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ORIGINAL ARTICLE

Confocal Microscopy Study of Neurovascular Distribution in Facial Port Wine Stains (Capillary Malformation)

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Background/Purpose: Vascular ectasia observed in port wine stain (PWS) birthmarks might be secondary to localized reduction of neural innervation and associated loss of autonomic stimulation. Our objective was to investigate this theory and evaluate nerve density, blood vessel density, and average blood vessel size in untreated and pulsed dye laser with cryogen spray cooling (PDL-CSC) treated PWS skin.

Methods: Biopsy skin specimens were taken from 14 adults with a PWS, categorized by: uninvolved skin; untreated PWS skin; PWS skin with a history of good blanching, and PWS skin with a history of poor blanching both in response to PDL treatment. Seven specimens of normal, unaffected skin were used as the experimental control group. Indirect immunohistochemistry was performed on all specimens followed by confocal microscopy imaging with computer analysis to determine nerve density, blood vessel density, and average blood vessel size.

Results: Nerve density was significantly decreased in all PWS sites as compared to uninvolved skin ($p < 0.01$). Average blood vessel diameter was larger in untreated as compared to treated PWS sites and varied between different sites within a single PWS.

Conclusion: Nerve density was decreased in all evaluated PWS sites, and this may be a factor in lesion pathogenesis. PWS blood vessel size correlated with the PDL blanching response and may prove to be a useful prognostic indicator of therapeutic outcome. [*J Formos Med Assoc* 2008;107(7):559–566]

Key Words: blood vessels, confocal microscopy, nerve supply, port wine stain, pulsed dye lasers

Port wine stains (PWS) are congenital vascular malformations characterized by multiple dilated vessels in the dermis which become larger or more ectatic with age. These lesions, which are benign, disfiguring cutaneous vascular birthmarks, occur with an incidence of 0.3% in the population.[1–4] As two-thirds of these malformations occur on the face, PWS are a clinically significant problem. PWS should not only be considered a cosmetic problem *per se*, but a disease with potentially devastating psychological and physical complications. Personality development is adversely influenced in virtually all patients by the negative reaction of others to a "marked" person.[5–7] In the initial stages, most PWS appear as faint, pink macules, but the lesions tend to darken progressively, displaying a reddish-purplish nature.[8] The subsequent hypertrophy of underlying bone and soft tissue further disfigures the facial appearance of many patients. Histopathologic studies of PWS show a normal epidermis overlying an abnormal plexus of subsurface blood vessels located in the upper dermis. Vascular ectasia occurs in the most superficial 800 μm of the skin and involves mature

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dermal vessels that can be identified using immunohistochemistry.[9]

Previous studies proposed that the pathogenesis of PWS is related to a reduction in neural innervation around the ectatic blood vessels.[6–10] The neural defect is likely to be autonomic in nature, as there have not been cases of sensory loss experienced by the patient affected by PWS. Blood flow in the absence of tonic modulation is thought to produce PWS vascular ectasia. Consistent with this theory, ectasia is progressive with age, resulting in lesion darkening from pink to purple hues.[2]

The development of lasers, namely their ability to damage PWS blood vessels selectively, have undoubtedly offered a promising treatment option. The pulsed dye laser (PDL)[11–14] along with the concept of selective photothermolysis[15,16] significantly improved PWS treatment. Yellow light ($\lambda = 585–595$ nm) emitted by the PDL is preferentially absorbed by hemoglobin (the major chromophore in blood) in the PWS vessels and, after being converted to heat, causes thermal damage and thrombosis. The PDL produces reasonably good results in a select population of PWS patients due to its ability to selectively destroy cutaneous blood vessels.[17–20] However, very few patients achieve complete PWS blanching despite receiving multiple laser treatments.[21–24] Furthermore, while there are some factors (including anatomic location, size, color, nodularity, age) that appear to influence treatment response, none of these have been accepted as a reliable prognostic indicator of therapeutic outcome to date.[18]

The purpose of our study was to compare average nerve density, blood vessel density, and average blood vessel size in uninvolved versus involved (lesion) skin from PWS patients to investigate the proposed role of neural innervation. These parameters were also compared between untreated and treated PWS sites.

