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TITLE

PRECLINICAL AND TRANSLATIONAL RESEARCH FOR NEW TECHNOLOGIES IN VASCULAR SURGERY: USE OF EXTRACELLULAR VESICLES DERIVED FROM AUTOLOGOUS SERUM FOR THE HEALING OF CHRONIC VENOUS ULCERS UNRESPONSIVE TO CONVENTIONAL TREATMENTS

Candidate: Dr Lorenzo Gibello

Tutor: Prof. Fabio Verzini

Ph.D. Coordinator: Prof. Gianluca Ciardelli

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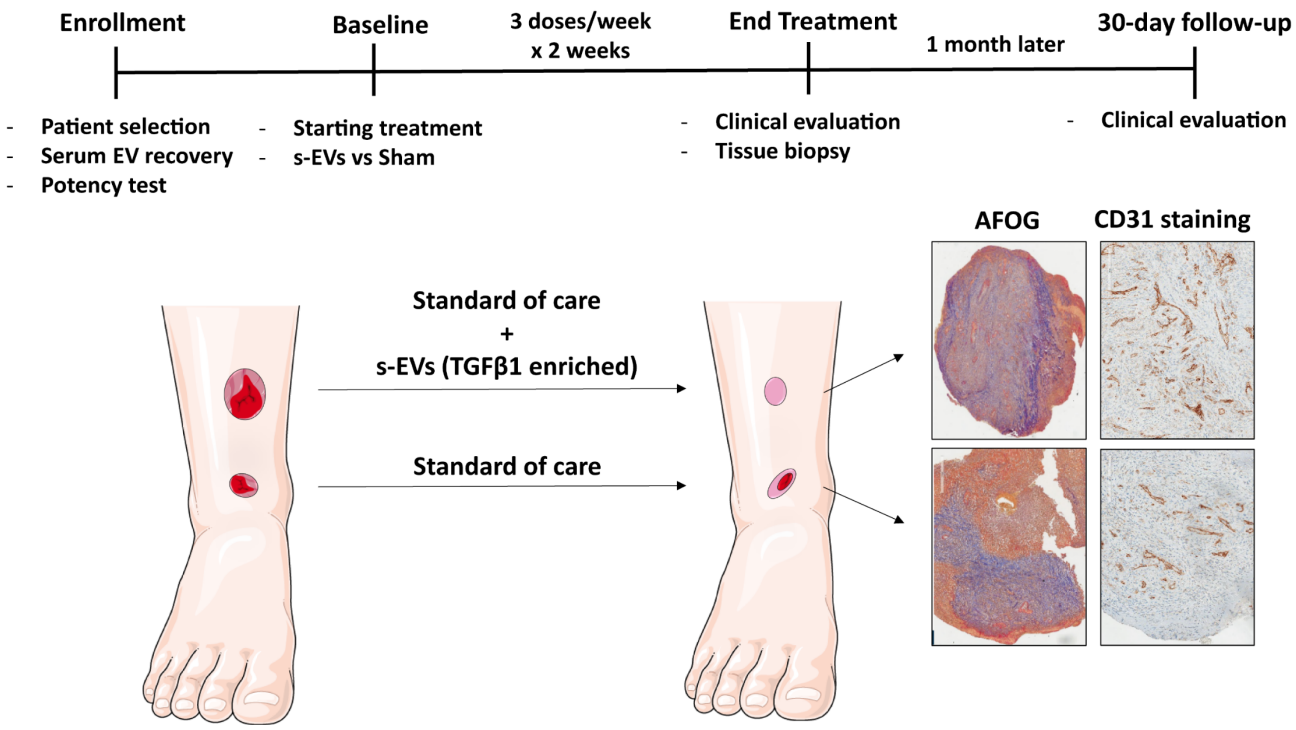
ABSTRACT

Current therapeutic approaches for chronic venous ulcers (CVUs) still require evidence of effectiveness. Diverse sources of extracellular vesicles (EVs) have been proposed for tissue regeneration, however the lack of potency tests, to predict *in-vivo* effectiveness, and a reliable scalability have delayed their clinical application. This study aimed to investigate whether autologous serum-derived EVs (s-EVs), recovered from patients with CVUs may be a proper therapeutic approach to improve the healing process. A pilot case-control interventional study (CS2/1095/0090491) has been designed and s-EVs recovered from patients. Patient eligibility included two or more distinct chronic lesions in the same limb with 11 months as median persistence of active ulcer before enrollment. Patients were treated three times a week, for 2 weeks. Qualitative CVU analysis demonstrated that s-EV-treated lesions displayed a higher percentage of granulation tissue compared to the control group (Sham) (s-EVs 3 out of 5: 75-100% *vs* Sham: none), further confirmed at day 30. s-EVs-treated lesions also displayed higher sloughy tissue reduction at the end of treatment and at day 30. Additionally, s-EV treatment led to a median surface reduction of 151 mm^2 compared to 84 mm^2 in the Sham group, difference even more evident at day 30 (s-EVs 385 mm^2 *vs* Sham 106 mm^2 *p=0.004*). Consistent with the enrichment of transforming growth factor-β1 in s-EVs, histological analysis revealed an increase of vessel number and regenerative tissue.
This study first demonstrates the clinical effectiveness of autologous s-EVs in promoting the healing process of CVUs unresponsive to conventional treatments.

