser Doppler

Introduction

Impaired and heterogeneous microcirculatory blood flow is a common feature in severe sepsis and during hemorrhage [14, 21], and is increasingly recognized as the cause of organ dysfunction. Although impaired microcirculatory perfusion can be detected clinically, quantification of the degree of hypoperfusion without the use of specific tools is difficult.

Several direct and indirect methods are available for quantitative assessment of microcirculatory perfusion in the clinical setting. These include laser Doppler flow

(LDF), tissue tonometry, oximetry and spectrophotometry, magnetic resonance imaging (MRI), and microdialysis. All of these methods have drawbacks, such as lack of calibration/arbitrary units (LDF, MRI), interference with concurrent changes in metabolism (tonometry, spectrophotometry), derangement by artefacts (oximetry), need for high resource allocation (MRI) and invasiveness (microdialysis).

Orthogonal polarization spectral (OPS) imaging is a relatively new technique, which allows direct visualization of the microcirculation. The OPS system has been tested in a variety of conditions and locations, both clinically and experimentally [4, 5, 17, 23]. However, analysis of the generated images (movies) can be difficult. In the case of intestinal microcirculation, all sequences have to be analyzed manually, since no software for full quantification is available. There is disagreement regarding the way in which the information from OPS images should be addressed and translated into flow [1, 4, 8, 11, 12, 15–18, 23, 24]. Proposed criteria include quantification of visible capillaries and/or their diameter, scoring different flow types in vessels of different sizes, and (semi-) quantitative assessment of flow or blood cell velocity [4, 8, 10, 15–20, 23, 24]. It is very likely that such different ways of quantifying the microcirculatory perfusion will result in different findings when several investigators are assessing the same OPS sequence. A less-than-perfect image quality—for instance as a result of moving tissue—may leave some room for interpretation and further increase the variability between investigators' assessments.

The aim of this study was to assess the ability of two blinded investigators to quantify microcirculatory perfusion changes using OPS. The issue is whether different observers are able to generate consistent results that yield the same interpretation. We hypothesized that (1) semi-quantitative OPS analysis is able to detect gut hypoperfusion and (2) decreasing perfusion may influence the variability between OPS assessments of the two investigators. Data from this manuscript have been published in abstract form [3].

Materials and methods

This study was performed according to the National Institutes of Health guidelines for the care and use of laboratory animals, and the protocol was approved by the Animal Care Committee of the Canton of Bern, Switzerland. Hemodynamic and metabolic parameters of these animals have been published previously [9]. A description of anesthesia and monitoring is provided in the electronic supplement.

Surgical preparation

A gastric tube was inserted orally to drain gastric fluids. Through right and left cervical incisions, catheters were inserted into the carotid artery, pulmonary artery, and superior vena cava. A midline laparotomy was performed. The superior mesenteric artery (SMA) was identified at its origin, and both a vascular occluder and an ultrasonic flow probe (Transonic® Systems Inc., Ithaca, NY, USA) were placed around it. A second flow probe was placed around the portal vein. Through an antimesenteric incision, three small custom-made LDF probes (Oxford Optronix, Oxford, UK) were sutured on the distal jejunal mucosa close to each other and three more LDF probes were sutured on the serosa in close proximity. In addition, microdialysis catheters (CMA/20, CMA Microdialysis AB, Stockholm, Sweden) and tonometers (Tonometrics, TRIP NGS Catheter, Datex-Ohmeda Division, Instrumentarium Corporation, Helsinki, Finland) were inserted into the distal jejunal and proximal duodenal walls, and lumen, respectively. A jejunostomy was performed to assess the mucosa for OPS measurements and to drain gases and fluids. The intestinal mucosa was cleaned with warm water at the location of OPS measurement. A small canula for blood sampling was inserted into a distal jejunal mesenteric vein. All bowel incisions were closed with continuous sutures, and the abdominal wall was reapproximated with clamps. After completion of the surgical preparation, the animals were allowed to recover for 60 min before the experimental protocol was started.

Experimental protocol

The animals were divided into two groups. In animals with blood flow reduction ($n = 8$), the vascular occluder around the SMA was inflated, and SMA blood flow was reduced in steps of 15% of baseline flow. At each step, stable blood flow was maintained for 45 min. Blood and microdialysis samples were taken at the end of each step. In controls ($n = 5$), the same samples were taken at corresponding time points without altering SMA blood flow. At the end of the experiments, the pigs were killed with an infusion of intravenous potassium chloride.

