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SHORT REPORT

# Histological and Immunofluorescent Analysis of a Large Tributary of the Great Saphenous Vein Treated with a 1920 nm Endovenous Laser: Preliminary Findings☆

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**Objectives:** To analyse the biological effects of a 1920 nm endovenous laser (EVL) on extra-fascial great saphenous vein (GSV) *in vitro*.
**Methods:** A 10 cm length of a large tributary bypassing a hypoplastic segment of the GSV (sometimes called an "extra-fascial GSV") was obtained during routine varicose vein surgery. The length was treated in five sections with different LEEDs (0 (control), 20, 40, 60, and 80 J/cm) with a 1920 nm EVL at 4W power, in a novel *in vitro* treatment model. The biological effects were assessed by histological staining of the samples for haematoxylin and eosin (HE) and Martius Scarlet Blue (MSB), and by immunofluorescent detection of p-p53 and VCAM-1.
**Results:** Histological analysis showed significant structural damage at LEEDs above 60 J/cm, especially in the intima and media, with the treatment at 80 J/cm causing perforation of the vein wall. In addition, there was a significant increase in p-p53 expression in treated tissue at 60 and 80 J/cm.
**Conclusions:** Using this *ex vivo* model, the results indicate that *in vitro* treatment with a 1920 nm EVL, at or above an LEED of 60 J/cm and 4 W power, causes significant vein wall cell death reaching deep into the media by a combination of direct thermal damage and apoptosis. A wavelength of 1920 nm appears to be effective for the endovenous ablation of truncal veins.

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## OBJECTIVES

Over the last two decades varicose vein surgery has progressed from invasive open surgical techniques to minimally invasive endovenous surgery performed under duplex ultrasound guidance. Radiofrequency ablation (RFA) was the first successful percutaneous endovenous thermal ablation technique to be administered for truncal venous reflux. More recently, endovenous lasers (EVL) have been used in EVL ablation (EVLA), targeting haemoglobin and more recently water as the chromophore for the laser energy.

There have been many clinical studies looking at the success rates of EVLA relative to open surgery. Research has negatively favoured the latter, with reports of revascularisation, neovascularisation, and, consequently, high rates of varicose vein recurrence.[1] By comparison, the post-operative pain, scarring, bruising, and overall recovery time has been reported to be significantly lower with RFA and EVLA.[2] However, a recent meta-analysis looking at the results of treatment of the great saphenous vein (GSV) 5 years after open surgery, EVLA, and ultrasound foam sclerotherapy, has shown that these advantages of EVLA over open surgery appear to be early, and outcomes at 5 years appear to be similar.[3] Thus, endovenous thermal ablation has been the recommended treatment for truncal venous reflux by the National Institute of Health and Care Excellence[4] since 2013 and by the European Society for Vascular Surgery guidelines in 2015.[5]

Although the exact biological mechanism of thermal in induced venous occlusion has not yet been demonstrated, it has been proposed that transmural vein wall cell death is essential for fibrotic occlusion after treatment. Following recent findings regarding sclerotherapy, apoptosis has been proposed as a cell death mechanism after thermal ablation.[6] The objective of this study was to analyse the biological effects of a 1920 nm EVL on an *ex vivo* large tributary bypassing a hypoplastic segment of the GSV, often called an