GRAPHICAL ABSTRACT



INTRODUCTION

Chronic venous ulcer (CVU) is the most common type of ulcer affecting the lower extremity, with a prevalence of 2% in general population and increasing to 5% in individuals over the age of 65-year-old[1-2]. Up to 93% of CVUs will heal in 12 months, with 7% remaining unhealed after five years[3]. Moreover, recurrence rate within 3 months after wound closure is as high as 70%[4]. Chronic wounds have also a significant social and economic burden, both for the severe deterioration of patient's quality of life and for healthcare costs[5]. The associated costs for CVU care are just over $15,000 but increase significantly for patient who have delayed healing and can result in costs as high as $34,000 per patient per year[6,7]. In this population, indirect costs connected with temporary work disability and productivity loss are largely underestimated[5].

CVU healing process is affected by both systemic and local factors. Among the systemic factors, nutritional status plays a crucial role. In fact, wound healing is an energy-demanding process and several studies demonstrated that malnutrition alters the inflammatory response and collagen synthesis which are fundamental for tissue repair[8]. Considering local factors, two main conditions are responsible for the persistence and, in some cases, worsening CVUs: wound infection and edema. Although CVU bacteria colonization is common, and of little clinical significance, the creation of a resistant extracellular biofilm protecting pathogens and impairing the activation of the inflammatory response in surrounding tissues delay ulcer healing[9-10]. The routinely use of systemic antimicrobials is not beneficial for CVUs, as suggested by a Cochrane review, while a prompt recognition and treatment of active infection is mandatory[11]. Chronic edema is the underlying pathogenetic mechanism for CVUs and is responsible for the high recurrence rate. For this reason, guidelines recommend with the highest level of evidence, the use of multilayer or inelastic compression for the treatment of active venous leg ulceration[12].

Over the past decades, growth factors have increasingly been used to promote CVU healing process. Despite interesting preliminary results, a recent meta-analysis showed how suggested benefits for this approach are still weak based on low quality of evidence[13]. Currently, there is no consensus on how much growth factors improve tissue repair compared to standard of care. This evidence appears relevant to justify higher costs of these treatments compared to more affordable conventional compression stockings and standard wound dressings.

Recently, extracellular vesicles (EVs) derived from different sources have been proposed for tissue regeneration[14-15]. EVs act as cell-to-cell paracrine or endocrine-like communication mechanisms, and, in the last decades, several pre-clinical studies have provided conceivable rational for their clinical application[14-16]. The therapeutic potential of EVs depends on the transfer of their cargo (proteins, active lipids, mRNA and ncRNA to name a few) to target cells. Our previous

preclinical study demonstrated that serum-derived EVs (s-EVs) recovered from healthy donors are enriched in Transforming Growth Factor-β (TGFβ1) and several transcripts associated with its functional activities[17]. We also set-up an *in-vitro* potency test able to predict the ability of s-EVs to induce neo-angiogenesis and prevent muscle cell damage in an *in-vivo* mouse model of acute hind limb ischemia[18]. Additionally, it has been reported that TGFβ1 s-EV content can predict s-EV angiogenic potential in high-risk cardiovascular patients[17]. Currently, the foremost barriers for EV clinical application consist of the lack of potency tests, their potential immunogenicity, and clinical scalability[19], theoretically defeated by serum-derived autologous products.

Based on these premises, this study aimed to evaluate the healing properties of autologous s-EVs in CVU patients resistant to conventional treatments. We designed a pilot case-control interventional study to compare the healing process of CVU lesions treated with s-EVs + standard of care *vs* standard of care alone, at the end of treatment and one month later.

MATERIALS AND METHODS

Study design and participants

The executive committee designed and oversaw the trial procedures and analysis. The trial and the study protocols were approved by the Ethics committee at the Città della Salute e della Scienza Hospital. All procedures agreed with the principles of the Helsinki Declaration and all participants provided written informed consent.

