TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE

FEASIBILITY STUDY ON SHEEP

REV.	REDACTOR	APPROVER
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B	THOMAS CHARREL CSO, STUDY INVESTIGATOR 06/10/2021	BERNARD GREILLIER PRESIDENT AND CEO 08/10/2021

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SUMMARY

The purpose of this nonclinical study was to evaluate the performance (in terms of vein occlusion) and the local tissue effects of echo-guided HIFU (High Intensity Focused Ultrasound) treatments in sheep's saphenous veins.

HIFU treatments were used to treat 4 saphenous veins. Two HIFU doses were evaluated 4s/60J and 7s/105J with and without tumescence injection in perivenous tissues. Before and after treatment, ultrasound scans were performed (at Day 0, 3, 7 and 21). Sheep were then euthanized at D21. Local tissue effects were evaluated based on the macroscopic observations, while the evaluation of the device performance was based on ultrasonic scan.

Macroscopically throughout the study duration, for dose of 4s/60J and with tumescence, significant lumens diameter reduction of 84% was observed without skin burns. For doses of 4s/60J without tumescence and 7s/105J skin burns of slight to severe grade was observed along the vein, while no thrombus nor lumen reduction were observed.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
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1 INTRODUCTION

The Chronic Venous Insufficiency (CVI) is one of the major pathologies in the world, affecting 20% of the industrialized country population (and predominantly Caucasian) [1-5]. While current market intelligence indicates that more than 400,000 procedures are performed per year in the US, it has to be noted that 150,000 procedures are performed in South Korea [6].

Varicose Vein Treatment Market Expected to Behold a CAGR of 6.4% through 2016-2026. Towards the end of 2026, the global market for varicose vein treatment will have reached value of US\$ 931.4 M, registering a robust growth at 6.4% CAGR [7].

Current medical procedures for varicose vein treatment aiming at the obliteration of the affected veins, such as sclerotherapy, thermal ablation, are currently recommended over to ligation and stripping. Since Fallon's work [8] in the 70s, HIFU (High Intensity Focused Ultrasound) applied to the treatment of veins and arteries has been studied by multiple international teams, and by the INSERM-LabTAU [9, 10]. VeinSound has partnered with LabTAU to demonstrate that superficial vein occlusions (up 3mm in diameter) could be achieved in vivo.

Based on the promising results of the Proof Of Concept and the pre-clinical trial previously performed on small animals, we anticipate that obliteration of large veins such as saphenous, perforator and tributary veins can be achieved in humans and offer a "non invasive" varicose vein treatment alternative to current endovenous treatments.

The purpose of this animal study is to evaluate the performance (in terms of vein occlusion) and the local tissue effects of echo-guided HIFU (High Intensity Focused Ultrasound) treatments in sheep's saphenous veins. VeinSound confirms that the conduct of this study does not unnecessarily duplicate previous experiments.

2 BACKGROUND & SIGNIFICANCE

The work carried out by many international teams shows an advanced understanding of the ultrasonic mechanisms in the veins. In a recent review, Serrone et al. [11] have modeled the different mechanisms of ultrasonic action leading to the occlusion of a vein. Obliteration mechanisms have now been clearly identified and equivalences can be drawn between HIFUs and other current methods. HIFU thermal effect is similar to the endo-ablation (RF, LASER or Vapor) to the extent that it leads to tissue contraction by collagen denaturation and "vein diameter reduction" – The body will then naturally digest the treated vein. Yet HIFU based therapies requires no catheter insertion, multiple "stab incision" and skin scaring and in the case of LASERs no skin discoloration or pigmentation.

Previous preclinical proof of concept performed on the rabbit (funded by AVIESAN), have been executed in collaboration with LabTAU and finalized in Q2 2017. Eighteen (18) rabbits have been subject to VeinSound's HIFU procedures and followed over a one (1) month period. The treated venous sections were marked / localized / identified and the obliterations were evaluated under a macroscopic examination confirmed by a high-frequency Doppler ultrasound scan (Cermep, Lyon, France)¹.