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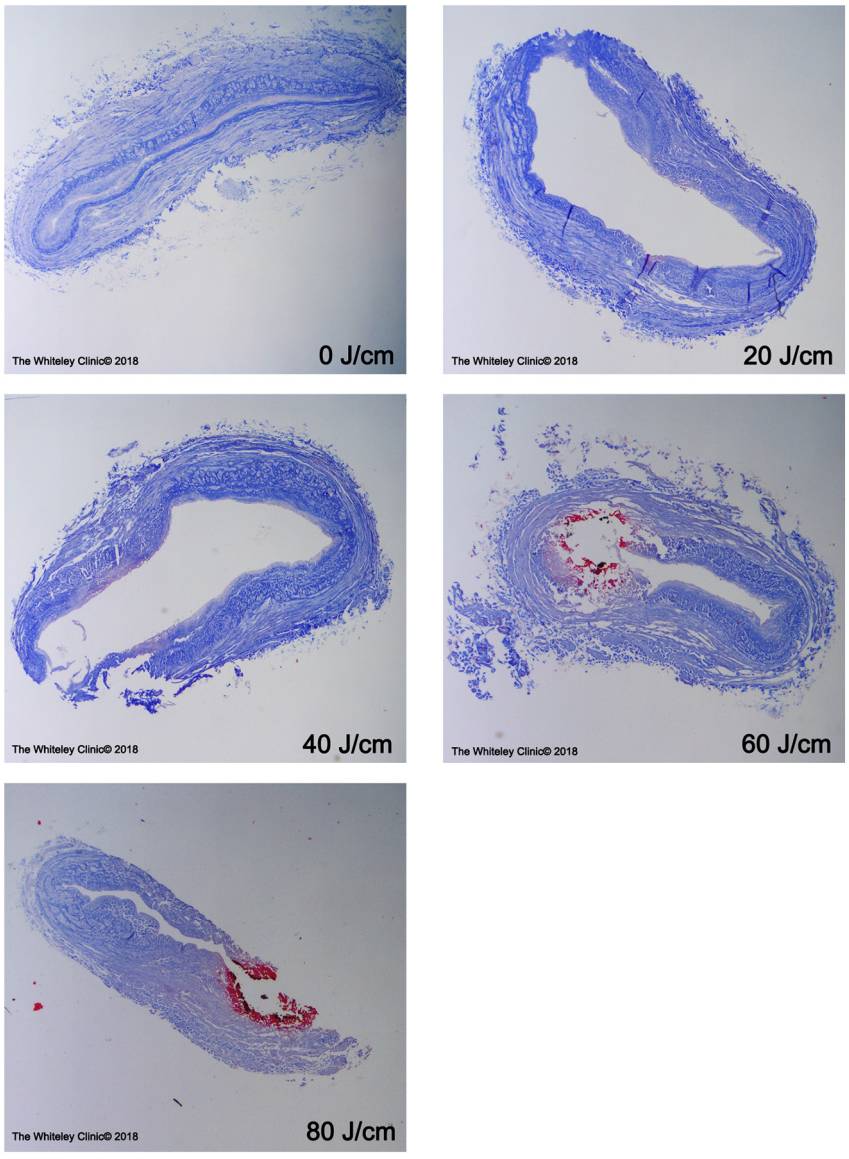


**Figure 1.** Histological analysis of a large extra-fascial tributary bypassing a hypoplastic segment of the great saphenous vein (GSV) treated with varying LEEDs by a 1920 nm endovenous laser. Stained with Martius Scarlet Blue. Connective tissue appears blue and fibrin red. Pictures taken at ×40 magnification.

"extra-fascial GSV," treated *in vitro*, by histology and immunofluorescence.

## MATERIALS AND METHODS

A 10 cm length of a large tributary of the GSV was obtained from a patient undergoing routine varicose vein surgery. After extraction, the length was immediately placed in a Petri dish containing phosphate buffered saline (PBS). The vein length was split into five equal sections. A jacket tipped laser fibre (NeverTouch Direct, AngioDynamics, Albany, NY, USA), attached to a 1920 nm EVL (AngioDynamics, Albany, NY, USA) was inserted into the open end of each section, and passed up the length of vein. One section was untreated, to act as a control, and the other four were treated with the 1920 nm EVL at 4W, with four different pull back speeds; 5, 10, 15, and 20 s/cm. Thus, the five sections taken from the length of vein were treated with five different linear endovenous energy densities (LEEDs): 0, 20, 40, 60, and 80 J/cm. After treatment, the sections were immediately halved.