A single center case-control interventional study (Clinical Trial number: CS2/1095/0090491) has been designed to evaluate whether s-EVs treatment accelerates healing of CVUs compared to conventional treatment. Patients were eligible if showed two or more distinct chronic lesions in the same limb. Chronic lesion was defined as the loss of skin tissue without any tendency to heal in the past 3 months. Inclusion criteria for the study can be grouped in three domains: demographics and risk factors, wound condition, and laboratory tests, as summarized in Table 1.

Briefly, participants undergo a blood sample for s-EV isolation and the preparation of 6 doses of active s-EVs. Protocol: the CVU with bigger surface was treated with s-EVs + standard wound dressing, while the smaller one with standard wound dressing alone (Sham). Multilayer bandage was made to guarantee homogeneous elastic compression throughout the limb. Medications were renewed 3 times per week for two weeks. A 4-mm punch biopsy was performed in the center of ulcer bed at the end of the treatment for histological analysis in patient 4 and patient 5. A picture of the lesion was

acquired at every stage to evaluate progression of the ulcer healing as well as at day 30 follow-up (continuing with standard treatment alone for both lesions).

	INCLUSION CRITERIA	EXCLUSION CRITERIA
Demographics & risk factors	• Age >18 and < 85 • Absence of peripheral arterial disease*	• Cancer • Diabetes
Wound condition	• Ulcer in granulation phase§	• Active wound infection° • Tendon or bone exposure
Laboratory tests	• Hb > 10 mg/dl • Plts > 100.000 mg/dl • Positivity to the potency test	• HBV + • HCV+ • HIV +
Study consent	• Written agreement to participate to study protocol	

Table 1. Inclusion and exclusion criteria.
*PAD was defined as absence of distal arterial pulses or ankle-brachial index < 0.9.
§ granulation phase: small amount of fibrin, no necrotic tissue
°Active wound infection was clinically determined as increased amount of exudate, malodorous exudate, poor pain control.

s-EV isolation, characterization, and potency test

A 50 ml whole blood sample was necessary to obtain 17.5 ml of serum after precipitation as previously described[20]. All operations were performed in compliance with the Good Practices Guidelines (GPGs) and according to the "Guide to the preparation, use and quality assurance of blood components into the Blood Bank laboratory" by the European Directorate for the Quality of Medicines (EDQM) in a grade A laminar flow hood placed inside a grade D environment in accordance with the requirements of Good Manufacturing Practice (GMP). Whole blood was obtained by a venipuncture in class two sterile tubes without anticoagulant (VI 2 PRP BiomedDevice, Italy) and left to coagulate at least one hour at room temperature. The tubes were then centrifuged at 1,500 g (2,800 RPM) for 15 minutes. The supernatant serum was transferred into new tubes of the same type and was centrifuged at 3,000 g (3,900 RPM) for 30 minutes to remove debris. A solution of clinical grade PEG 400 and protamine hydrochloride was added to the serum in a 1:4 ratio and incubated at 4 °C overnight. Therefore, after a centrifugation at 1,500 g (2,800 RPM) the supernatant was discarded, and the pellet was resuspended in 17.5 ml of sterile saline solution. This final product was divided in 6 aliquots of about 3 ml and frozen at -80 °C. Each dose was thawed at the day of application and used within 6 hours. The potency test was performed as previously described.[17]

Vascular endothelial growth factor (VEGF) served as positive control. The test was considered positive when both the angiogenesis and proliferation value exceeded 50% of VEGF activity (Table 2).[18]

Patients	Angiogenesis assay (%)	Proliferation assay (%)	Average (%) compared to VEGF activity
1	61.5± 2.8	51± 1.3	57.75±1.07
2	64.2±1.9	77.6±1.4	70.9±1.65
3	74.3±2.3	59.5±1.1	66.9±1.04
4	68.5±2.4	52.3±1.5	60.4±1.95
5	66.2±2.2	72.1±1.8	69.15±2

Table 2. Potency test of patients enrolled in the study. Values exceeding 50% of VEGF activity for angiogenesis and proliferation were considered effective.

