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Editor's Choice

Characterization of Laser-Resistant Port Wine Stain Blood Vessels Using *In Vivo* Reflectance Confocal Microscopy

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Background and Objectives: Port wine stain (PWS) is a congenital vascular malformation of the human skin. Laser is the treatment of choice for PWS. Laser-resistant PWS is one crucial factor accounting for inadequate treatment outcome, which needs to be fully characterized. This study aims to quantitatively characterize the morphology of laser-resistant PWS blood vessels in the upper papillary dermis using *in vivo* reflectance confocal microscopy (RCM).

Study Design/Materials and Methods: A total of 42 PWS subjects receiving laser treatment from August 2016 through July 2018 were enrolled into this study. Thirty-three subjects had facial PWS; nine had extremity PWS. All subject's PWS received multiplex 585/1,064 nm laser treatment. RCM images were taken before and after treatment. The density, diameter, blood flow, and depth of PWS blood vessels were analyzed.

Results: We found 44.4% PWS on the extremities (four out of nine subjects) were laser-resistant, which was significantly higher ($P < 0.001$) when compared with those PWS on the face (15.2%, 5 out of 33 subjects). The laser-resistant facial PWS blood vessels had significantly higher blood flow (1.35 ± 0.26 U vs. 0.89 ± 0.22 U, $P < 0.001$), larger blood vessel diameters (109.60 ± 18.24 μm vs. 84.36 ± 24.04 μm, $P = 0.033$) and were located deeper in the skin (106.01 ± 13.87 μm vs. 87.82 ± 12.57 μm, $P < 0.001$) in the skin when compared with laser-responsive PWS on the face. The average PWS blood vessel density ($17.01 \pm 4.63/mm^2$ vs. $16.61 \pm 4.44/mm^2$, $P = 0.857$) was not correlated to the laser resistance.

Conclusions: Laser-resistant PWS blood vessels had significantly higher blood flow, larger diameters, and were located deeper in the skin. RCM can be a valuable tool for a prognostic evaluation on laser-resistant lesions before treatment, thereby providing guidance for tailored laser treatment protocols, which may improve the therapeutic outcome. The limitations for this study include relative small sample size and acquisitions of different blood vessels before and after 2 months of treatment. Lasers Surg. Med. © 2019 Wiley Periodicals, Inc.

Key words: port wine stain; laser; resistant; reflectance confocal microscopy

INTRODUCTION

Port wine stain (PWS) is a congenital progressive vascular malformation of human skin, resulting from differentiation-impaired endothelial cells (ECs) in dermis with a progressive dilatation of immature venule-like vasculatures [1]. PWS occurs in estimated 3–5 infants per 1,000 live births [2–4].

PWS can exist alone or in association with many other vascular malformations, such as Sturge-Weber syndrome, Parkes-Weber syndrome, Klippel-Trenaunay syndrome, and arteriovenous malformations [5]. PWS initially appear as flat red macules in childhood; lesions tend to darken progressively to purple and, by middle age, PWS often become raised as a result of the development of vascular nodules, which may spontaneous bleed or hemorrhage [6,7]. Moreover, PWS is a disease with potentially devastating and lifelong psychological and social complications that can greatly impair the quality of life for afflicted individuals [8–10].

Pulsed dye laser (PDL) irradiation at 585–595 nm is the treatment of choice for PWS. Yellow light absorption by hemoglobin converts incoming photo energy to heat thus inducing blood vessel wall necrosis [11–17]. Unfortunately, complete PWS clearance after PDL treatment occurs in <10% of patients [18–20]. Therefore, laser-resistant PWS blood vessel is a crucial factor accounting for the inadequate treatment outcomes. However, the morphological and molecular phenotypes of laser-resistant blood vessels have not been fully characterized. This knowledge gap hampers the development of more effective treatments for laser-resistant lesions.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported.

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Reflectance confocal microscopy (RCM) is a non-invasive imaging system that has been used to evaluate cutaneous pathology with cellular level magnification and resolution in clinical practice at the bedside [21]. RCM can record dynamic processes in human skin *in situ*, such as the cutaneous microcirculation. Various parameters such as skin thickness, epidermal cell types and sizes, blood vessel density, size, shape, and depth can be imaged and analyzed by RCM [21,22]. These features make RCM a useful tool to evaluate the *in vivo* dynamic features of PWS blood vessels before and after laser treatment.

In this study, we aimed to characterize the general morphology of laser-resistant PWS blood vessels using RCM. Our results showed that laser-resistant facial PWS blood vessels had significantly higher blood flow, larger diameters, and were located deeper in the skin when compared with laser-responsive PWS on the face. Our data also suggested that RCM could be a valuable tool for a prognostic evaluation on laser-resistant lesions before treatment, thereby providing guidance for tailored laser treatment protocols and improving therapeutic outcome.