Methods

Twenty-one male subjects, aged from 18 to 58 years (mean age, 25.6 years), were included in this study (IRB:96-0615C). Fourteen subjects had PWS lesions on the face and had all received three single PDL-CSC (cryogen spray cooling) treatments, thus leaving the remaining seven patients with normal skin to serve as the study control group. The parameters of PDL-CSC, ScleroPLUS® (Candela, Wayland, MA, USA) PDL were as follows: 585 nm wavelength, spot size 7 mm, pulse duration (τ_p) 1500 μs, and energy densities ranging from 7 to 10 J/cm^2. Cryogen spurt duration (30 ms) and delay between cryogen delivery and laser irradiation (20 ms) were controlled with a programmable digital delay generator. PDL-CSC treatments were administered at three intermittent intervals of 6–8 weeks, for a total of 6 months. The first treatment of the series was given to 14 patients and there was a 6–8 week interval before the second treatment was undertaken. After the second and third applications of the PDL-CSC treatment, recording of the laser response was taken after another 6–8 weeks. Seven average, healthy volunteers were used as the experimental control group. Skin biopsy specimens (3 mm diameter punches) were taken under local anesthesia (2% xylocaine) from the following sites: normal skin (7 patients, 14 specimens in total to use as a control); uninvolved skin (14 patients, 14 specimens in total); pretreated PWS skin with good blanching response (7 patients, 14 specimens); pretreated PWS skin with a poor blanching response (7 patients, 14 specimens); posttreatment PWS skin with a good blanching response (7 patients, 14 specimens); and posttreatment PWS skin with a poor blanching response (7 patients, 14 specimens). Based on comparisons between the pre- and posttreatment index of their hemoglobin, each patient's PWS was assigned a blanching response of "poor" when the index was < 25%. The DermaSpectrometer (Cortex Technology, Smedevaenget 10, 9560 Hadsund, Denmark)[25] performed calculations of the index of hemoglobin and concurred that 14 PWS specimens had physical signs of a good blanching response (index of hemoglobin > 25%).

Specimens were fixed and sectioned, and immunohistochemical staining for pan-neuronal and pan-endothelial markers was performed according

to the compulsory protocol. Specimens were immediately fixed in cold (4°C) Zamboni's fixative[24] and left overnight. The next day, specimens were transferred to 20% sucrose phosphate-buffered saline (PBS) and refrigerated for 24 hours (or until processing). Thick (100 μm) sections were cut with a frozen, sliding microtome (American Optical Co., Buffalo, NY, USA), placed in spot plates (petri dishes), and flooded with 0.05 mol/L PBS (pH 7.4) containing 0.3% Triton-X-100 (PBS/TX) (Sigma Chemical Co., St. Louis, MO, USA) and 5% normal donkey serum (NDS) as a blocking serum for 1 hour to avoid nonspecific background staining. Subsequently, the floating sections were incubated for 16 hours at 4°C on a rotating table with the following primary antibodies: (a) pan-neuronal marker for nerves, protein gene product 9.5 (PGP 9.5)[26] (Ultraclone, Wellow, UK) at a working dilution of 1:1000; and (b) pan-endothelial marker CD31 (Dako Corp., Carpinteria, CA, USA)[27] at a working dilution of 1:80.

Primary antibodies were visualized with cyanine 2 or cyanine 3.18 fluorophores conjugated to the appropriate mouse or rabbit secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). For secondary antibodies, floating sections were incubated overnight at 4°C on a rotating table and then washed with 1% NDS in PBS/TX for four successive incubations. Washed sections were mounted without drying on coverslips, dehydrated in alcohol, cleared with methyl salicylate (Fisher Scientific, Pittsburgh, PA, USA), and mounted in DPX (a mixture of distyrene, a plasticizer, and xylene) (Fluka, Ronkonkoma, NY, USA) for microscopy.