Treatment protocol and ulcer assessment

CVU with the bigger surface area was treated with s-EVs and standard of care, the smaller one was treated with standard of care alone (Sham). s-EVs were applied all along the wound edges with a 5 ml syringe and 25-gauge needle in a sterile setting. Standard of care consisted of wound cleansing, irrigation, and disinfection. Hydrofiber Aquacel Ag Extra Plus dressing (ConvaTec, Reading, Berkshire UK) was used to maintain ulcer's moisture balance and to guarantee anti-biofilm activity. A multilayer bandage was then applied for a graduated limb compression. Medication was repeated three times per week for a fortnight. At the end of treatment, a 4-mm punch biopsy was obtained at the level of the wound bed of both lesions. Samples were stored in formalin and sent for histological analysis. At the end of the two weeks, a weekly standard of care medications was maintained for all patients, in a wound care nursing ambulatory until complete healing. The safety of clinical treatment was monitored after each application and over 30 days after the last s-EV administration, when a final appointment was accomplished. Ulcer characterization was performed at every step of the protocol by evaluating both clinical and morphological parameters. Clinical parameters included the evaluation of the amount of lesion exudate, type of tissue, peri-wound skin condition, and signs of local infection. Exudate amount was classified in none, low, moderate, and high according to the clinical experience. The type of tissue was evaluated by estimating the percentage of necrotic tissue, sloughy tissue, and granulation tissue in a scale from none, <25%, 25-50%, 50-75% to 75-100%. Peri-wound skin condition was classified in normal, erythematous,

edematous, and macerated. Signs of local infection were considered positive in the presence of redness onset, heat, fever, pain, foul odor at the level of the wound or around it. Morphological parameters were evaluated by taking a picture of both lesions throughout all medications and at follow-up. Images were stored in a database, while estimation of wound surface area performed using ImageJ2 software (Scientific Computing Facility, MPI-CBG Dresden).

ELISA assay

Relative quantification of EVs-TGFβ1 from patients' serum was performed using DuoSet ELISA Development Systems (R&D Systems) according to manufacturer instructions. Then, 25 μg of total proteins were used for the ELISA assay. Sera from healthy patients (3 samples) served as internal control.

Histological examination

All samples were formalin-fixed and paraffin-embedded (FFPE). Three micron-thick paraffin sections for each case were collected onto charged slides and stained with haematoxylin-eosin (H&E), Masson's trichrome and acid fuchsine orange G (AFOG). In addition, in order to evaluate the microvascular proliferation, a serial paraffin section was processed by immunohistochemistry for CD31 (clone JC70, prediluted. Ventana Medical Systems, Tucson, AZ, USA) using an automated platform Ventana BenchMark AutoStainer (Ventana Medical Systems, Tucson, AZ, USA). Subsequently, histological sections were digitalized using the Aperio Scanner ScanScop XT (Wetzlar, Germany). On the slide immuno-stained with CD31, the area of microvascular proliferation was calculated in relation to the total area of the examined histological section.

Statistics

Categorical variables are expressed as frequencies (%), continuous variables are expressed as median and interquartile range (IQR). The Fisher's exact probability test was used to compare categorical variables. Comparison of continuous variables was performed using Pearson's chi-squared test. Statistical significance was set at $p < .05$. All statistical analyses were conducted with R version 3.6.2 (R foundation for statistical computing, Wien, Austria). In vitro results are representative of at least 3 independent experiments.

RESULTS

Patient characteristics

Between January 2020 and October 2022, four patients and five case-control lesions were enrolled in the study. One patient was enrolled twice due to the onset of bilateral multiple lesions that were treated with a 3-month interval. The enrollment procedure and treatment protocol are summarized in Fig. 1. Study population had 77-year median age (IQR 18) and 3 (75%) were females. Among risk factors for CVUs two patient (50%) presented with body mass index > 25, two patient (50%) had history of saphenectomy for varicose veins, one patient (25%) had experienced the occurrence of deep venous thrombosis, and one patient (25%) had history of recurrent chronic venous ulcers. All patients presented with multiple CVUs in the same limb, median persistence of active ulcer condition before enrollment was 11 months (IQR 8.5). Baseline CVU characteristics were similar in s-EV and Sham groups with absence of necrotic tissue, similar percentage of granulation tissue and absence of signs of infection. Lesions in s-EV group presented with a higher exudate amount (2/5 high exudate in s-EVs vs none in Sham control) and a more edematous peri-wound skin condition (3/5 in s-EVs vs none in Sham control) as reported in Table II. Median surface area at baseline was 758 mm^2 (IQR 1411 mm^2) for s-EV group and 182 mm^2 (IQR 429 mm^2) for the Sham group (Table 3).