One half was immediately immersed in 10% buffered formalin and fixed for 24 hours. Following fixation, the tissue samples were placed into an automatic tissue processor (Shandon Citadel, Thermo Electron Corporation, Runcorn, UK) and processed routinely. The samples were then embedded into paraffin wax blocks using an embedding station (Raymond Lamb Blockmaster II, Thermo Fisher Scientific, Loughborough, UK). A microtome (Reichert 2040 Microtome, Leica Biosystems, Milton Keynes, UK) was then used to cut 5 µm sections and these were placed onto Superfrost slides. The slides were stained with haematoxylin and eosin (H&E) and Martius Scarlet Blue (MSB). The staining was performed using an Autostainer (Sakura Tissue-Tek DRS 2000, Sakura, Alphen aan den Rijn, Netherlands), to

ensure conformity. Images were taken at ×40, ×100, and ×200 magnifications using an EVOS microscope (Electron Microscopy Sciences, Hatfield, PA, USA).

The other half of each vein section was taken, snap frozen and mounted in optimal cutting temperature (OCT) compound (Sakura). Sections 6 μm thick were then cut from these samples using a microtome cryostat (Hyrax C 25, Zeiss, Oberkochen, Germany) and the sections were taken onto Superfrost Plus slides (Thermo Fisher Scientific). These slides were fixed in ice cold methanol/acetone (50:50) for 9 minutes and were washed with PBS three times. The slides were then incubated, in a dark humid chamber, with 2% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. After a subsequent wash with PBS, the slides were incubated with a polyclonal rabbit anti-VCAM-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) 1:100 in 0.2% BSA in PBS for 1 hour at room temperature in a dark humid chamber. After washing with PBS, the slides were incubated in the same way with a fluorescein isothiocyanate (FITC) labelled anti-rabbit IgG antibody (Santa Cruz Biotechnology Inc.) 1:250 in 0.2% BSA in PBS.

The same slides were then double stained; using a goat anti-p-p53 antibody (Santa Cruz Biotechnology Inc.) 1:100 in 0.2% BSA in PBS for 1 hour and a cyanin-3 (Cy3) labelled anti-goat IgG antibody (Santa Cruz Biotechnology Inc.) 1:250 in 0.2% BSA in PBS. Five minutes prior to the end of the last incubation, one drop of 4,6-diamidine-2-phenylinole chloride (DAPI) was added (1 μg/mL in 0.2% BSA in PBS).

After incubation, coverslips were mounted onto the slides using Mowiol solution (10% Mowiol [Sigma—Aldrich, Poole, UK] in 25% glycerol with 0.1% p-phenylenediamine). Visualisation of the fluorescence was undertaken using a fluorescence microscope (Olympus BX61, Olympus, Hamburg, Germany). Quantification of these images was then performed using software (SimplePCI, Hamamatsu, Shizuoka, Japan) to measure the intensity of fluorescence in each section.

The study received a favourable ethical opinion from the NRES Committee South East Coast — Surrey (13/LO/0058) and the University of Surrey Ethics Committee (EC/2013/23/FHMS/FS). All human tissue samples were used and stored in accordance with Human Tissue Act (HTA) 2004 UK guidelines. Patient consent was collected and patient information was stored in accordance with the Data Protection Act (UK, 1998). Generation of graphs and all statistical analysis were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

## RESULTS

Histological analyses showed no significant damage at 0, 20, and 40 J/cm. Significant asymmetrical thermal damage was observed throughout the intima and media at 60 J/cm and extensive damage throughout the entirety of the vein wall at 80 J/cm (Fig. 1). At 80 J/cm perforation of the vein wall was observed.

Visual analysis of the immunofluorescence showed no difference in p-p53 or VCAM-1 expression between the control and vein segments treated at 20 and 40 J/cm. Observation indicated an increase in p-p53 expression in the endothelium of the vein sample treated at 60 J/cm, but no difference for VCAM-1. Furthermore, vein sections treated with an LEED of 80 J/cm showed an upregulation of p-p53, with no change in VCAM-1 expression (Fig. 2).

Quantitative analysis of p-p53 fluorescence intensity showed no significant difference in expression among the control, 20, and 40 J/cm ($p > .05$), confirming the visual analysis. There was a significant increase in the expression of p-p53, compared with the control, at 60 and at 80 J/cm ($p = .0077$ and $p = .0465$, respectively), both in the endothelial cell layer alone and across the vein wall. Quantification of VCAM-1 fluorescence showed no significant difference in the expression throughout the vein wall (Fig. 3).