Ultrasonic transit time flowmetry

SMA and portal venous blood flows were measured throughout the experiment with ultrasonic transit time flowmetry using an HT 206 flowmeter (Transonic® Systems Inc., Ithaca, NY, USA).

Laser Doppler flowmetry

Microcirculatory blood flow in the mucosa and muscularis of the jejunum were measured continuously using a multichannel laser Doppler flowmetry (LDF) system (Oxford Optronix, Oxford, UK). The microsurface probes

have a sampling depth of 0.5–1.0 mm, resulting in a sampling volume of 0.3–0.5 mm^3. We did not record biological zero flows for the LDFs at the end of the experiments. To assess the between-flow probe variability, we used the *relative* flow changes.

Orthogonal polarization spectral imaging

To always be able to measure at the same region of the gut, the OPS imaging probe (Cytoscan™, Rheologics, Inc., Exton, PA, USA) was fixed to a customized holder. The holder was equipped with three joints, which made it possible to position the probe in close proximity to the jejunal mucosa without squeezing it. In circumstances in which the probe still exerted pressure against the intestinal wall, its position was gently adjusted. At each step of SMA blood flow reduction, microcirculatory blood flow images in a single region were recorded for at least 1 min on a videotape for later processing. The images were only recorded when the resolution of the image was judged to be good.

The videotapes were afterwards cut into three video sequences of equal length (15–20 s) per step of blood flow reduction. A random number was assigned to each sequence and the sequences were handed to the two investigators (HB and VK). The film data corresponding to the video sequences were processed with special analysis software (Capiscope Vers. 3.9.3.0, KK Technologies, Devon, England). One video sequence consisted of 400–500 single picture frames, from which a subsequence of 30–60 (2–3 s) picture frames was selected. This subsequence was played in a loop for analysis. Both investigators were free to choose a sequence representing in their view the best image quality of that sequence (i.e., clear identification of villi, vessels, and blood flow; good contrast; and the least possible movement). Accordingly, both investigators analyzed all sequences for each flow condition, but the selection of frames in the specific sample to represent each flow condition was left to the individual investigator. For quantification of perfusion, we refer to the electronic supplement.

Calculations and statistics

The LDF coefficient of variation was calculated as the standard deviation of the three mucosal LDF values divided by the associated average. Absolute values were used. Similarly, coefficients of variation were also used to assess "interobserver variability" and "intraobserver variability" for the OPS measurements. The term "interobserver variability" is used for all comparisons between the two observers, although the comparisons have several different components: these include selection of frames that are considered representative, selection of the ROI within the frame considered representative, and the "interobserver variability" in assessing exactly the same ROI. Likewise, the term "intraobserver variability" reflects the differences between two assessments within the same sequence (but not necessarily the same frame), and within the same frame (but not necessarily within the same ROI), for the same observer.

Further statistical remarks can be found in the electronic supplement. Data are presented as median and range. Sigma Stat version 3.1 (Systat Software Inc., Richmond, CA, USA) and SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis.

Results

In the flow reduction group, blood flow in the SMA was reduced stepwise from 24 (18–31) ml/kg/min to 3 (2–5) ml/kg/min, while it remained constant in group C (see Table ES1 in the electronic supplement). Systemic hemodynamics are also displayed in the electronic supplement (Table ES1). Heart rate increased in both groups, while the increase in cardiac index was only significant in the control group. Core temperature was 38.7°C (37.5–40.1°C) in the control group and 39.1°C (36.5–40.1°C) in the flow reduction group (n.s.). Signs of ischemia in the venous effluent (increased L/P ratio, lactate release) appeared at 60% of flow reduction [9].

Laser Doppler flowmetry

Although all three LDF probes demonstrated a similar reduction in blood flow in the flow reduction group and unchanged perfusion in the control group (see Figure ES1 in the electronic supplement), differences in relative blood flow changes between the three measurement sites confirmed heterogeneous microcirculatory perfusion [coefficient of variation for relative flow reduction: 0.26 (0.14–0.52); median (range)].