MATERIALS AND METHODS

Subjects

The study protocol (#16095) was approved by the ethics committee of the Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, China. Forty-two subjects (25 women and 17 men) receiving laser treatments from August 2016 through July 2018 were enrolled into this study. Thirty-three subjects had facial PWS; nine subjects had extremity PWS. Signed written consent forms were obtained from each subject before entrance into the study. Subjects ranged in age from 1 to 48 years with an average age of 14.3 years. Exclusion criteria included pregnant and lactating women; subjects sensitive to light or photosensitizers; subjects with skin inflammatory lesions from bacterial, fungal, or viral infections; and subjects with complications such as severe heart, liver, and kidney disease.

Treatment

All subjects' PWS received multiplex 585/1,064 nm laser treatment (Cynosure, Inc, Westford, MA), which delivered sequential PDL and Nd:YAG wavelengths. The energy densities were 6.5 and 35 J/cm^2 with pulse durations of 0.5 and 15 ms for the 585 and 1,064 nm wavelengths, respectively, delivered on a spot size of 7 mm. A cooling device was applied during laser treatment. The entire PWS was treated with no overlap of individual spots. Subjects received 4–6 laser treatment sessions with an interval of 4 weeks between successive procedures. The rationales for using 585/1,064 for treatment of PWS in this study are (i) 1,064 nm can target blood vessels suiting deeply in dermis where the 585 nm cannot reach effectively and (ii) 585 nm first converts hemoglobin to methaemoglobin that has an absorption coefficient closely matching with 1,064 nm, rendering a synergistic effect for PWS treatment by using dual wavelengths.

RCM Imaging

RCM imaging was performed on the exact same PWS site from every subject before treatment at baseline and again 2 months after the final laser treatment using a VivaScope® 1500 (Caliber Imaging and Diagnostics; Lucid Inc., Rochester, NY) system. This RCM uses 830 nm light, which will penetrate to a maximal depth of 250–300 μm into the human skin with a resolution of <1.25 μm horizontally and <5.0 μm vertically. The maximal scanning depth of 150 μm used in this study was to ensure the optimal resolution of PWS blood vessels in the papillary dermis. The scanning depth was pre-set at 100 μm in the upper papillary dermis. The scanning was then increased stepwise at 3.05 μm up or down for several layers from the initial depth of 100 μm. Two to three layers with the highest number of dilated PWS blood vessels were imaged by RCM scanning. The PWS blood vessels showing dark tubular or circular structures with white blood cell movement was observed during live RCM video.

The average PWS blood vessel depth was determined by the location where the RCM images showing the highest density of blood vessels. The average blood vessel density was calculated by counting blood vessel numbers from 6 to 8 images in a 0.5 mm × 0.5 mm area from 2 to 3 layers above and below 100 μm within the upper papillary dermis. PWS blood vessels at that depth were used for subsequent analysis of blood vessel diameters and relative blood flow. The average blood vessel diameters were analyzed on RCM images using ImageJ (2017 edition, Java-based freeware; National Institutes of Health, Bethesda, MD) software.

PWS blood flow was determined based on the calculation of peaks of luminous intensity of blood cells in the vessels with the following modifications [22]. A blood vessel perfusion value can be represented by the peak pixel luminous intensity of blood cells. Changes in frequency of the blood cells peak pixel intensity per image composed in the RCM video could be used to reflect the relative velocity of vessel perfusion; these arbitrary units were calculated and subsequently applied as indexes for relative blood flow in PWS blood vessels.

Clinical Outcome Evaluation

Digitalized high-resolution photographs were taken at baseline, immediately after each treatment and 2 months after the final laser treatment. Clinical evaluations after laser treatments were performed by two independent dermatologists not involved in the study. The primary efficacy measure, namely PWS clearance in response to laser treatment, was scored into four categories: 4, complete response (90–100% clearance); 3, response (60–89% clearance); 2, improvement (20–59% clearance); and 1, ineffective (<20% clearance). The effective rate was the sum of the complete response, response, and improvement rates.

Statistical Analysis

The data was analyzed with the SPSS 22.0 software (IBM Inc, Armonk, NY). t Test and χ^2 test were