## DISCUSSION

This is a preliminary study, in one vein from one patient, which has outlined the biological effects of a 1920 nm EVL using an *ex vivo* model of EVLA treatment on the tributary vein wall. By performing both histological and immunohistochemical analysis on vein samples treated *in vitro*, this work has been able to show the extent of heat induced cell damage caused at different LEEDs, and confirm the presence of apoptotic cell markers within the vein wall. The results clearly show that the extent of thermal damage to the vein wall increases, as the LEED increases, with significant structural damage to the vein wall observed with treatments above 60 J/cm using a power of 4W. This is in keeping with work done by the present authors in the same model but with the more commonly used 1470 nm EVLA, which has been presented internationally and is being submitted for publication.

p53 is a tumour suppressor gene, which is activated by pro-apoptotic signals, to p-p53. Observations of the activation of p53 to p-p53 at 60 and at 80 J/cm, imply a role for apoptosis in fibrotic occlusion and atrophy after EVLA of the GSV. Furthermore, by obtaining a large tributary bypassing a hypoplastic segment of the GSV (the “extra-fascial GSV”), rather than using superficial tributaries obtained during phlebectomies, the analysis is more comparable with clinical treatment of an incompetent GSV. Anatomical studies on the structure of the tributaries that bypass hypoplastic sections of the GSV have shown that the vein wall is similar in structure to the truncal GSV above the hypoplastic segment. However, the vein wall of the tributary is somewhat thinner - 89% of the GSV wall thickness in one study[7] and 53% in the mid-thigh in another.[8]

Possible further limitations of this model include that being *ex vivo*, the vein is not surrounded by tissue and so the adventitial vena vasorum are empty and not flowing, and there is no blood within the vein lumen during treatment. As regards the former point, a vein treated *in vivo* is surrounded by tumescence and so this vein suspended within tumescence fluid during treatment is not in a dissimilar environment. The presence of blood within the lumen is regarded as important in some models,[9] but when

