***In vivo* reflectance-mode confocal microscopy assessments: impact of overweight on human skin microcirculation and histomorphology**

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Abstract. Reflectance-mode confocal microscopy (RCM) enables *in vivo* assessment of the human skin. Impact of overweight on both human skin microcirculation and histomorphology has not been investigated *in vivo*. The purpose of this study is to evaluate both microcirculation and histomorphology *in vivo* in overweight. In 10 normotensive overweight nondiabetic individuals (OW-group, BMI 29.1 ± 2.7 kg/m²) and 10 age- and sex-matched healthy lean controls (CO-group, BMI 20.4 ± 1.9 kg/m²) the following parameters were evaluated using RCM: dermal blood cell flow (DBCF), density of dermal capillaries (DDC), epidermal thickness (ET), and epidermal cell size (ECS). DBCF was counted at 63.11 ± 4.14 cells/min in OW-group and at 51.06 ± 3.84 cells/min in CO-group ($P < 0.05$). DDC was reduced in OW-group (4.91 ± 0.39 capillaries/mm²) compared to the controls (6.02 ± 0.64 capillaries/mm², $P < 0.05$). Histometric evaluation of ET reveals thickening in OW-group compared to the CO-group (54.79 ± 4.25 μ m versus 44.03 ± 3.11 μ m, $P < 0.05$). ECS differed significantly ($P < 0.05$) in OW-group (821.3 ± 42.02 μ m²) compared to the controls (772.6 ± 34.79 μ m²). Inverse correlation of dermal capillary density and overweight point to reduced total tissue perfusion while positive related blood cell flow reveals vasodilatation. Increase of both ET and cell size indicates remodeling of cutaneous histomorphology, maybe as an early stage of adiposity-related skin condition. © 2016 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.21.3.036009]

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1 Introduction

In the past, studies evaluated the amount of weight and the relation to cardiac output, hypertension, atherosclerosis, angiopathy, vascular reactivity, and endothelial function.^{1–4} Moreover, it was demonstrated that the human skin is even involved in systemic diseases including hypertension, hypercholesterolemia, cardiovascular disease, diabetes mellitus, and obesity.^{5–8} However, overweight-associated effects on *in vivo* cutaneous microcirculation has not been investigated *in vivo*. Given that adequate microvascular perfusion is essential for cellular integrity, little attention has been given to *in vivo* histomorphology of the human skin. Hitherto, several noninvasive measuring techniques have been used to assess skin microvascular status including laser Doppler flowmetry, plethysmography, orthogonal polarization spectral imaging, transcutaneous oxygen measurements, electromagnetic flowmetry, or hemoglobin oxygenation.^{9–14} While all of these monitoring devices have their specific applications and limitations, none of them is able to evaluate both the microvascular function and the local morphological pattern. Both parameters are important, however, to assess restoration of cellular homeostasis. Reflectance-mode confocal microscopy

(RCM) is a noninvasive imaging technique to observe the human skin *in vivo* and in real-time. This high-resolution microscope opens a window in to living tissue and enables investigation of cutaneous microcirculation and histomorphology on cellular and subcellular levels.^{15–20} The aim of the present study was to evaluate the impact of overweight on *in vivo* microcirculation and histomorphology of the human skin using RCM.

2 Methods

2.1 Volunteers

Ten normotensive overweight nondiabetic individuals (BMI 29.1 ± 2.7 kg/m²) and 10 age- and sex-matched healthy lean controls (BMI 20.4 ± 1.9 kg/m²) all with skin type III (Fitzpatrick Classification Scale) were enrolled into the study and assigned to one of the following groups.

- OW-group: overweight individuals (4 m; 6 f, mean age 39.1 ± 9.6 years)
- CO-group: control lean individuals (4 m; 6 f, mean age 35.8 ± 8.4 years)

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2.2 Protocol

In a standardized session, RCM measurements were performed in both groups on the volar aspect of a randomly selected forearm. All measurements were performed in a fasting state. Thirty minutes prior to measurements, the subjects were recumbent in a room for acclimatization without physical or psychological stress. Prior to the measurements, heart rate, blood pressure, and plasma glucose level were measured as normal. Drugs, creams, ointments, or other cosmetic products were avoided 48 h before starting the study. This study was approved by the local ethics committee and all patients accepted to take part in the study with a written informed consent. In a standardized session, a magnetic tissue ring of the RCM was applied to the skin to stabilize the imaging skin site. The corresponding magnetic objective head of the microscope was positioned over the ring to capture the tissue ring without applying pressure. Moreover, the investigated area in the center of the tissue ring was anytime free of pressure.