Specimens were initially examined with a Nikon (Melville, NY, USA) epifluorescence microscope. The sections were then imaged with an MRC-1000 scanning confocal microscope system equipped with a krypton/argon ion laser (Bio-Rad Life Science, Hercules, CA, USA). A series of optical sections (20× magnification) were acquired at 2 μm intervals through the entire thickness of the specimen (approximately 60 μm) and then projected into a single-focus image with the software supplied (Confocal Assistant 4.02, a free 2D and 3D animation software created and copyrighted by Todd Clark Brelje).

The densities of nerves and blood vessels ($\mu m^3/\mu m^3$ tissue) were quantified for all samples using the three-dimensional software Image3 LLC Velocity^2 Pro (Salt Lake City, UT, USA). This software uses the digital images acquired by the confocal microscope to create a three-dimensional model of the nerves and blood vessels, which can then be used to calculate density values. Measurements of blood vessel diameters were made for all samples using the public domain NIH Image program (U.S. National Institutes of Health, available on the Internet at: http://rsb.info.nih.gov/nih-image/). Measurements were taken of the diameter of the dermal vessels (superficial or deep) in four random, 20× magnification fields per skin biopsy sample.

Nerve density and blood vessel density of uninvolved skin of PWS subjects were compared to that of normal healthy skin in our controls along with untreated PWS facial sites (Table). Then following PDL-CSC treatment of these groups, these calculations were further compared between the PWS skin specimens with a history of good blanching in response to PDL treatments, along with those that had a history of poor blanching response to PDL treatments. Once determination of blanching response was recorded, specimens from skin with a good blanching history and with a poor blanching history were further analyzed for consistency in the PDL-CSC response.[14,24,28] Statistical analysis was performed and recorded after the third intermittent interval using a two-sample Student's *t* test by means of the computer software Stat View® version 5.0.1. (SAS Institute Inc., Cary, NC, USA).

Results

Papillary and reticular dermal blood vessels were included for calculation of average vessel diameters (Table). Uninvolved skin from PWS patients had average nerve and blood vessel densities of $18.51 \pm 1.02 \times 10^{-3}\ \mu m^3/\mu m^3$ tissue and

Table. Blood vessel and nerve density findings*

Biopsy group/skin categorizations	Nerve density (10^{-3} μm^3/μm^3 tissue)	Blood vessel density (10^{-3} μm^3/μm^3 tissue)	Blood vessel diameter (μm)
Control group w/normal healthy skin ($n=14$)	11.87 ± 0.72	6.39 ± 0.31	17.33 ± 0.98 (7.82 ± 0.41 to 30.01 ± 1.52)
Uninvolved skin of PWS patients ($n=14$)	18.51 ± 1.02	6.74 ± 0.35	15.53 ± 0.94 (6.38 ± 0.30 to 28.91 ± 1.43)
Pretreated PWS: blanching response			
Good ($n=14$)	0.81 ± 0.36†	22.37 ± 1.36†	57.85 ± 2.15 (8.65 ± 0.53 to 189.58 ± 6.14)
Poor ($n=14$)	0.65 ± 0.02†	24.71 ± 1.52	87.72 ± 3.21 (28.23 ± 1.31 to 227.81 ± 13.26)
Posttreatment PWS: blanching response			
Good ($n=14$)	1.92 ± 0.06†	6.93 ± 0.37	28.53 ± 1.56 (8.67 ± 0.55 to 81.35 ± 3.17)
Poor ($n=14$)	0.38 ± 0.01†	31.94 ± 1.96†	49.37 ± 2.03 (26.93 ± 1.17 to 283.05 ± 18.03)