The proposed project aims to confirm those previous results on a larger animal model (closer to Human) with the development of a dedicated therapy probe.

¹ Data on File – VeinSound preclinical study on small animal – June 2017

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

3 MATERIALS

VeinSound, in collaboration with the LabTAU (INSERM 1032 unit), have developed a custom HIFU probe (V-PROB) and computer-controlled system (V-SYS) to monitor treatment during preclinical study. The V-PROB is made of two confocal ceramics (MEGGITT, Denmark) working at 3.3MHz. The space between the two ceramics integrates an USB linear imaging probe (SP-L01, Interson corp., USA). Imaging and therapeutic plane are mechanically aligned. The V-SYS is based on a computer platform (PXIe-1073, NI, USA) and a custom LabView program to pilot therapeutic sequence in time and energy. The HIFU signal is amplified through RF power amplifier (1140LA) to supply the V-PROB, which converts electrical energy into ultrasonic energy. HIFU energy is then targeted on the vein using monitoring interface (Figure 1).

Figure 1 – Picture of the V-PROB and V-SYS in the operating room before surgical procedure (left) and user-interface to align focal beam (red) to the targeted vein (green).

A dedicated ultrasound scanner with Doppler mode (SmartUS, Teled, Lithuania), is used for imaging tissues at D0, D3, D7 and D21 for performance follow-up.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

4 METHODS

4.1 ANIMAL MANAGEMENT

Sheep will be housed in groups in cages identified by a card indicating the study number, animal numbers, sex, dates of beginning and end of experimental in-life phase (Table 1). The housing conditions may be adapted in case of aggressive behavior or clinical indication. Sheep will be kept under laboratory conditions. The housing room temperature and relative humidity will be recorded daily. The recommended temperature range for the room is 10 - 24°C. The light cycle will be controlled using an automatic timer (12 hours of light, 12 hours of dark). Only healthy previously unused animals will be selected. Conditions will conform to the European requirements (Directive EU/2010/63).

Table 1 – Description of species and management characteristics

Species	Sheep (<i>Ovis aries</i>)
Strain/Breed	Blanche du Massif Central (BMC)
Source	Bergerie de la Combe aux Loups, France
Sex	Female
Acclimation Period	Minimum 5 days
Number of Animals	2 for thermal HIFU treatment
Identification Method	Individual number on ear tag

Standard hay will be provided ad libitum and supplemented with a commercially available pelleted sheep feed (Special Diet Services, France). Minerals will be provided ad libitum (Sodimouton, Salins Agriculture). Potable water will be delivered ad libitum through species appropriate containers or delivered through an automatic watering system. No contaminants present in the feed or water are expected to adversely impact the results of this study. Experimental platform is an AAALAC International accredited facility and is registered with the French Department of Agriculture for animal housing, care and investigations.

This protocol will be approved by an Ethical Committee. Each protocol is part of a project authorization that is reviewed every five years by the Ministry of Education, Higher Education and Research. Any significant changes to this protocol are approved prior to conduct.

4.2 STUDY DESIGN

Two sheep, 4 saphenous veins identified S1 to S4, will be treated with VeinSound echo-guided HIFU in each saphenous vein according to the allocation table (Table 2). The sheep is recommended in the ISO 10993 standard, Part 6 (2016) as an appropriate animal to evaluate the performance and local tissue effects of vascular treatments. The sheep's vein morphology and size is similar to human's and this model have been described in the literature as an appropriate model to evaluate venous occlusion². The bilateral saphenous treatment was chosen to reduce the number of sheep used. The time-period was chosen based on the expected time for observation of occlusion.