Orthogonal polarization spectral imaging

Of 268 recorded films, investigator 1 excluded 21 (8%) and investigator 2 excluded 7 (3%) due to insufficient quality (mainly low contrast). Investigator 1 detected no significant changes in absolute vessel density in either group, while investigator 2 found a decrease in both groups. Accordingly, vessel density differed at various time points between the investigators, notably already at baseline (Fig. 1a, b).

In contrast to vessel density, significant reduction in perfused villi was detected by both observers at 75% Q_{sma} reduction ($P = 0.017$; Fig. 1c, d). Under control conditions, the flow pattern was judged as unchanged over time

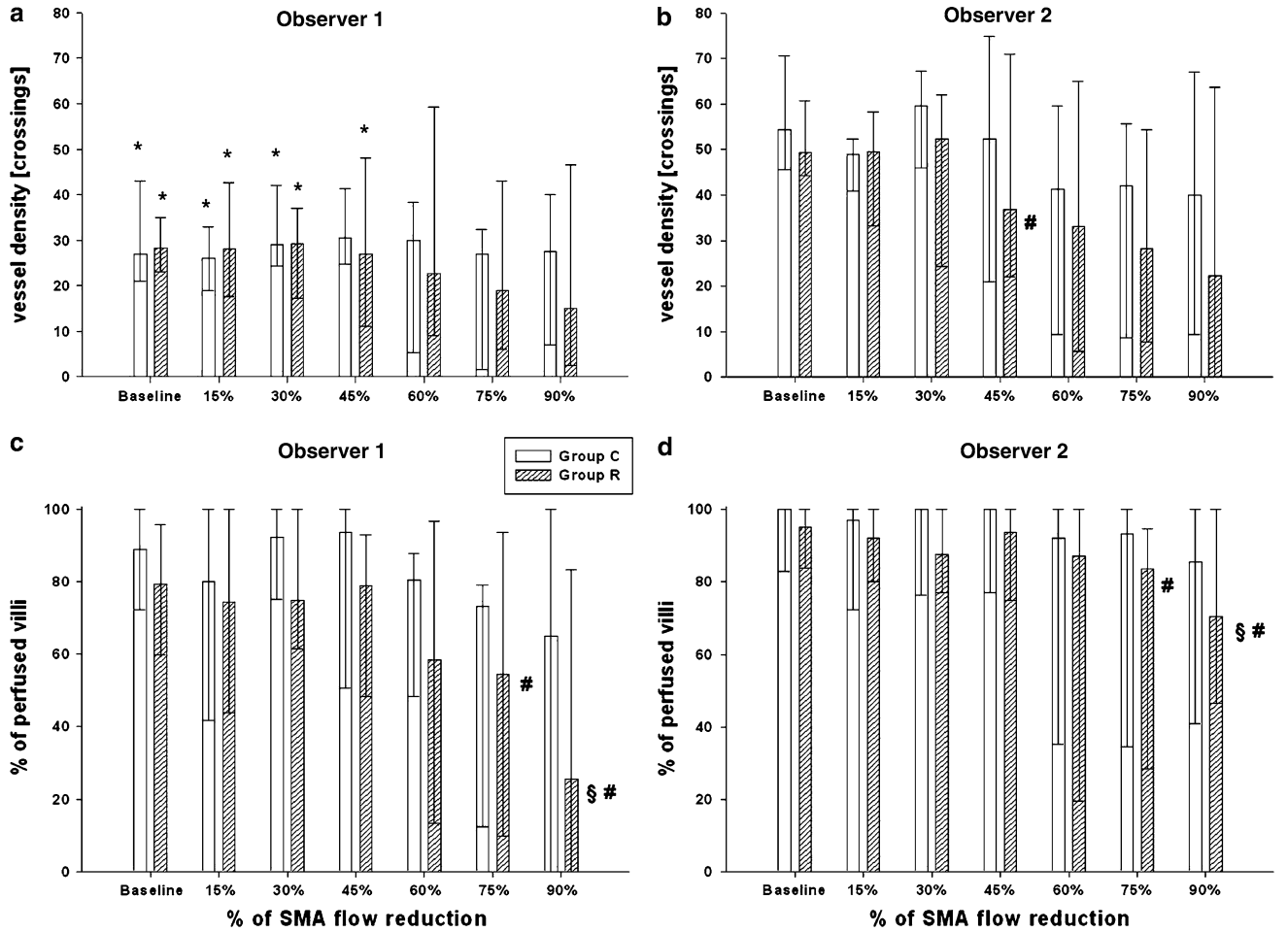

Fig. 1 a–d Vessel density and percentage of perfused villi. In group R ($n = 8$) SMA flow was reduced in six steps of 15% using an occluder. In group C ($n = 5$), the occluder was placed around the superior mesenteric artery (SMA) but was not inflated. Results are presented as median (range). [§]Friedmann test for repeated measurements, [#]Wilcoxon signed rank test versus baseline, first $P < 0.05$, *Mann–Whitney Rank sum test, compared to observer 2, $P < 0.05$

by both investigators (see Figure ES2 in the electronic supplement), although observer 2 counted a significantly higher fraction of high flows (and a lower fraction of intermediate/stagnant flow). A shift from high to intermediate or no flow was detected by both observers at 60–75% Q_{sma} reduction. Both investigators observed trends toward increased flow heterogeneity during progressive mesenteric arterial flow reduction (see Figure ES3 in the electronic supplement).

Data on "interobserver variability" and "intraobserver variability" for vessel density, number of perfused villi, and total number of villi are presented in Table 1.