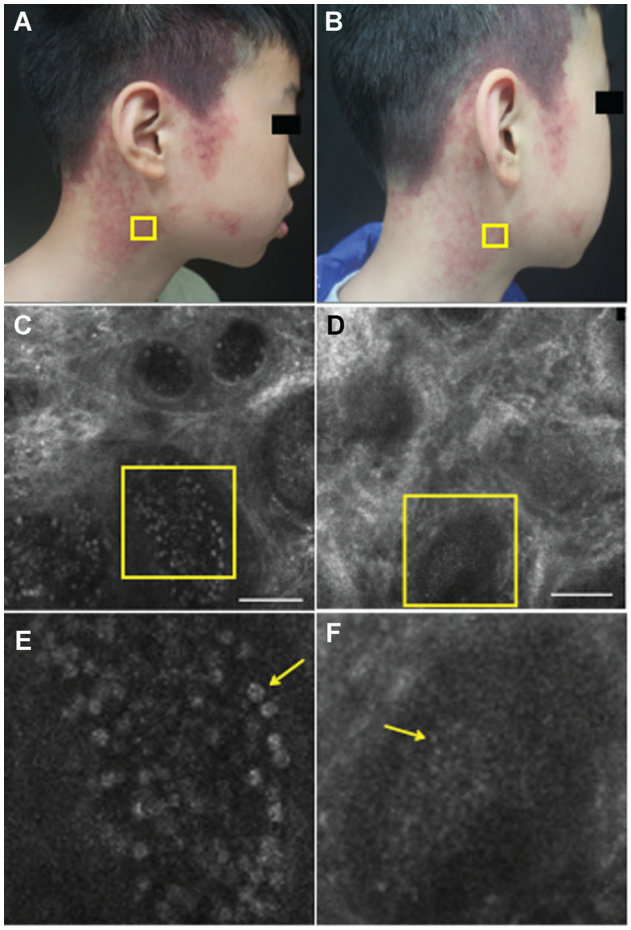

Fig. 1. A representative image of a male child (age 15) with laser-responsive port wine stain (PWS) on his right side of face and neck before (**A**) and after (**B**) multiplex 585/1,064 nm laser treatment. The reflectance confocal microscopy (RCM) images were taken from the same lesional area before (**C**) and after (**D**) treatment. (**E**) and (**F**) are higher magnifications from the boxed blood vessels in (**C**) and (**D**), respectively. The PWS blood cells were indicated by yellow arrows. The depths of imaging planes were 75 (before) and 100 μm (after treatment). The diameters of the highlighted blood vessels were 223 (before) and 160 μm (after treatment). To be noted that the PWS blood cells formed clusters in (**E**). Scale bar = 100 μm.

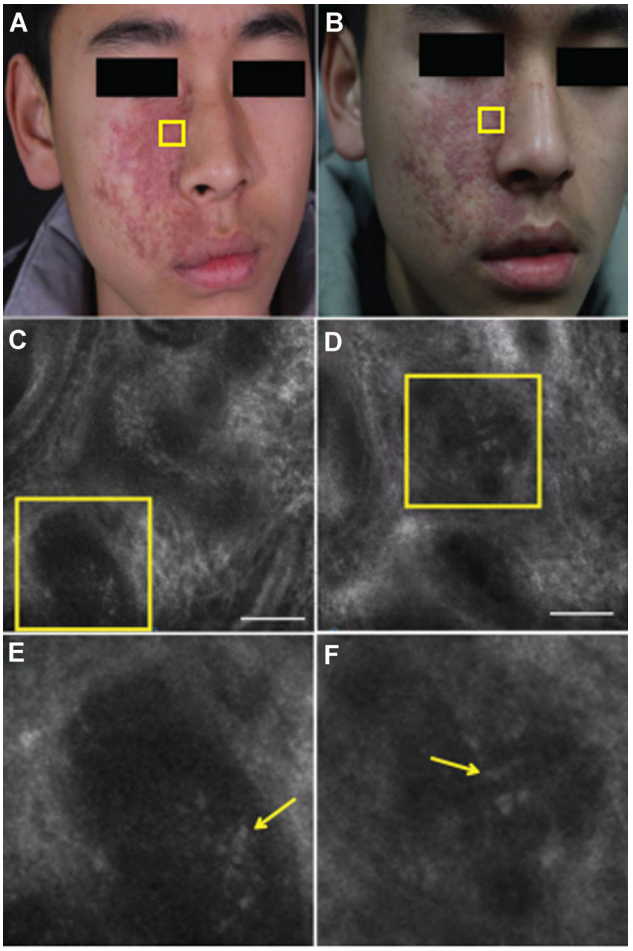

Fig. 2. A representative image of a young man (age 13) with laser-resistant port wine stain (PWS) on his right side of face before (**A**) and after (**B**) multiplex 585/1,064 nm laser treatment. The reflectance confocal microscopy (RCM) images were taken from the same lesional area before (**C**) and after (**D**) treatment. (**E**) and (**F**) are higher magnifications from the boxed blood vessels in (**C**) and (**D**), respectively. The PWS blood cells were indicated by yellow arrows. The depths of imaging planes were 100 (before) and 130 μm (after treatment). The diameters of the highlighted blood vessels were 236 (before) and 205 μm (after treatment). Scale bar = 100 μm.

used to analyze the data for statistical analysis. $P < 0.05$ were considered statistically significant.