² Data on File – VeinSound animal model evaluation – June 2018

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

Table 2 - Study Design and Allocation Table

Sheep Number	Dose	Tumescence	Time-period			
			D0	D3	D7	D21
1	105J for 7s	Without	Ultrasound scan + HIFU treatment	Ultrasound scan	Ultrasound scan	Ultrasound scan Termination and sampling
2	60J for 7s	With				
3	60J for 4s	Without				
4	60J for 4s	With				

4.3 PRE-OPERATIVE PROCEDURE

Within one day prior to surgery, animals were weighed, the treatment area was clipped free of wool and depilatory cream (Vichy) was applied onto the shaved area to clean any remaining wool. The sheep were fasted overnight. On the day of treatment (Day 0), pre-medication was performed by intravenous injection of a mixture of diazepam® (TVM) and butorphanol (Torphasol®, Axience). Anesthesia was induced by intravenous injection of propofol (Propovet®, Zoetis). Each sheep was intubated, mechanically ventilated and placed on isoflurane inhalant anesthetic (IsoFlo®, Zoetis) for continued general anesthesia. An intravenous infusion with a suitable electrolyte solution (Ringer lactate®, CEVA SA) was performed during surgery. A neutral ophthalmic ointment (Ocrygel®, TVM) was applied to both eyes to protect the corneas from drying and was re-applied as needed. The sheep was placed in lateral position on a warmed pad. A rectal temperature probe and a rumen tube were placed during surgery. Electrocardiogram (ECG), peripheral non-invasive arterial blood pressure and oxygen saturation were monitored.

4.4 TREATMENT PROCEDURE

The treatment was performed by VeinSound staff and under their own responsibility. Before treatment, a coupling gel (Alcyon) was spread onto the probe and onto the sheep's leg. An ultrasound scan was performed to measure the diameter of the saphenous vein, to measure and to mark the treatment area (approximately 10 cm, in the superficial saphenous vein). Perivenous tumescent infiltration of sodium chloride (0.9%, NaCl Baxter) was applied around the saphenous vein of few sites for thermal protection of peripheral tissues for S2 and S4 only (Figure 2). Each vessel was divided in three sections (L1 to L3 see Figure 3). The operator placed the V-PROB on L1. Once the target area was identified, the operator applied HIFU treatment from upstream to downstream venous flow. The V-PROB was manually displaced per step of 5mm, guided by a ruler placed along the vein. The contralateral saphenous vein was treated similarly according to allocation table (Table 2).

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

Figure 2 – Photo of the tumescence injection performed before S2 and S4 treatments (a). Ultrasound images comparison before (b) and after tumescence injection (c). Tumescence can easily be identified through hypo-echogenic layer formation (*) increasing distance between skin and vein tissues.

Down stream

Figure 3 - Scheme of the sheep leg and 3 treated sections L1, L2 and L3 of saphenous vein.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

4.5 POST-OPERATIVE PROCEDURE OBSERVATIONS AND EVALUATIONS

Sheep was moved to a recovery area and monitored for recovery from the anesthetic until sternal recumbency was achieved. After recovery, sheep were returned to their cage and observed for general health. Sheep were observed daily for general health and to detect mortality and morbidity. When any animal exhibited adverse clinical signs, it was examined and treated as needed.

The vein occlusion was evaluated at D3, D7, and D21 after treatment. This evaluation was performed by duplex ultrasound control. The following parameters were evaluated and compare with D0 pre-treatment observations:

- Vein diameter D (Figure 4)
- Occlusion evolution with Doppler mode

Additionally, macroscopic observations of the skin on two parameters (erythema and edema) were evaluated using the following scoring system:

- 0: Nothing to report
- 1: Slight grade
- 2: Moderate grade
- 3: Marked to sever grade

Figure 4 – D: Diameter of the vein measured on ultrasound image in transverse (left) and longitudinal (right) section of the vein