With respect to measurement site, a statistically significant interobserver variability for the number of vessels, number of villi, and number of perfused villi with high mean coefficients of variation was found. In contrast, in sublingual OPS images from eight volunteers, investigator 1 counted 43 (36–57) and investigator 2 counted 44 (37–56) vessel crossings (n.s). The interobserver coefficient of variation was 0.03. The two observers agreed completely on flow and flow pattern in each of the images. Representative pictures of identical sequences and ROI from gut and sublingual mucosa analyzed by both observers are shown in Figure ES4 of the electronic supplement. The interobserver variabilities for vessel crossings and perfused villi were 0.59 (−0.80 to 0.95) and 0.53 (0.00–1.20), respectively, when the regions of interest were chosen individually by the investigators, and 0.44 (−0.29 to 0.74) and 0.53 (0.14–1.25), respectively, when the same regions of interest were used. Mean intraobserver differences for the various conditions were small, with a low coefficient of variation.

Table 1 Interobserver and intraobserver variability in postanalysis and in sublingual OPS in volunteers

	Interobserver variability				Intraobserver variability			
	Mean	Mean difference	Mean CV	*P* value	Mean	Mean difference	Mean CV	*P* value
Same sequences used by both observers					First versus second analysis			
Vessels	40 (5–64)	25 (−4 to 45)	0.59 (−0.80 to 0.95)	$P < 0.001$	46 (3–81)	−1 (−13 to 19)	0.00 (−0.40 to 0.86)	$P = 0.951$
Number of villi	13 (9–20)	5 (−1 to 12)	0.43 (−0.06 to 0.86)	$P < 0.001$	13 (8–21)	0 (−4 to 7)	0.00 (−0.33 to 0.42)	$P = 0.966$
Number of perfused villi	10 (2–20)	6 (0–10)	0.53 (0.00–1.20)	$P < 0.001$	11 (2–20)	0 (−7 to 5)	0.00 (−0.74 to 0.34)	$P = 0.212$
Same sequences and same region of interest (ROI) used by both observers					Second versus third analysis			
Vessels	40 (11–66)	19 (−5 to 43)	0.44 (−0.29 to 0.74)	$P < 0.001$	42 (16–58)	−5 (−62 to 44)	−0.27 (−1.21 to 1.67)	$P = 0.416$
Number of villi	13 (9–20)	6 (−6 to 9)	0.40 (−0.38 to 0.80)	$P < 0.001$	13 (7–19)	−1 (−9 to 1)	−0.07 (−0.72 to 1.00)	$P = 0.992$
Number of perfused villi	10 (5–15)	5 (−2 to 12)	0.53 (0.14–1.25)	$P < 0.001$	10 (4–16)	0 (−13 to 12)	0.00 (−1.38 to 1.33)	$P = 0.946$
Sublingual OPS in volunteers								
Vessels	44 (37–57)	0.5 (−2 to 6)	0.01 (−0.05 to 0.12)	$P = 0.357$				