**Data presented as mean ± standard deviation or mean ± standard deviation (range); †$p < 0.01$ vs. uninvolved skin.*

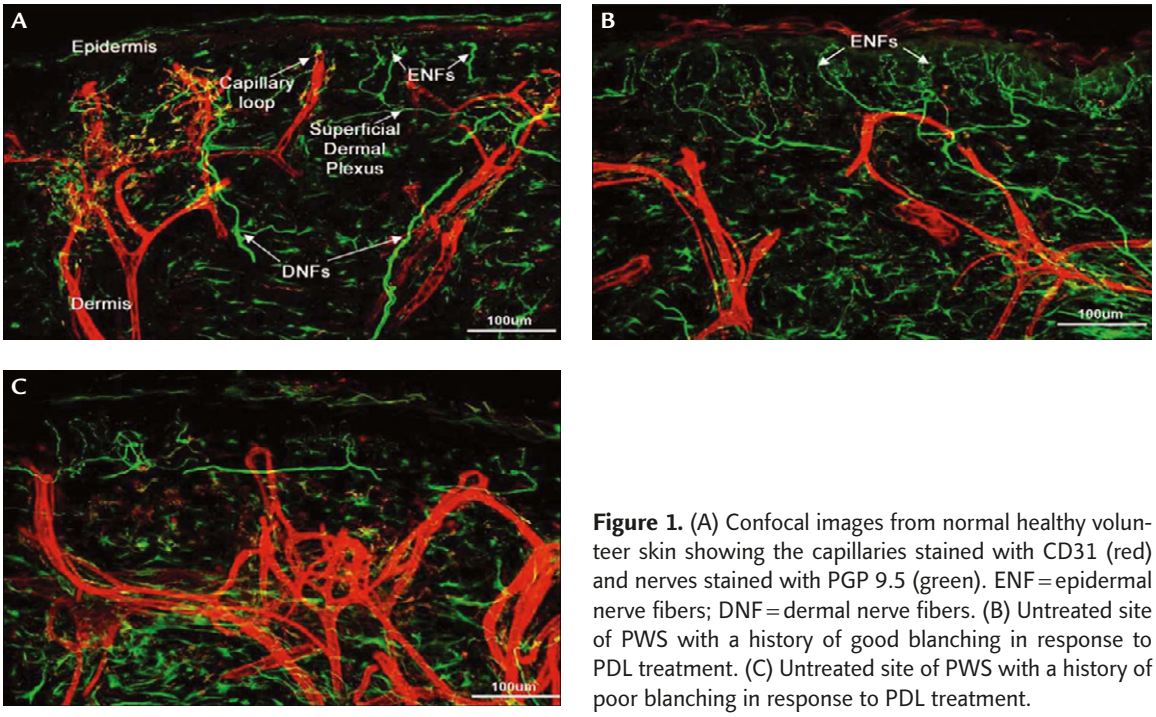

Figure 1. (A) Confocal images from normal healthy volunteer skin showing the capillaries stained with CD31 (red) and nerves stained with PGP 9.5 (green). ENF = epidermal nerve fibers; DNF = dermal nerve fibers. (B) Untreated site of PWS with a history of good blanching in response to PDL treatment. (C) Untreated site of PWS with a history of poor blanching in response to PDL treatment.

6.74 ± 0.35 × 10^{-3} μm^3/μm^3, respectively. These values were not statistically different from samples of the control group (normal skin) where average nerve and blood vessel densities were 11.87 ± 0.72 × 10^{-3} μm^3/μm^3 and 6.39 ± 0.31 × 10^{-3} μm^3/μm^3 respectively. The average blood vessel diameter in uninvolved skin from PWS patients was 15.53 ± 0.94 μm with a range of 6.38 ± 0.30 to 28.91 ± 1.43 μm (Table; Figures 1A and 1B). Nerve density was significantly decreased (range, 0.38–1.92 × 10^{-3} μm^3/μm^3) in all PWS sites as compared to the net results observed in uninvolved skin specimens ($p < 0.01$). Blood vessel density was variable as described below. Average blood vessel diameter was greater in all PWS sites as compared to that of uninvolved skin. Histologic