4.6 TERMINAL PROCEDURES

The sheep were weighed within one day before termination and were fasted overnight for food and for water. Sheep were anesthetized as described in Section 5.4. The sites were examined by ultrasound analysis as described in section 5.7.2 and the saphenous vein was exposed and macroscopically observed. The sheep were then heparinized by injection of heparin solution (Heparine choay®, Sanofi) to prevent post-mortem clotting. Under deep anesthesia, Sheep D8091 was exsanguinated 6 minutes after heparin injection, while Sheep D8089 and D8090 were terminated by a slow intravenous injection of embutramide-mebezonium-tetracaine (T61®, MSD) and then the vein was exsanguinated (see Section 8 – 2). Skin tissue of each analyzed site was sampled, at the study director's discretion and in agreement with the Sponsor for further histopathology analysis.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

5 RESULTS

5.1 MACROSCOPIC ULTRASOUND OBSERVATIONS

Before each treatment no difficulty was observed in the ultrasound scan of each saphenous vein performed by the Sponsor. Each vein was then divided in three sections treated independently. Thermal effect on the vein appears significantly for a dose of 4s/60J, with tumescence (S4). In this case, the lumen diameter is reduced from 5 to 0.8mm (Figure 5), leading to partial obliteration of the vein. The blood flow is still measurable using the Doppler mode (Figure 6). No significant diameter reduction is measured with other protocols (Appendix 1).

Figure 5 – Intraluminal diameter evolution at D21 after HIFU treatment S4

S4 (Right)

Figure 6 – Reconstruction of the saphenous vein S4 along L1, L2 and L3 at D0, D3 and D21 with a significant lumen reduction and residual blood flow (white arrow) concluding to partial obliteration.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

5.2 MACROSCOPIC OBSERVATIONS DURING THE FOLLOW-UP

For S1, a dose of 7s/105J without tumescence, showed the most severe erythema (graded from slight to marked/severe) and occasionally presented slight signs of edema. However, for treatment with tumescent infiltration (S2 and S4), erythema was observed in the early time only with a slight grade, then this observation did not persist after 10 days. The individual macroscopic scores of erythema and edema are presented in Appendix 2.

Global safety and performance data's are summarized in Table 3 below.

Table 3 – summary of the results if the Safety and Performance at D21

Saphenous vein	Dose	Tumescence	Safety at D21 Efficacy at D21
S1 (Left)	7s / 105J	No	Safety: important burn on the skin Efficacy: not evaluated
S2 (Right)	7s / 105J	Yes	Safety: burn on the skin Efficacy: No Obliteration
S3 (Left)	4s / 60J	No	Safety: burn on the skin Efficacy: No Obliteration
S4 (Right)	4s / 60J	Yes	Safety: Redness on the skin Efficacy: Important lumen reduction with partial obliteration

6 CONCLUSION

In this animal study on four saphenous veins, we explore the possibility to reduce vein diameter with thue use of a custom hand-piece (V-PROB) and HIFU console (V-SYS). The dose of 4s/60J seems promising even if the venous occlusion is partial, a significant lumen diameter of 84% has been achieved. Skin safety is improved with the usage of tumescence. Based on these first results on big animals, design of the V-PROB will be optimized to reduce HIFU energy in the skin entry plane and thermal elevation on skin per se. The computer-controlled system V-SYS will be integrated to improve treatment sequence. Finally the treatment dose will be adjusted to optimized treatment duration and validated in-vivo.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