Inter-observer and intraobserver variability of the 24 equal sequences of the post-analysis. *Mean* is the mean value of both observers for each parameter, *mean difference* is the mean difference of both observers, and *CV* is the mean coefficient of variation for the matched paired data. The *P* value describes the performed Wilcoxon signed rank test for matched pairs

Jejunal tissue lactate/pyruvate ratios and mucosal-arterial pCO_2 gradients

Jejunal tissue lactate/pyruvate ratios increased from 8.6 (8.0–14.1) to 20.3 (10.5–37.4) after 60% SMA flow reduction, and to 40.7 (31.1–51.9) after 90% SMA flow reduction, and remained unchanged in controls. Jejunal mucosal-arterial pCO_2 gradients increased from 1.3 kPa (0.4–3.5 kPa) to 10.8 kPa (4.8–18.5 kPa) after 75% SMA flow reduction, and to 17.2 kPa (10.6–18.5 kPa) after 90% SMA flow reduction, while no changes occurred in the control group.

Discussion

We acknowledge upfront that there are limitations to the methods applied in this manuscript, since we do not have a "gold standard" to compare our OPS data against. However, this will likely be the case in any clinical application of this technique to evaluate the impact of a medical condition on microvascular perfusion. The critical issue is whether different observers in the same laboratory or in different laboratories are able to generate consistent results that yield the same interpretation.

This study highlights a number of methodological issues in assessment of the microcirculation. Under steady-state hemodynamic conditions, assessment of gut microcirculation with OPS imaging is strongly influenced by selection of the video sequence, selection of sample frames within the sequence, and definition of the region of interest where the qualification and quantification of the flow are performed. These sources of variability are further amplified by intraobserver and interobserver variability in assessment of exactly the same region of interest.

Capillaries are identified by passing red blood cells. If a plasma gap lies on the grid line, an observer may not count this capillary unless one or more red blood cells are very close to the line. This leaves room open for subjective decisions. We found that neither of the two blinded investigators was able to detect the differences between the groups with and without blood flow reduction using OPS in terms of vessel density, while both determined a significant reduction of perfused villi after 75% blood flow reduction. We also found that when all the sources of variability listed above were allowed to accumulate by letting the investigators choose the sample they considered representative, there was a large variability in the results obtained by the two investigators for all OPS-derived parameters. In contrast, when OPS imaging was assessed in healthy volunteers from sublingual measurements and exactly the same region of interest, a very low interindividual variability was confirmed.

OPS data for functional vessel density, diameter of microvessels, and red blood cell velocity correlated well with data obtained by fluorescence microscopy [7, 13]. Despite the use of OPS imaging in a variety of conditions and organs [8, 11, 12, 16, 24], and the attractiveness of direct visualization of microcirculation, the quantification of changes and their physiologic and clinical relevance remains controversial [1, 4, 8, 11, 12, 15–18, 23, 24]. The proposed criteria include visible capillaries, clearly visible flow of individual erythrocytes versus large thrombosed vessels coursing in a criss-cross fashion [11, 12], vessel diameter, red blood cell velocity [10, 19, 20], and functional capillary density [8, 15, 16, 24]. Also, for quantitative or semiquantitative analysis, several different approaches have been used: scoring different flow types attributed to different sizes of vessels [18], counting vessel crossings among vertical and horizontal lines and then deriving vascular density, and categorization of flow [4, 17, 23].

Likewise, various approaches to evaluate the reproducibility of OPS results have been used. De Backer et al. [5] reanalyzed three sublingual OPS recording sequences and found good reproducibility. The reproducibility of the sublingual recordings was confirmed recently by others [2, 22], and in the present study by us. Despite differences in assessing the sublingual OPS (only microvascular flow but not vessel density, short sequences, stationary conditions vs. interventions), it seems reasonable to conclude that sublingual OPS measurements are far less problematic than measurements at the gut mucosa.

Despite both investigators' experience with OPS and the precautions taken to reach consensus, we found an obvious systematic difference between the two investigators with regard to the vessel density. This unexpected finding underlines the complexity of using OPS to assess gut microcirculation. The quality of the images in the gut mucosa may sometimes leave open to interpretation where a villus or a vessel starts or stops or what should be interpreted as an artefact. We assume that there would have been better agreement with more reassessments and comparisons between the two investigators. The excellent agreement between the same two observers in sublingual OPS analysis speaks against a systematic difference in the way the images were analyzed. Further possible explanations for the differences between investigators' assessments include temporal flow variability, spatial variability and/or heterogeneity during the flow recordings, and compression of the microcirculation. Nevertheless, we suggest that investigators using OPS should be trained systematically and in depth in all conditions where they intend to use the device to minimize differences in individual interpretations of OPS images.