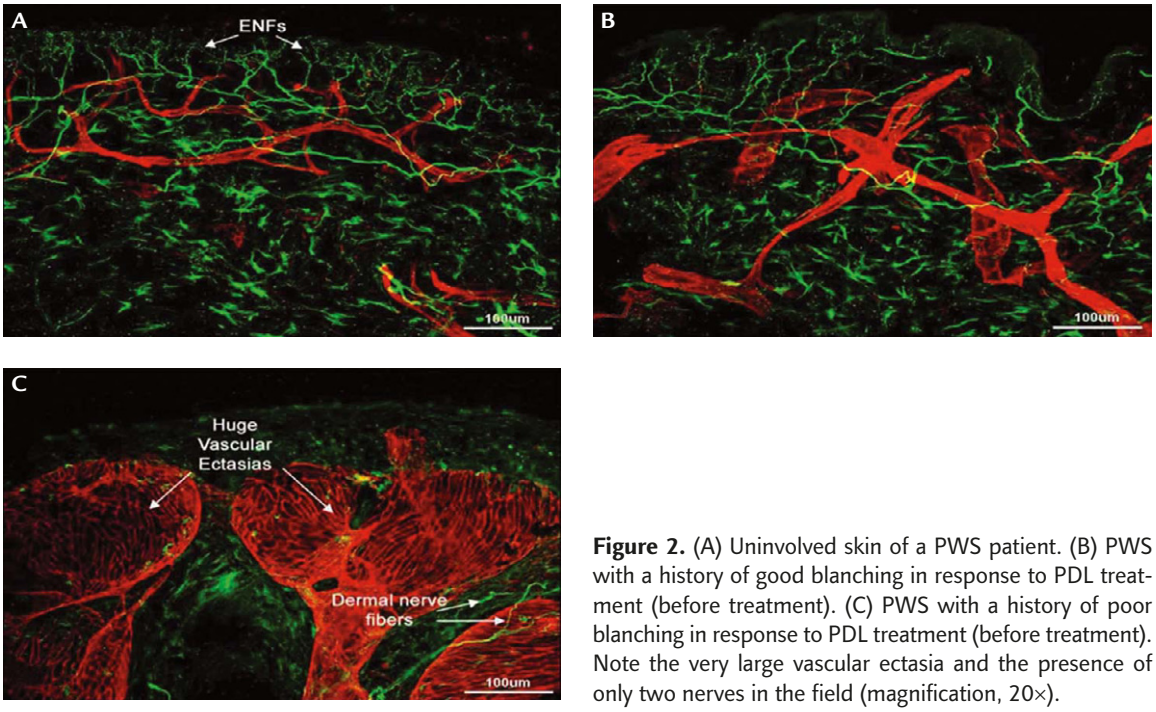

Figure 2. (A) Uninvolved skin of a PWS patient. (B) PWS with a history of good blanching in response to PDL treatment (before treatment). (C) PWS with a history of poor blanching in response to PDL treatment (before treatment). Note the very large vascular ectasia and the presence of only two nerves in the field (magnification, 20×).

examination of pretreatment PWS sites of patients revealed a decrease in nerve density as compared to uninvolved skin from the same patient. Average nerve density in the pretreated sites of the poor blanching response lesions was $0.65 \pm 0.02 \times 10^{-3}\ \mu m^3/\mu m^3$. Blood vessel density was increased in pretreated PWS specimens to $24.71 \pm 1.52 \times 10^{-3}\ \mu m^3/\mu m^3$. Average vessel diameter was increased in pretreated PWS specimens with poor blanching response and had an average vessel diameter of $87.72 \pm 3.21\ \mu m$.