RESULTS

Three out of 33 subjects with facial PWS showed a complete response, 12 showed manifested responses (Fig. 1A and B), 13 showed improvements, and 5 showed an ineffective response (Fig. 2A and B). The overall effective rate was 84.85% (Table 1). Blood vessels in facial PWS that were laser-resistant showed a significantly higher blood flow (1.35 ± 0.26 U vs. 0.89 ± 0.22 U, $P < 0.001$), larger blood vessel diameters (109.60 ± 18.24 μm vs. 84.36 ± 24.04 μm, $P = 0.033$), and were located deeper in the skin (106.01 ± 13.87 μm vs. 87.82 ± 12.57 μm, $P < 0.001$) when compared with laser-responsive PWS on the face (Fig. 3). Laser-responsive PWS on the face showed significantly lower relative blood flow (0.57 ± 0.21 U vs. 0.89 ± 0.22 U, $P < 0.001$), less blood vessel densities (8.25 ± 2.19/mm^2 vs. 16.61 ± 4.44/mm^2, $P < 0.001$), and smaller blood vessel diameters (49.04 ± 19.41 μm vs. 84.36 ± 14.04 μm, $P < 0.001$) as well as an increase in blood vessel depth (107.57 ± 12.38 μm vs. 87.82 ± 12.57 μm, $P < 0.001$) after treatment when compared with baseline (Fig. 3). Laser-resistant facial PWS did not show any statistically significant differences in those morphological features of blood vessels after treatment when compared with baseline (Fig. 3).

In the extremity PWS group, no subject showed a complete response, two showed manifested responses (Fig. 4A and B), three exhibited improvements, and four

TABLE 1. Comparison of Treatment Efficacies Among Patients in Two Groups

PWS group	Complete response	Response	Improved	Ineffective	Overall response rate (%)
The facial PWS	3	12	13	5	84.85%
(n = 33)	9.09%	36.36%	39.39%	15.15%	
The extremity PWS	0	2	3	4	55.55%*
(n = 9)	0%*	22.22%	33.33%	44.44%*	

PWS, port wine stain.
*$P < 0.001$ in the facial PWS group vs. the extremity PWS group.

showed an ineffective response (Fig. 5A and B). The overall effective rate was 55.6%, which was statistically significantly lower than that observed for facial PWS ($P < 0.01$) (Table 1). Blood vessels in the laser-resistant extremity PWS showed a statistically significant higher relative blood flow (1.33 ± 0.29 U vs. 0.97 ± 0.14 U, $P = 0.042$) when compared with laser-responsive extremity PWS (Fig. 6). The laser-responsive extremity PWS blood vessels showed statistically significant lower relative blood flow (0.97 ± 0.14 U vs. 0.74 ± 0.16 U, $P = 0.041$) and higher blood vessel densities (15.02 ± 1.58/mm^2 vs. 10.01 ± 1.00/mm^2, $P < 0.001$) after treatment when compared with baseline (Fig. 6). Laser-responsive extremity PWS blood vessels also showed smaller blood vessel diameters (108.50 ± 17.69 μm vs. 121.50 ± 23.81 μm, $P = 0.291$) and deeper blood vessel depths (109.50 ± 18.91 μm vs. 107.50 ± 11.90 μm, $P = 0.823$), which were not statistically significant, after treatment when compared with baseline (Fig. 6), probably due to the limited sample size in this group. Laser-resistant extremity PWS did not show any statistically significant changes in those blood vessels features after treatment when compared with baseline (Fig. 6).

Moreover, PWS blood vessels showed statistically significant larger diameters and depth in extremity lesions when compared with facial lesions (Table 2). There were no statistically significant differences in relative blood flow and blood vessel densities between these two groups (Table 2).