When the investigator-dependent variation in selecting the representative sample of gut mucosal flow for analysis was excluded, the variation between the results obtained by the two observers remained high. This underscores the difficulty in objectively classifying the capillaries and their flow characteristics in the gut mucosa. Nevertheless, both observers agreed in classifying the transition to a more heterogeneous flow pattern during flow reduction.

A significant reduction in the number of perfused villi was observed later than a significant increase in the L/P ratio. Although we suspect that this is at least in part a consequence of the suboptimal sensitivity of OPS in detecting the perfusion changes, an alternative explanation is preserved perfusion of the villi and production of lactate in submucosal gut layers.

Limitations of the study

Our study has both strengths and important limitations. Strength is the large sample size of OPS images for the comparison between observers. Furthermore, the study design reflects the real-life difficulties that every investigator is confronted with in choosing a sample that should be representative. A major limitation is that our model was solely designed for the assessment of regional microcirculatory blood flow changes during gradual mesenteric ischemia. The results may be different in other clinically relevant scenarios, such as hemorrhagic shock, sepsis, or local microvascular obstruction. Another major limitation is the semiquantitative quantification of OPS images. The size of the capillaries and the resolution, contrast, and motion artefacts precluded the use of the built-in functions of the OPS device to quantify capillary density and flow. If the OPS data were to be quantified as relative changes—as is done, for example, when laser Doppler flowmetry is used—our study would have demonstrated a strong trend toward a progressive decline in both groups over time. Moreover, calculation of "no flow" vessels in the density assessment probably overestimated the real perfusion in OPS.

In contrast to the sublingual images obtained by the same device in volunteers, the quality of OPS images was relatively poor and inferior to what has been obtained by other investigators. This may in part be related to the multiplicity of measurements acquired over a short period of time in our experiment. Limiting the number of measurements and measurement techniques may be useful to allow acquisition of better image quality in subsequent trials. Furthermore, the sequences from the OPS recordings we analyzed were short and do not allow precise description of the type of flow in a vessel. Interestingly, despite the short sequences, both investigators detected a shift from high to intermediate or no flow and increased flow heterogeneity during progressive mesenteric blood flow reduction. However, there is another explanation for the differences in interobserver agreement between sublingual and intestinal mucosa: the sublingual bed—in contrast to the gut mucosa—is composed of a high proportion of venules (yielding high contrast due to higher

hematocrit than capillaries). Accordingly, vessels may be identified more readily in the sublingual capillary region. Differences in identifying capillaries in sublingual tissue have little impact on interobserver differences, because the high number of venules will strongly influence the results. Various semiquantitative approaches have been used by others, mostly for sublingual tissues [2, 4, 6, 17]. The sublingual microcirculation has a net-like pattern, whereas the microcirculation of the villi demonstrates an organized capillary structure. This is likely to explain why alternative semiquantitative methods have been used by others as well. Tugtekin at al. [23] classified microvascular blood flow in the villi only by perfusion patterns. Verdant et al. reported a good correlation between microcirculation alterations in the sublingual area and in villi in an experimental model of septic shock using computer-assisted quantitative methods [25].

Until problems related to image quality and flow and vessel quantification have been solved, assessing gut microcirculation with OPS remains problematic. The local variability in flow, the variability over time even in steady-state macrohemodynamic conditions, and the difficulties in defining a representative sample are further amplified due to variability between observers in quantifying the same region of interest. In this respect, the gut mucosa is much more difficult to assess than the sublingual mucosa.

In conclusion, OPS seems to have important drawbacks when used for the assessment of intestinal microcirculatory perfusion. The image quality in terms of contrast and brightness is in our opinion very important for the reliable assessment of OPS images. The "crossing line" semiquantitative method, although it has good reliability in sublingual studies, does not seem to be useful for villi microvasculature evaluation. New attempts to solve this problem are needed.

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