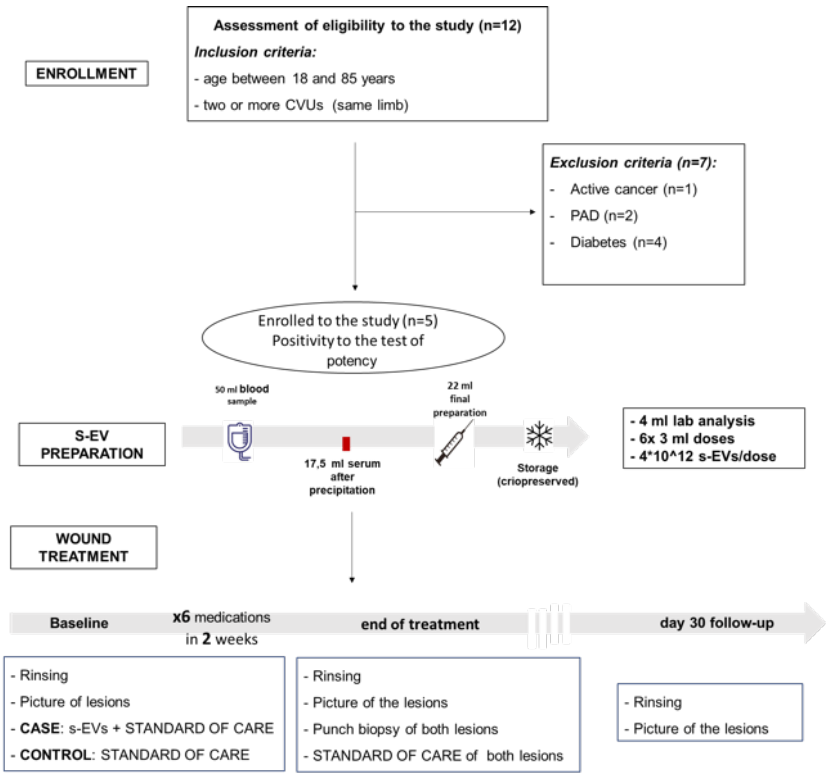


Fig. 1. Clinical trial protocol. Patients included in the study were assigned to s-EV-treatment or saline (Sham)-procedures (see Methods). s-EVs were collected from blood samples as indicated.

s-EVs isolated from CVU patients are enriched in TGβ1

s-EVs derived from serum of all patients were analyzed by NanoSight, TEM (Fig. 2A), and MACSPlex kit (Fig. 2B). As shown in Fig. 2C, exosomal markers CD63, CD9, and CD81 were detected, while GM130 protein served as s-EV negative marker. Moreover, using the MACSPlex kit we further confirmed the expression of exosomal markers and the enrichment of platelet and endothelial markers (2B). Finally, as previously reported[17] all patient's-EVs were enriched in TGFβ1 (Fig. 2D).