2.3 Instrument

RCM was performed using a commercially available confocal microscope (Vivascope1500, Lucid Inc., Rochester, New York). This device enables high resolution and noninvasive imaging of the human skin without the necessity of external fluorescence and without the need to process the tissue by freezing, sectioning, or staining. This confocal model operates with a special gallium-arsenide laser source emitting in a long wavelength band at 830 nm. Since this wavelength is in the “optical window” of the human skin, optical sectioning of the epidermis and dermis, including the upper dermal plexus is achievable up to a controlled depth of 350 μm . High-resolution imaging on cellular and subcellular levels is feasible owing to a vertical resolution of 1.9 μm and a lateral resolution of 0.4 μm . By generating 20 frames per second, real-time imaging is achievable. This means the feasibility of observing dynamic processes of microcirculation in real-time such as blood cell flow. Due to the laser's low power of 30 mW at the skin surface, no tissue damage occurs. The single frame field of view is 500 $\mu\text{m} \times 500 \mu\text{m}$.

2.4 Parameter

In RCM images, the epidermal–dermal junction reflects strongly due to its melanin content, as bright circles of basal layer with the dark focus corresponding to the dermis. The circles are surrounded by the spinous layer of the epidermis. In the focus of the dermal papillae, the lumina of capillary loops are visible as black holes. Blood cell flow can be clearly observed in real-time imaging as brightly reflecting erythrocytes circulating through the capillary loops (Fig. 1). *In vivo* RCM was performed to evaluate dermal blood cell flow (DBCF), density of dermal capillaries (DDC), epidermal thickness (ET), and epidermal cell size (ECS).

- DBCF was evaluated by counting the number of circulating cells in the dermal capillaries of four dermal papillae in four fields of view (500 $\mu\text{m} \times 500 \mu\text{m}$) in each volunteer during a total of 30 s of RCM real-time imaging.

Fig. 1 In horizontal, *en face* virtual sectioning, confocal imaging depicts the epidermal–dermal junction as bright circles of basal layer with the dark focus corresponding to the dermis. In the focus of the circles (dermal papillae), the lumina of capillary loops are visible as black holes (arrows). Blood cell flow can be clearly observed in real-time imaging as brightly reflecting erythrocytes circulating through the capillary loops. The field of vision measures 500 $\mu\text{m} \times 500 \mu\text{m}$.

- The DDC per area was assessed in real-time RCM imaging by counting the number of capillaries indicating blood cell circulation in four fields of view in each volunteer.
- The ET was measured as the distance between skin surface and the apical plane of the dermal papillae; in each volunteer two measurements per field of view were taken in four fields of view.
- The ECS was determined by evaluating RCM images. In four fields of view from the corners of the virtual quadrilateral field (500 μm per side), four cells were measured using an image analysis program; Image Tool (see details in section data and statistical analysis). The ECS was measured only of the apical plane of the epidermal granular layer to avoid measurement errors.

2.5 Statistical Analysis

The RCM images were evaluated in a blinded manner using a free available image analysis program “ImageTool” (Version 3.0, UTHSCA, San Antonio, Texas).²¹ This software provides several image analysis tools, such as area or distance measurements. For statistical analysis SPSS version 16.0 (SPSS, SPSS Inc., Chicago, Illinois) software for Windows was used. The distribution of variables was tested for normality using Kolmogorov–Smirnov test. Comparisons between the groups were performed using independent *t*-test (two-sided). *P*-values below 0.05 were considered statistically significant.

3 Results

Based on more than 4800 s real-time RCM imaging and evaluation of more than 160 offline confocal images following data were obtained.

3.1 Dermal Blood Cell Flow

In real-time RCM imaging, DBCF was counted at 63.11 ± 4.14 cells/min in OW-group and thus was over 19% higher compared to the controls at 51.06 ± 3.84 cells/min ($P < 0.05$) (Fig. 2). These findings are based on counting more than 4500 blood cells.

3.2 Density of Dermal Capillaries

DDC was determined at 4.91 ± 0.39 capillaries/mm² in OW-group and at 6.02 ± 0.64 capillaries/mm² in CO-group ($P < 0.05$) (Fig. 3); thus capillary density was reduced over 22% in OW-group compared to the controls.

3.3 Epidermal Thickness

Histometric evaluation of the ET pointed to 54.79 ± 4.25 μm in OW-group, and thus reveals nearly 20% thicker epidermis in OW-group compared to the CO-group (44.03 ± 3.11 μm , $P < 0.05$) (Fig. 4). ET results are based on over 160 measurements in over 80 fields of view.

3.4 Epidermal Cell Size

Area measurements of over 320 cells in over 80 confocal images indicate ECS at 821.3 ± 42.02 μm^2 in OW-group and at

Fig. 2 DBCF was counted in real-time confocal imaging at the level of the papillary dermis in the capillary loops of the upper dermal plexus. Forearm blood cell flow was significantly ($*P < 0.05$) elevated in overweight (OW-group) compared to lean controls (CO-group).