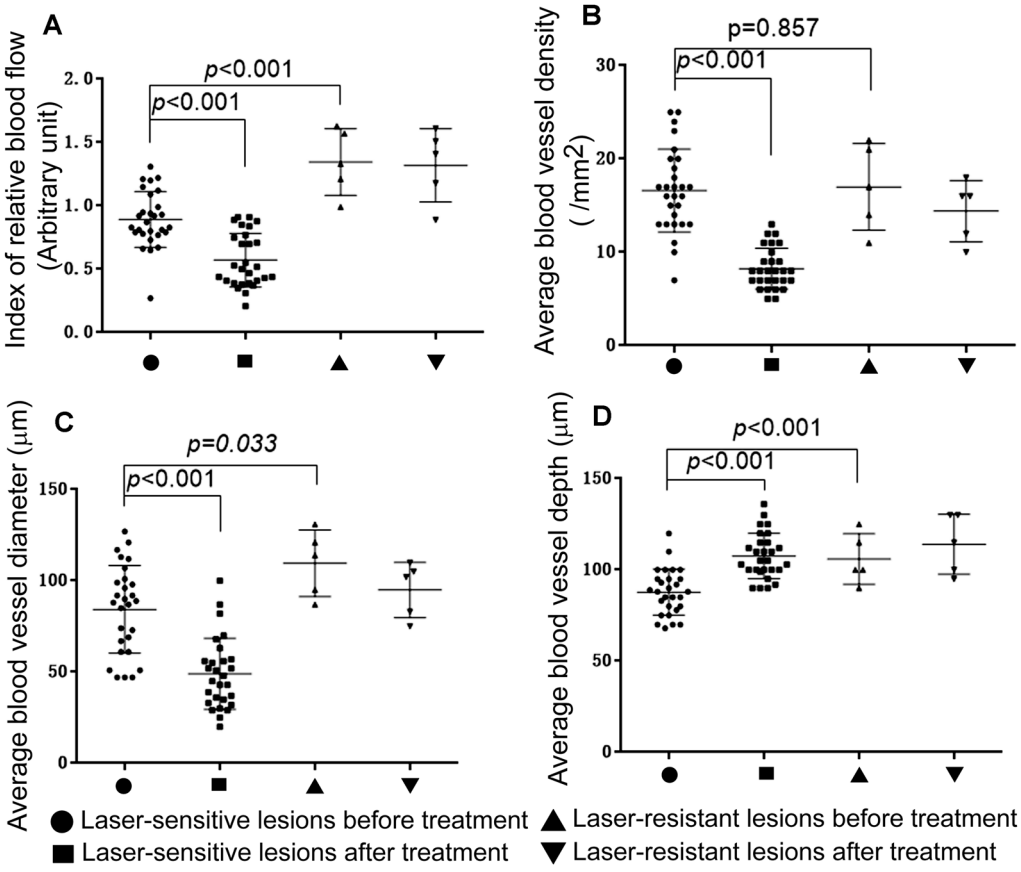

Fig. 3. xQuantitative analysis of relative blood flow (**A**), average blood vessel density (**B**), diameter (**C**), and depth (**D**) in facial port wine stain (PWS) lesions before and after multiplex 585/1,064 nm laser treatment.

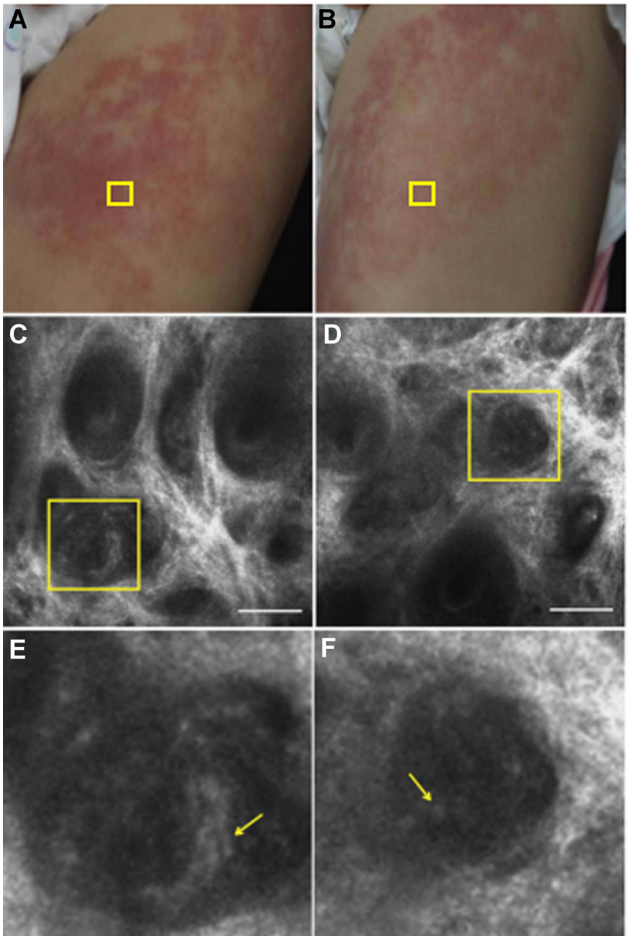

Fig. 4. A representative image of a female child (age 3) with laser-responsive port wine stain (PWS) on her left leg before (**A**) and after (**B**) multiplex 585/1,064 nm laser treatment. The reflectance confocal microscopy (RCM) images were taken from the same lesional area before (**C**) and after (**D**) treatment. (**E**) and (**F**) are higher magnifications from the boxed blood vessels in (**C**) and (**D**), respectively. The PWS blood cells are indicated by yellow arrows. The depths of imaging planes were 95 (before) and 110 μm (after treatment). The diameters of the highlighted blood vessels were 172 (before) and 117 μm (after treatment). Scale bar = 100 μm.