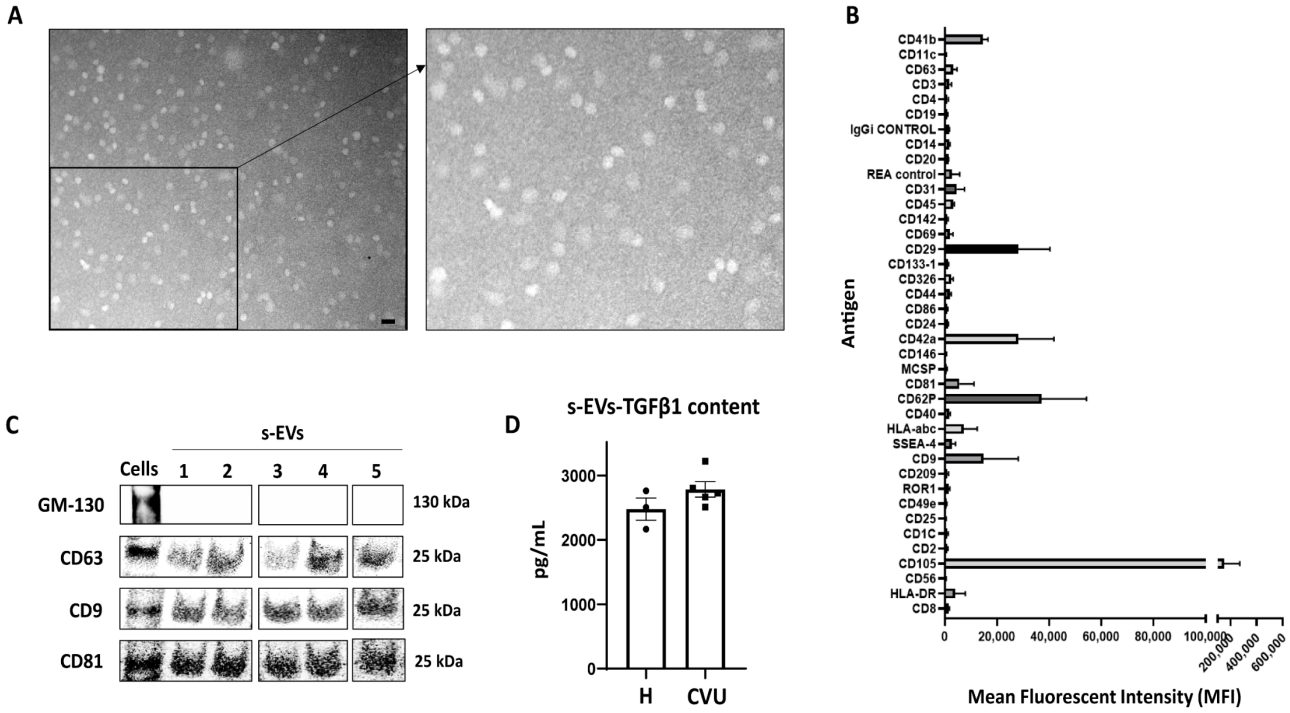


Fig. 2. s-EV characterization. (**A**) Representative images of TEM. Original magnification 140 K, scale bar: 100 nm. (**B**) FACS with MacsPlex kit on s-EVs recovered from all patients (**C**) Western blot analysis of exosome markers (CD63, CD9, and CD81) and negative s-EV marker (GM130). (**D**) TGFβ1 s-EV content in patients (CVU) and healthy subjects (H).

Autologous s-EVs promote CVU healing

All patients successfully completed the treatment. No adverse local or systemic reactions or adverse events have been recorded. None of the lesions presented signs of local infection during treatment and at the follow-up. Two lesions in the Sham group healed at follow-up. Qualitative CVU analysis showed how lesions treated with s-EVs were characterized by a higher percentage of granulation tissue compared to the Sham group (3 out of 5 lesions with 75-100% of granulation tissue in s-EVs *vs* none in the Sham group). This result was confirmed at day 30 (4 out of 5 lesions with 75-100% of granulation tissue in s-EV group). At baseline, sloughy tissue was higher in the s-EV group

compared to the Sham control (2 out of 5 lesions with 75-100% sloughy tissue for s-EVs compared to none in Sham group). However, lesions treated with s-EVs revealed a higher reduction of sloughy tissue already at the end of treatment (2 out of 5 lesions presented with more than 25% sloughy tissue in s-EVs compared to 3 out of 5 lesions in the Sham group). At 30-day follow-up, both untreated and treated lesions displayed <25% of sloughy tissue (Table 3). Surface analysis revealed that lesions treated with s-EVs presented a median surface reduction of 151 mm^2 compared to 84 mm^2 in the Sham group. This difference was even more evident at follow-up (385 mm^2 for s-EVs *vs* 106 mm^2 for the Sham group; *p=0.004*) (Table 4). Surface area normalization did not show statistical difference in terms of surface reduction between the groups. Representative images are reported in Fig. 3.

	s-EVs +				**Sham**				*p-value*
CHARACTERISTICS		**BASELINE**	**END TREATMENT**	**30-DAY FOLLOW-UP**		**BASELINE**	**END TREATMENT**	**30-DAY FOLLOW-UP ***	
Amount of exudate	low		1 (20%)	3 (60%)	low	3 (60%)	3 (60%)	1 (33%)*	*ns*
	moderate	3 (60%)	3 (60%)	2 (40%)	moderate	2 (40%)	2 (40%)	1 (33%)*	
	high	2 (40%)	1 (20%)		high			1 (33%)*	
% of necrotic tissue	none	5 (100%)	5 (100%)	5 (100%)	none	5 (100%)	5 (100%)	3 (100%)*	*ns*
	<25%				<25%				
	25-50%				25-50%				
	50-75%				50-75%				
	75-100%				75-100%				
% of sloughy tissue	none			2 (40%)	none	1 (20%)	2 (40%)	2 (66%)*	*ns*
	<25%	1 (20%)	3 (60%)	3 (60%)	<25%			1 (33%)*	
	25-50%		2 (40%)		25-50%	1 (20%)	1 (20%)		
	50-75%	2 (40%)			50-75%	3 (60%)	2 (40%)		
	75-100%	2 (40%)			75-100%				
% of granulation tissue	none				none				*ns*
	<25%	2 (40%)			<25%	2 (40%)	1 (20%)		
	25-50%	2 (40%)	1 (20%)		25-50%	1 (20%)	2 (40%)		
	50-75%	1 (20%)	1 (20%)	1 (20%)	50-75%	1 (20%)	2 (40%)	1 (33%)*	
	75-100%		3 (60%)	4 (80%)	75-100%			2 (66%)*	
Signs of infection	N° of infected lesions	0	0	0	N° of infected lesions	0	0	0	*ns*
Peri-wound skin condition	normal	1 (20%)	4 (80%)	3 (60%)	normal	1 (20%)	4 (80%)	2 (66%)*	*ns*

Table 3. Characterization of lesions according to clinical parameters. *Two lesions completely healed at day 30 follow-up. Sham corresponds to the lesion treated with saline.