The DermaSpectrometer was used on the patients to determine the extent of blanching response, and, based on comparisons between the pre- and posttreatment index of their hemoglobin, each patient's PWS was assigned a blanching response, with "poor" when the index was < 25%. The instrument took calculations of the index of posttreatment hemoglobin and concurred that 14 PWS specimens had physical signs of a good blanching response (index of hemoglobin > 25%), due to a decrease in the average nerve density of $1.92 \pm 0.06 \times 10^{-3}\ \mu m^3/\mu m^3$ ($p < 0.01$) while there was a relatively normal blood vessel density (mean, $6.93 \pm 0.37 \times 10^{-3}\ \mu m^3/\mu m^3$) as compared to that of the uninvolved skin (Table; Figures 2A–C and 3A–C). Blood vessel diameter was relatively normal as compared to that in uninvolved skin (Table). The range of blood vessel diameters for these patients was 8.67 ± 0.55 to $81.35 \pm 3.17\ \mu m$ (mean, $28.53 \pm 1.56\ \mu m$). In all cases, use of PDL-CSC treatment was administered on each patient a total of three times to determine the effects of the laser on the PWS affected area.

Of the above mentioned 14 patients, the DermoSpectrometer further concluded that seven patients showed physical signs of poor blanching in response to PDL-CSC treatment. These seven patients had experienced a decrease in average nerve density of $0.38 \pm 0.01 \times 10^{-3}\ \mu m^3/\mu m^3$ ($p < 0.01$) and increased average blood vessel density of $31.94 \pm 1.96 \times 10^{-3}\ \mu m^3/\mu m^3$ ($p < 0.01$) as compared to the skin of uninvolved subjects (Table). Blood vessels were also enlarged as compared to uninvolved skin samples (Table; Figure 1). The range of blood vessel diameters was 26.93 ± 1.17 to $283.05 \pm 18.03\ \mu m$ (mean, $49.37 \pm 2.03\ \mu m$). Again, in all cases, use of PDL-CSC treatment was administered to each patient a total of three times over the course of about 6 months to determine the effects of the laser on the PWS affected area.