DISCUSSION

The PDL produces reasonably good clinical results in patients due to its ability to destroy superficial PWS blood vessels (<300 μm below skin surface) [11–17]. Currently, the vast majority of PWS patients receive a large number (>20) of treatments to achieve reasonably good clearance [18–20]. However, complete clearance occurs in <10% of patients treated [18–20]. PDLs cannot achieve the critical core temperature necessary to irreversibly destroy blood vessels seated at deeper locations (>300 μm). Furthermore, epidermal melanin, residing over the abnormal plexus of blood vessels, has relatively strong optical absorption at the 585–595 nm treatment wavelengths. Therefore, a large fraction of light intended to reach and destroy the targeted PWS blood vessels is absorbed by melanin, which prevents treatment of individuals with moderate-heavy pigmentation. In this study, the multiplex 585/1,064 nm laser was used to increase the optical penetration depth to reach deeper blood vessels as well as reduce light absorption by epidermal melanin. However, 44.4% of extremity PWS and 15.2% of facial PWS were resistant to laser treatment, suggesting that these blood vessels need tailored laser treatment protocols based on their morphological features. For example, a higher laser dosage can be considered for those laser-resistant facial PWS lesions with larger diameters of blood vessels.

There are many factors contributing to laser-resistant PWS, including lesion thickness, anatomical location, size, and patient age [23]. The morphological aspects of PWS blood vessels, namely depth (>400 μm), diameter (<20 μm), and wall thickness are among the factors that significantly impact treatment outcomes [24]. In the current study, we quantitatively evaluated the morphological features of PWS blood vessels *in vivo* by RCM. We found that PWS blood vessel flow, diameter, and depth, but not density, are the key factors contributing to the laser-resistance. In our study, we showed that the average diameter of laser-responsive facial PWS blood vessels decreased from 84.36 to 49.04 μm after laser treatment, suggesting that the remaining PWS blood vessels (diameters < 49.04 μm) were more resistant to laser treatment. Furthermore, we found that many PWS blood vessels with larger diameters were also resistant to laser treatments (Figs. 3 and 6), suggesting that molecular phenotypes rather than morphological features are possibly underlying the laser-resistant mechanisms for those blood vessels. We recently characterized a spectrum of pathological and molecular phenotypes of PWS blood vessels (i) the alterations of entire physiological milieu of human skin, including ECs, smooth muscle cells and extracellular matrix, in infantile PWS [25]; (ii) the activation of the mitogen-activated protein kinases, PKCα, and phosphoinositide 3-kinase in PWS blood vessels [26,27]; (iii) upregulation of membrane trafficking and exocytosis of PWS ECs [28]; and (iv) expression of surface markers of EphB1/EphrinB2/CD133/CD166 on PWS ECs [1]. These are the first steps toward uncovering the molecular pathogenesis of PWS, which are likely associated with laser-resistant PWS phenotypes.

Recently, PDL in combination with anti-angiogenic agents has been evaluated for treatment for laser-resistant lesions and PWS recurrence. Molecularly, laser-induced local hypoxia leads to upregulation of hypoxia-inducible factor 1α and vascular endothelial growth factor [29–31], causing activation of angiogenesis pathways including phosphorylation of the mammalian target of rapamycin and p70S6 kinase. As a result, more angiogenic genes are transcribed and translated, leading to reformation, and reperfusion of blood vessels. We attempted to block this pathway using angiogenic inhibitors, such as rapamycin and axitinib [32–35], post-PDL treatment. In rodent skin, both topical rapamycin and axitinib showed effective inhibition of the early stages of angiogenesis induced by PDL, but blockage of the late