		PATIENTs						
	Time surface measurement	**1**	**2**	**3**	**4**	**5**	**Median Baseline - end treatment**	**Median Baseline 30-day follow-up**
s-EVs +	**Baseline**	1331	2472	409	758	571	151 (IQR 544)	385 (IQR 681)
	End treatment	435	1800	318	630	420		
	30-day follow-up	250	1540	184	373	320		
	Baseline - end treatment	896	672	91	128	151		
	Baseline – follow-up	1081	932	225	385	251		
Sham	**Baseline**	77	44	319	661	182	84 (IQR 86)	106 (IQR 54)
	End treatment	59	43	235	433	78		
	30-day follow-up	0	0	188	471	76		
	Baseline-end treatment	18	1	84	228	104		
	Baseline- follow-up	77	44	131	190	106		
						p-value	*p*= 0.2	***p*= 0.004**

Table 4. Ulcer surface reduction. Ulcer surface is expressed as mm^2 at baseline, end of treatment, and at follow-up per each patient.

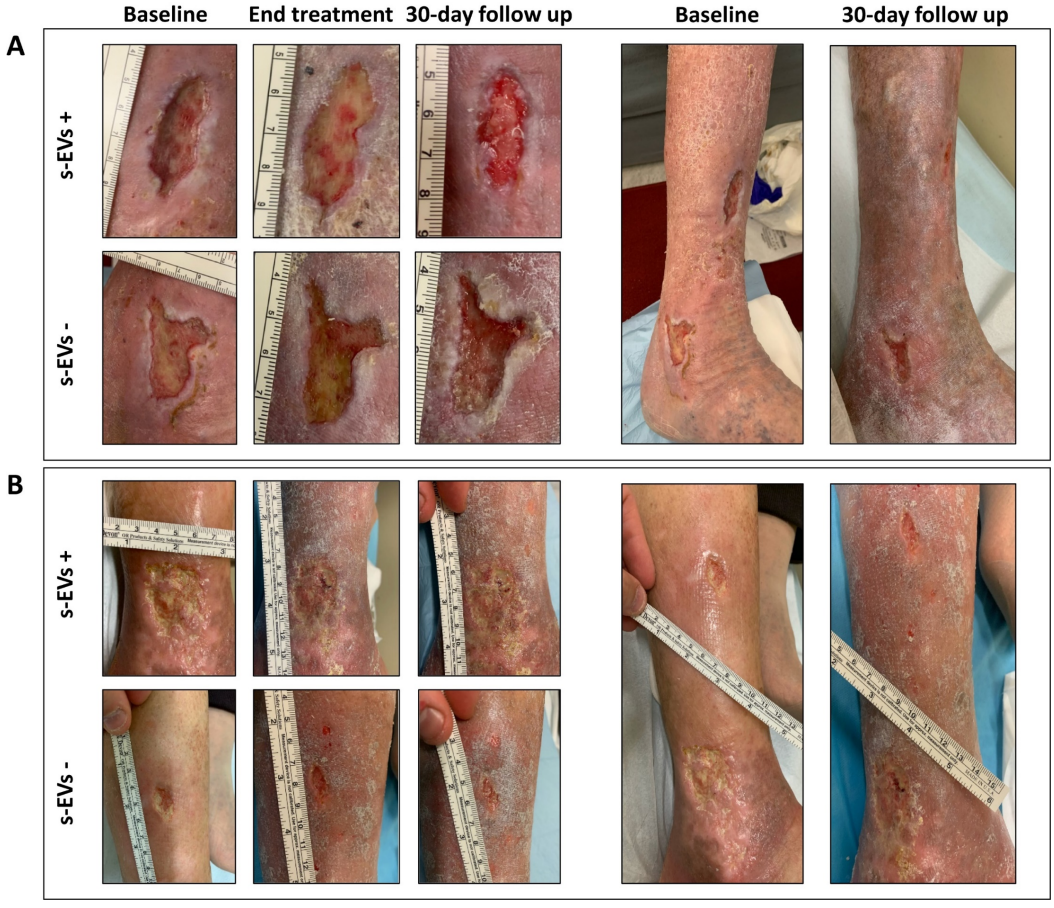


Fig. 3. Representative images of untreated or treated CVUs. (A-B) CVUs (from patient 4 and 5 respectively) were untreated (Sham) or s-EVs treated 3 times a week for two weeks (see MM). Images recorded at baseline, at the end of treatment, and at day 30 (follow-up) are reported.