Fig. 3 DDC was assessed in real-time confocal imaging by counting the number of capillaries indicating blood cell circulation. Capillary density was significantly ($*P < 0.05$) reduced in overweight (OW-group) compared to the control group (CO-group).

Fig. 4 Illustrated is the ET measured *in vivo* using confocal microscopy as the distance between skin surface and the apical plane of the dermal papillae. ET of the forearm was significantly ($*P < 0.05$) thicker in overweight (OW-group) compared to the controls (CO-group).

772.6 ± 34.79 μm^2 in CO-group ($P < 0.05$); thus ECS was nearly 6% higher in OW-group compared to the controls.

4 Discussion

The main finding of the present study is that overweight, compared with leans, is characterized by both impaired microvascular function and remodeling of skin histomorphology. Precisely, analysis of *in vivo* human skin microcirculation with respect to blood flow and capillary density indicate an inversely correlation of overweight and dermal capillary density while blood cell flow was positively related to overweight. In addition, increase of both ET and cell size indicates significant changes of the human skin histomorphology in overweight. Hitherto, a range of modalities have been used to evaluate human skin perfusion focusing on either blood flow or capillary density. RCM, however, enables separate observation of both blood cell flow and functional capillary density by real-time visualization of individual blood cell circulation through dermal capillaries. Hence, effects of vasoconstriction and vasodilatation on blood flow are just as feasible as assessments of total area perfusion. In the past, the amount of weight and the associated microcirculatory disturbances were discussed controversially. Our results obtained *in vivo* using RCM are consistent with previous studies on impaired microvascular function in obesity,²² however, we could already observe comparable microvascular dysfunction in overweight. Moreover, our findings extend previous reports as we are able to demonstrate morphological changes of the skin in overweight, maybe as a result of the evaluated impaired tissue perfusion. Nestel et al.²³ observed blood flow in the forearm microcirculation using plethysmography and found increased local sympathetic neuronal responsiveness and diminished nitric-oxide-mediated dilation in the forearm vasculature with increasing body adiposity. Our RCM results point to an increase of cutaneous blood cell flow of the forearm in accordance with the neuronal and mediator-related vasodilatation described by Nestel et al. In contrast, in a laser Doppler study, blood flow was reported to be reduced in forearm skin of obese nondiabetic individuals.²⁴ However, laser Doppler measures the total local microcirculatory blood perfusion of the tissue and summarizes blood cell flow and density of perfused capillaries. Hence, this does not seem to be a real inconsistency for our findings, as we may confirm reduced total tissue perfusion in terms of capillary density in overweight. By means of intravital video microscopy, Francischetti et al.²⁵ observed skin capillary density at rest and after postocclusive reactive

hyperemia. The authors observed structural and functional alterations in skin microcirculation that are proportional to the increase in the degree of obesity. In a similar model, using video microscopy Czernichow et al.²² reported resting capillary density was negatively related with obesity. Hence, the authors suggest that a lower baseline tissue perfusion was evident in obesity. Nailfold capillary density was measured using capillaroscopy and it indicated similar regulatory mechanisms of the dermal capillaries with reduced capillary recruitment in obesity.²⁶ Based on our *in vivo* RCM observations, we are able to confirm the regulation of dermal capillary density and consequently a lower total tissue perfusion even in overweight. In addition to the impaired skin microcirculation, our results strongly suggest that overweight is associated along with histomorphological remodeling of the skin as we could evaluate significant increase in both ET and cell size. Indeed, this is an interesting finding which has not been reported previously. It can only be speculated that the histomorphological changes observed are an early stage of adiposity-related skin conditions such as dermatitis, callus, corn, or acanthosis nigricans. The pathophysiological mechanism behind the histomorphological adaptation in overweight is probably multifactorial and based on hormonal, neural, and local control mechanisms similar to the cause of microvascular dysfunction. Moreover, it may be the result of a microvascular dysfunction itself. However, it should be emphasized that the design of the present study does not evaluate variables that may explain the relationship among these parameters. Moreover, the evaluated data of lean controls in this study are in accordance with previous reports on RCM measurements concerning skin microcirculation²⁷ and skin morphometry.²⁸ In conclusion, impaired local microvascular function along with histomorphological remodeling of the human skin was evaluated in overweight. Inversely correlation of dermal capillary density and overweight point to reduced total tissue perfusion while positively related blood cell flow indicate vasodilatation. In addition, increase of both ET and cell size reveals remodeling of the human skin histomorphology in overweight, maybe as an early stage of adiposity-related skin conditions. For the first time, the present study indicates the pathophysiological impact of overweight on both cutaneous microcirculation and histomorphology. Further RCM studies could be helpful to improve understanding of adiposity-related pathophysiological interactions by evaluating microcirculation and histomorphology for diverse degree of BMI.

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