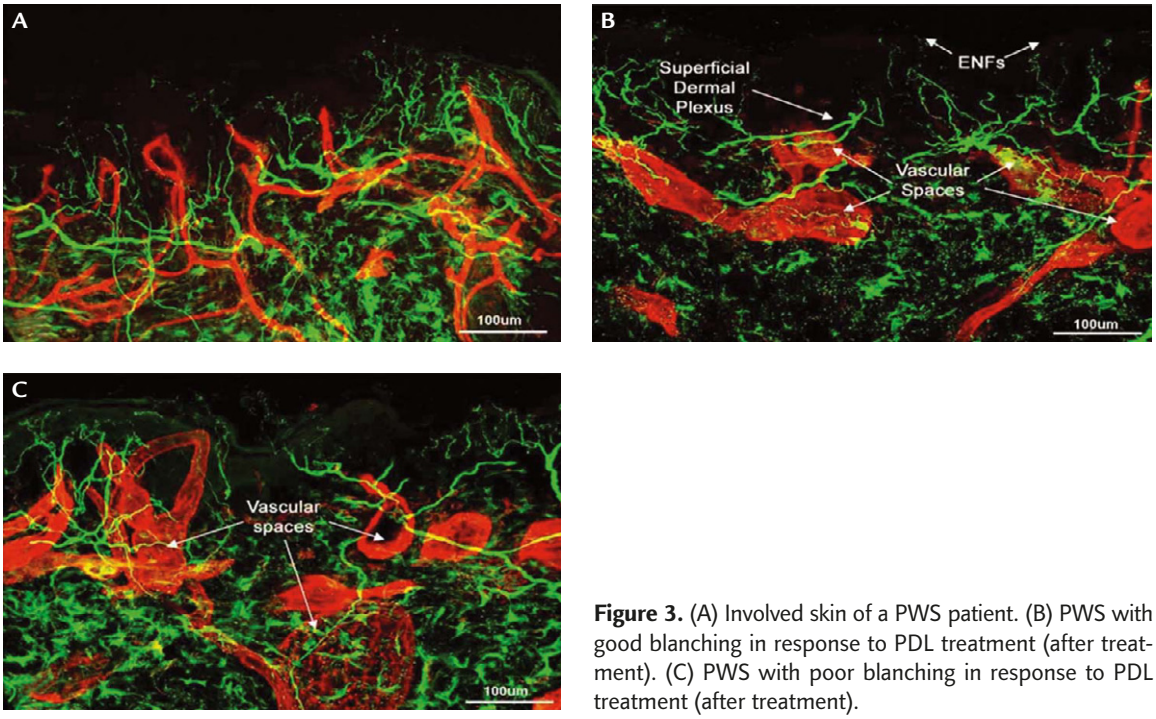

Figure 3. (A) Involved skin of a PWS patient. (B) PWS with good blanching in response to PDL treatment (after treatment). (C) PWS with poor blanching in response to PDL treatment (after treatment).

Discussion

PWS are progressive vascular lesions composed of ectatic dermal capillaries.[2] As expected, our data found the average blood vessel diameter to be larger in all PWS sites as compared to those of uninvolved skin. The pathogenesis of this vascular dilatation is unknown, but previous studies have proposed a reduction in neural innervation in areas of skin with PWS involvement.[5–7] Our confocal images confirmed a significant decrease in nerve density from samples of both pretreated and posttreated ($p < 0.01$) PWS skin compared to uninvolved skin. Our findings add support to the theory that PWS vascular ectasia may result from decreased neural innervation. From the tabulated results, it can be seen that as nerve density decreased the blood vessel diameter increased (Table). However, other possible explanations cannot be ruled out including the influence of a localized third factor (e.g. cytokine), which could form during fetal development, promoting both vessel dilatation and inhibition of nerve development. It is interesting to note the proposed role for decreased nerve density in lesion pathogenesis may be unique to PWS as compared to other vascular lesions. Different mechanisms have been proposed for the development of other vascular entities such as hemangiomas.[29] Further study is required.

Although the numbers of PDL-CSC treatments were the same, PWS of the two laser-treated groups (good blanching versus poor blanching) had contrasting results. The correlation between posttreatment blood vessel size and therapeutic outcome was consistent. Average vessel diameter was larger in areas with a history of poor blanching in response to PDL-CSC treatment as compared to areas with a good blanching response. This reaction may be due to the excessively large vessels requiring prolonged pulsed duration, posing difficulties in accurate photocoagulation. This relationship may be useful from a predictive standpoint as noninvasive imaging of PWS and determination of average blood vessel diameter prior to treatment may be used as a prognostic indicator of therapeutic outcome. Patient age and a number of PWS characteristics such as size, color and anatomic location have been considered as prognostic indicators of PDL-CSC therapy. However, to date, none of these variables have been accepted as a reliable indicator of therapeutic outcome.

Our data also revealed a trend towards larger vessels in pretreated versus posttreated PWS sites. As there were no pretreatment biopsies, we were unable to determine from this study if the decrease in average vessel size in treated PWS was a result of laser therapy. However, we believe this to be a likely explanation, which should be explored in future prospective studies.

In facial PWS, average vessel diameter varied depending on the location and size of the affected area. Variations in vessel size within the PWS have important implications for laser treatment. Currently, medical specialists most often treat an entire PWS lesion with identical laser parameters such as wavelength, pulse duration, energy density, and CSC (with use of spurt duration application).[30] However, PWS blood vessel size and depth affect the optical absorption and scattering properties of the skin and, if these factors vary, laser parameter adjustments for different areas within a lesion is likely to be beneficial. Diagnostic imaging methods for PWS characterization such as infrared tomography and optical Doppler tomography are being evaluated as a means of rapidly and noninvasively determining blood vessel size and depth to optimize laser treatment parameters on a site-to-site basis.[31–33]

In conclusion, nerve density was decreased in all evaluated PWS sites, and this may be a factor in lesion pathogenesis. PWS blood vessel size correlated with the blanching response of PDL treatment and may prove to be a useful prognostic indicator of therapeutic outcome. Our experimental results will be confirmed in a larger study in which we will follow serial biopsies (beginning at pretreatment) from multiple PWS sites obtained from both the same and different individuals.

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