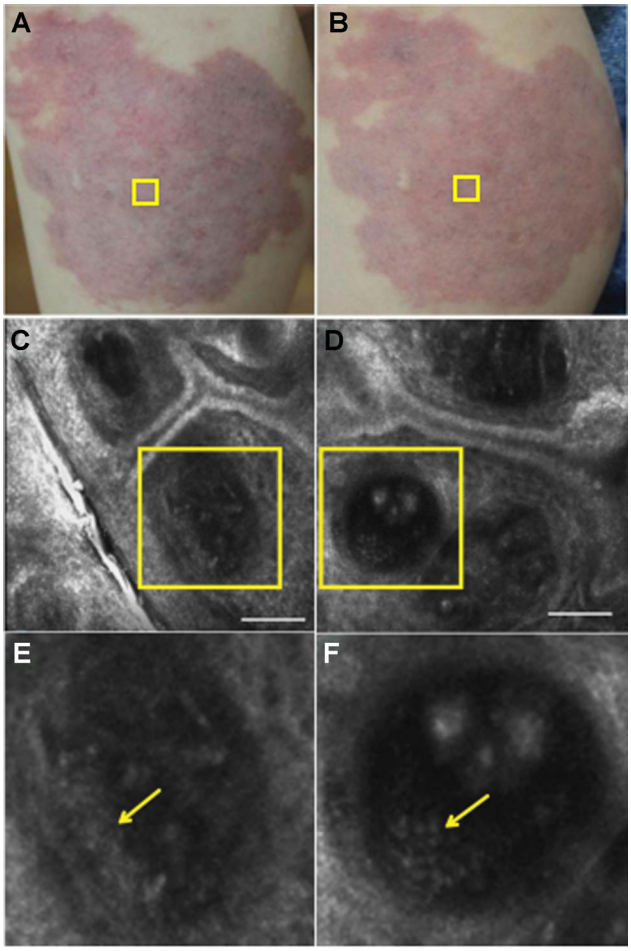

Fig. 5. A representative image of a female child (age 4) with laser-resistant port wine stain (PWS) on her left leg before (**A**) and after (**B**) multiplex 585/1,064 nm laser treatment. The reflectance confocal microscopy (RCM) images were taken from the same lesional area before (**C**) and after (**D**) treatment. (**E**) and (**F**) are higher magnifications from the boxed blood vessels in (**C**) and (**D**), respectively. The PWS blood cells were indicated by yellow arrows. The depths of imaging planes were 115 (before) and 110 μm (after treatment), respectively. The diameters of the highlighted blood vessels were 248 (before) and 227 μm (after treatment). Scale bar = 100 μm.

stages of angiogenesis was ineffective [34,35]. These results may explain why rapamycin improves PWS lesion blanching in a limited subpopulation (~10%) of patients [36,37]. However, there are still many gaps that need to be filled before this approach can be successfully translated to patients.

RCM is a non-invasive optical imaging system that enables visualization of cytomorphological features of skin over time [21,38]. Facial lesions, such as PWS, are general challenges for biopsies due to the cosmetically sensitive nature of the area. RCM offers unique advantages for recording the dynamics of the PWS microcirculation including blood vessel density, size, location, and flow. However, one major limitation of RCM is imaging depth. The RCM device (VivaScope) employed in this study uses 830 nm light, which can penetrate to a maximal depth of 250–300 μm in the human skin. We found that imaging quality significantly decreased when the penetration depth was deeper than 150 μm during measurements. Therefore, the maximal scanning depth of RCM used in this study was about 150 μm to ensure that blood vessel structures could be visualized and captured with sufficient resolution. We were aware that we only captured a small fraction of PWS blood vessels, which were located in the upper papillary dermis. A large fraction of PWS blood vessels in the reticular dermis were not imaged due to technical limitations. Therefore, the data in the current study cannot be considered to present the totality of PWS blood vessels in the skin. Another limitation of this study is that it is very challenging to image the same blood vessels before and after treatment using RCM. Furthermore, PWS lesions show blood vessel progressive dilations and soft tissue hypotrophy during aging, which become more resistant to laser treatment. In the current study, we were unable to differentiate and compare the morphological features of laser treatment-resistant vasculatures in older patients from young ones due to the limited sample sizes. We will attempt to increase sample sizes and address this issue in the future study.

Another limitation of this study is the semi-quantification approach for blood flow imaging using RCM. Altintas et al. [39,40] pioneered methods to measure the quantitative blood cell flow using RCM. Subsequently, Cinotti et al. [22] compared three methods to calculate the blood cells from RCM images: manual count, the number of peak luminous intensity of blood vessel areas, and the average intensity of the blood vessel areas. They found that evaluation of the intensity peaks had good intracapillary reproducibility and was the most reliable approach. In the current study, we compared the changes in frequency of intensity peaks of PWS blood vessel areas from RCM videos over time, which revealed an index of the speed of movements of red blood cells overall. However, we do not know if each peak corresponds to a single blood cell (Fig. 1F) or to a cluster of cells (Fig. 1E). Further studies should be performed using real-time interpretation of RCM videos by software programs as well as incorporation of the average intensity of blood vessel areas, which may comprehensively present the index of red blood cell numbers.

Other non-invasive imaging systems that have been used or currently under development for evaluation of dermal vasculatures include laser Doppler flowmeter, laser Doppler imaging, dermoscopy, cross-polarized diffuse reflection color imaging system, and optical coherence tomography [39–48]. The main feature of RCM is that it can directly reveal the dermal structure in a simple and fast way, which makes it be a promising bedside tool for prognostic evaluation. A dermatopathologist with a special training of recognizing featured skin structures under RCM will be required to operate RCM. RCM in general only detect a small size of lesions (4 mm^2) at one time in just a few minutes, but not the entire PWS lesion. One big limitation of RCM in the evaluation of vascular