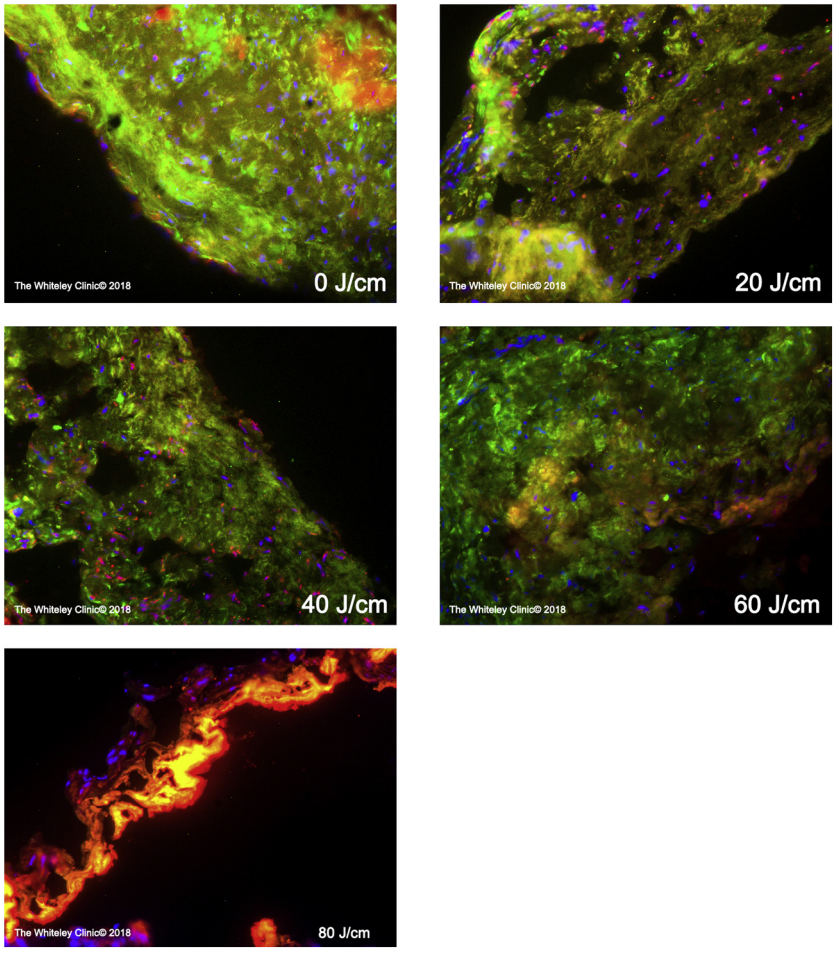


**Figure 2.** Immunohistochemical analysis of large extra-fascial tributary of GSV sections treated with varying LEEDs by a 1920 nm endovenous laser. Double stained with p-p53 appearing red, VCAM-1 appearing green, and DAPI staining the nuclei blue. Pictures taken at ×100 magnification.

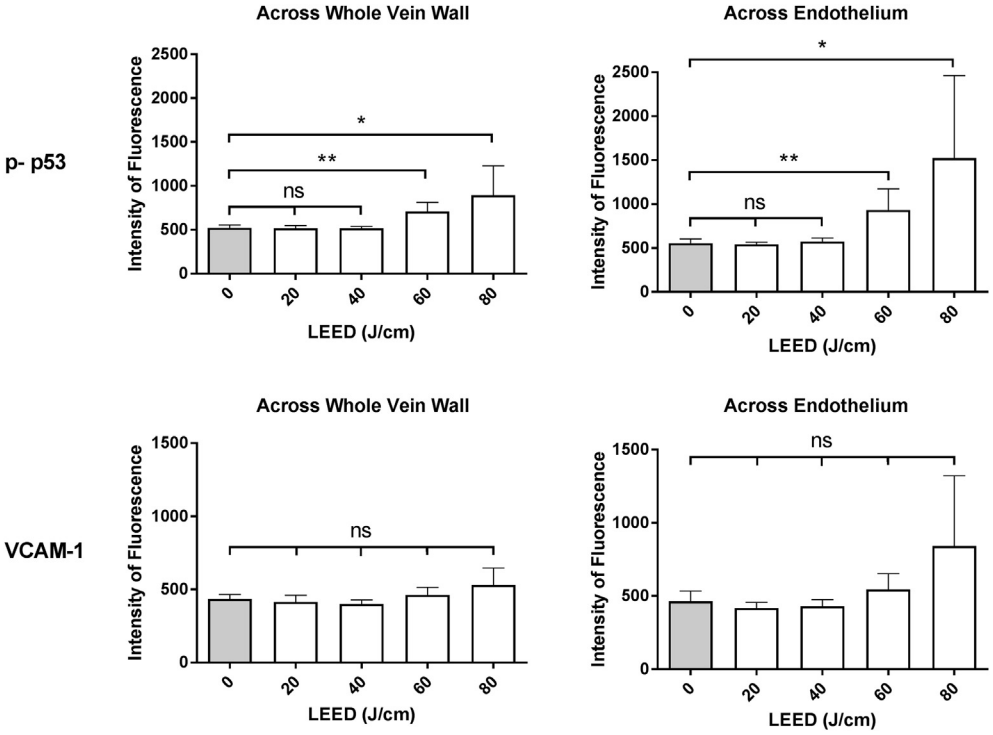


**Figure 3.** Quantification of the intensity of p-p53 and VCAM-1 fluorescence in vein sections treated with varying LEEDs by a 1920 nm endovenous laser. Data expressed as mean ± SD. $N = 5$. Comparisons made by unpaired $t$ test. $p < .05$. * < .0465, ** < .0077.

patients are in the Trendelenburg position and have tumescence fluid around the vein, there is little if any blood remaining in the vein at the time of treatment.[10]

In conclusion, the present *ex vivo* study shows that treatment of a large tributary bypassing a hypoplastic segment of the GSV, *in vitro*, with a 1920 nm EVL, at or above a LEED of 60 J/cm at 4 W, leads to significant cell death in the vein wall by a combination of direct thermal damage and apoptosis, which reaches deep into the media of the vein wall. Although only one vein was used in this preliminary study, this finding suggests that this wavelength could be used to ablate incompetent truncal veins at LEEDs similar to those used with the 1470 nm EVL.

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## CONFLICT OF INTEREST

None.

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