7 REFERENCES

- [1] M. H. Criqui, M. Jamosmos et A. Fronek, «Chronic Venous Disease in an Ethnically Diverse Population: The San Diego Population Study,» *American Journal of Epidemiology*, vol. 158, pp. 448-56, 2003.
- [2] L. Robertson, A. J. Lee et C. J. Evans, «Incidence of chronic venous disease in the Edinburgh Vein Study,» *Journal of Vascular Surgery: Venous and Lymphatic Disorders*, vol. 1, pp. 59-67, 2013.
- [3] Robertson L, Evans C and Fowkes F G R, «Epidemiology of chronic venous disease,» *Phlebology*, vol. 23, p. 103–111, 2008.
- [4] E. Rabe et F. Pannier, «What have we learned from the Bonn Vein Study?,» *Plebolympology*, vol. 13, pp. 188-94, 2006.
- [5] L. Mäkivaara, *Occurrence of varicose veins and bidirectional risk with cardiovascular diseases*, Tampere: Acta Universitatis Tampereensis 1350. Tampere University Press, 2008.
- [6] J. J.H., Varicose Vein Treatment in South Korea, Dardik A. (eds) Vascular Surgery. Springer, Cham, 2017.
- [7] A. Nikam, «Varicose Vein Treatment Market Expected to Behold a CAGR of 6.4% through 2016-2026,» future market insights, 5 02 2018. [En ligne]. Available: <https://www.latestmarketreports.com/2018/02/05/varicose-vein-treatment-market-expected-to-behold-a-cagr-of-6-4-through-2016-2026/>.
- [8] J. Fallon, «Effect of ultrasound on arteries,» *Arch Pathol*, vol. 94, pp. 380-8, 1972.
- [9] Y. C. Angel, S. Pichardo, R. Salomir, L. Petrusca et J.-Y. Chapelon, «Testing of a HIFU probe for the treatment of superficial venous insufficiency by using MRI,» chez *Conf Proc IEEE Eng Med Biol Soc*, 2006.
- [10] Pichardo, S.; Milleret, R.; Curiel, L.; Pichot O.; Chapelon, J-Y., «IN VITRO EXPERIMENTAL STUDY ON THE TREATMENT OF SUPERFICIAL VENOUS INSUFFICIENCY WITH HIGH-INTENSITY FOCUSED ULTRASOUND,» *Ultrasound in Med. & Biol.*, vol. 32, n° 16, pp. 883-91, 2006.
- [11] J. Serrone, H. Kocaeli, T. D. Mast et e. al., «The potential applications of high-intensity focused ultrasound (HIFU) in vascular neurosurgery (Review),» *Journal of Clinical Neuroscience*, vol. 19, pp. 214-21, 2012.

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEIN SOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

APPENDIX 1 – ULTRASOUND IMAGES

S1 (Left)

S2 (Right)

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

S3 Left

TITLE: HIFU VARICOSE VEIN TREATMENT USING VEINSOUND DEVICE - FEASIBILITY STUDY ON SHEEP	DATE: 06/10/2021
	REDACTOR: T. CHARREL	TR_CLI_001B

APPENDIX 2 - MACROSCOPIC EVALUATION OF EDEMA AND ERYTHEMA OF SITES

Allocation	S1 (Left)		S2 (Right)		S3 (Left)		S4 (Right)	
Time-Period	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema
Day 1	0	1	0	2	0	2	0	1
Day 2	0	1	0	1	0	2	0	1
Day 3	0	1	0	1	0	2	0	1
Day 4	0	1	0	1	0	2	0	1
Day 5	0	1	0	0	0	1	0	1
Day 6	0	1	0	0	0	1	0	1
Day 7	1	3	0	2	1	2	0	1
Day 8	1	3	0	2	1	2	0	1
Day 9	0	1	0	1	0	1	0	1
Day 10	1	2	0	1	0	1	0	1
Day 11	1	3	0	2	0	2	0	0
Day 12	1	3	0	2	0	2	0	0
Day 13	1	3	0	2	0	2	0	0
Day 14	1	3	0	2	0	2	0	0
Day 15	0	3	0	2	0	2	0	0
Day 16	0	3	0	2	0	2	0	0
Day 17	0	3	0	1	0	1	0	0
Day 18	0	3	0	1	0	1	0	0
Day 19	0	3	0	1	0	1	0	0
Day 20	0	2	0	1	0	1	0	0
Day 21	0	1	0	1	/	/	/	/