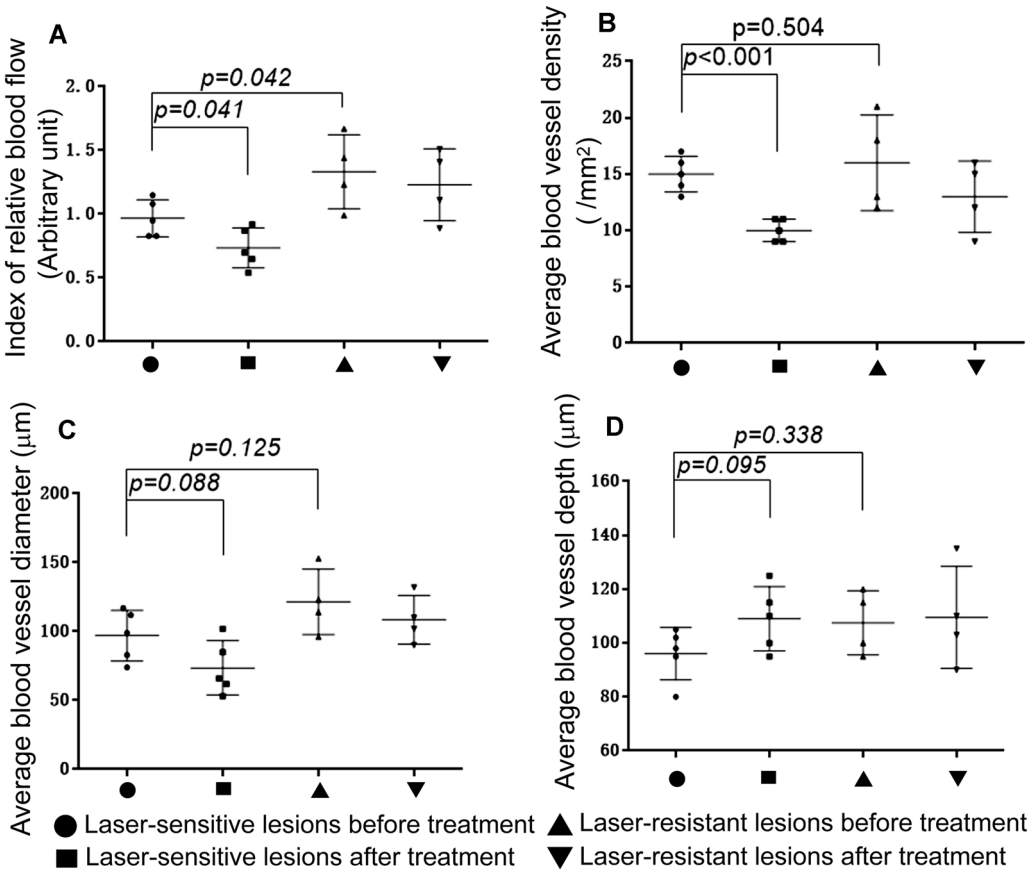

Fig. 6. Quantitative analysis of relative blood flow (**A**), average blood vessel density (**B**), diameter (**C**), and depth (**D**) in extremity port wine stain (PWS) lesions before and after multiplex 585/1,064 nm laser treatment.

malformations is the depth of blood vessels, which can be overcomed by the Doppler ultrasound. However, it is also challenging to evaluate the blood flow of small blood vessels in some patients using the Doppler ultrasound based on our experience. It will be worthy of investigating the potential applications in a combination of RCM and Doppler ultrasound in our future research. Furthermore, the current data are only based 585/1,064 dual wavelength-resistant PWS lesions. We are aware that a single wavelength laser treatment (such as 585 or 755 nm) resistant PWS blood vessels may have different morphological features, therefore can affect our conclusions.

In conclusion, we quantitatively characterized the general morphological features of laser-resistant PWS blood vessels, for example, blood flow, density, diameter, and depth, using *in vivo* RCM in this study. Our results have shown that laser-resistant facial PWS blood vessels had significantly higher blood flow, larger diameters, and were located deeper in the skin when compared with responsive PWS on the face. Our data also suggested that RCM can be a valuable tool for a prognostic evaluation on laser-resistant lesions before treatment, thereby providing guidance for tailored laser treatment protocols, which may improve the therapeutic outcome.

TABLE 2. Characteristics of PWS Blood Vessels in the Facial Lesions When Compared With Extremity Lesions

Lesion groups	Relative blood flow (arbitrary unit)	Blood vessel density (/mm^2)	Blood vessel diameter (μm)	Blood vessel depth (μm)
Facial PWS (n = 33)	0.96 ± 0.28	16.67 ± 4.40	88.18 ± 27.77	90.58 ± 14.18
Extremity PWS (n = 9)	1.13 ± 0.28	15.44 ± 2.88	107.89 ± 23.42	101.11 ± 11.71
P value	0.115	0.437	0.038	0.047

PWS, port wine stain.

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