Tissue regeneration and microvascular proliferation distinguish s-EVs treated CVUs

To further validate our clinical data, a 4-mm punch biopsy was performed at the end of treatment. Histologically, fibrinoid necrosis was more evident in Sham lesions compared to s-EVs treated ones (Fig. 4A-C and Fig. 5A-C) whereas fibrosis was more evident in s-EVs treated lesions, thereby demonstrating the presence of regenerative tissues (Fig. 4E-G and Fig. 5E-G). In addition, an increase in the microvascular proliferation areas in lesions subjected to s-EV treatment was observed (Fig. 4H and Fig. 5H). Specifically, in patient 1 the *ratio* between the area of vascular proliferation and the total area of the histological section examinated was 0.06 and 0.4 in the Sham group and s-EV treated lesions respectively (Fig. 6), while in patient 2, a ratio of 0.3 and 0.5 was calculated (Fig. 7).

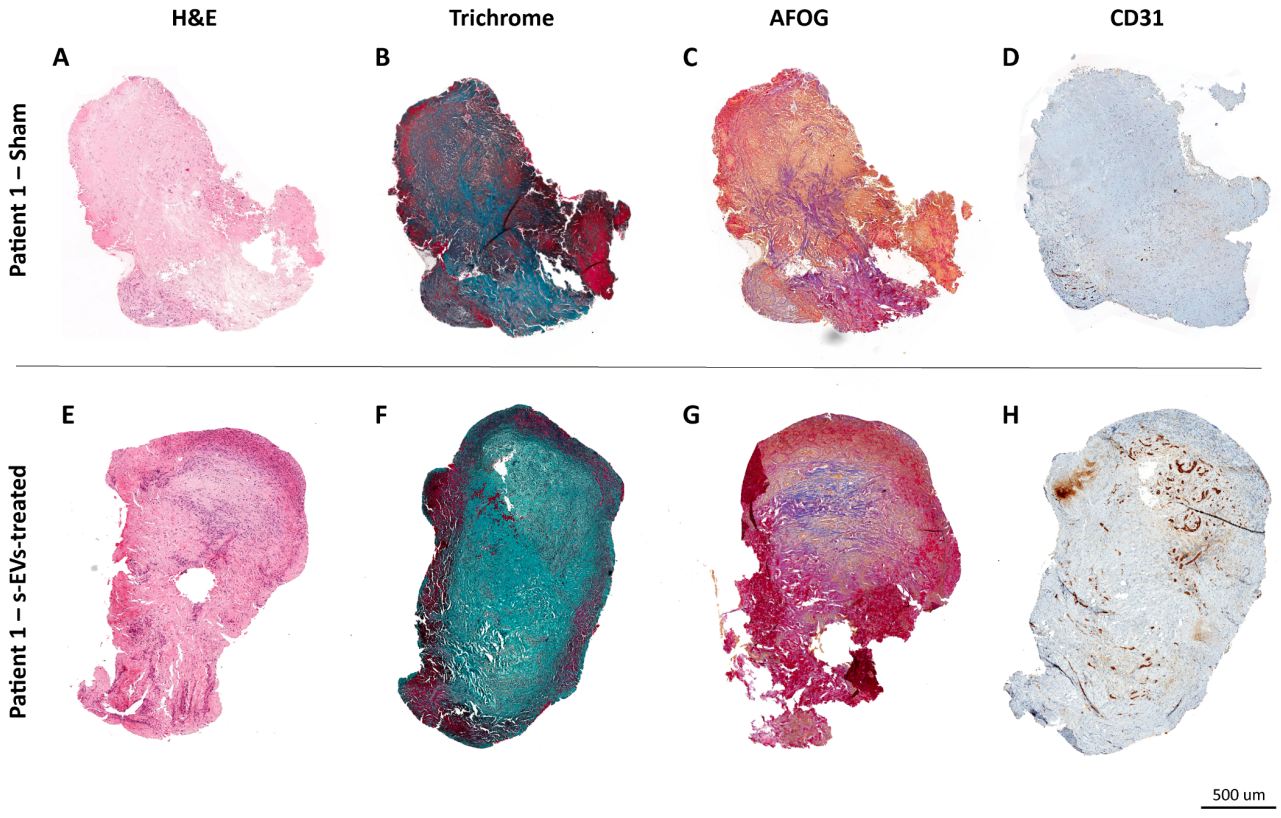


Figure 4. Histology of untreated (A-D) and s-EVs-treated lesions (E-H) in patient 1 (corresponding to patient 4). Sham lesion showed a prevalent fibrinoid necrosis (**A**: H&E, **B**: Masson's trichrome and **C**: AFOG) and a marginal *focus* of microvascular proliferation (**D**: CD31) whereas s-EVs-treated lesion exhibited a regenerative tissue with abundant fibrosis and neo-angiogenesis (**E**; H&E; **F**: Masson's trichrome; **G**; AFOG; **H**: CD31).

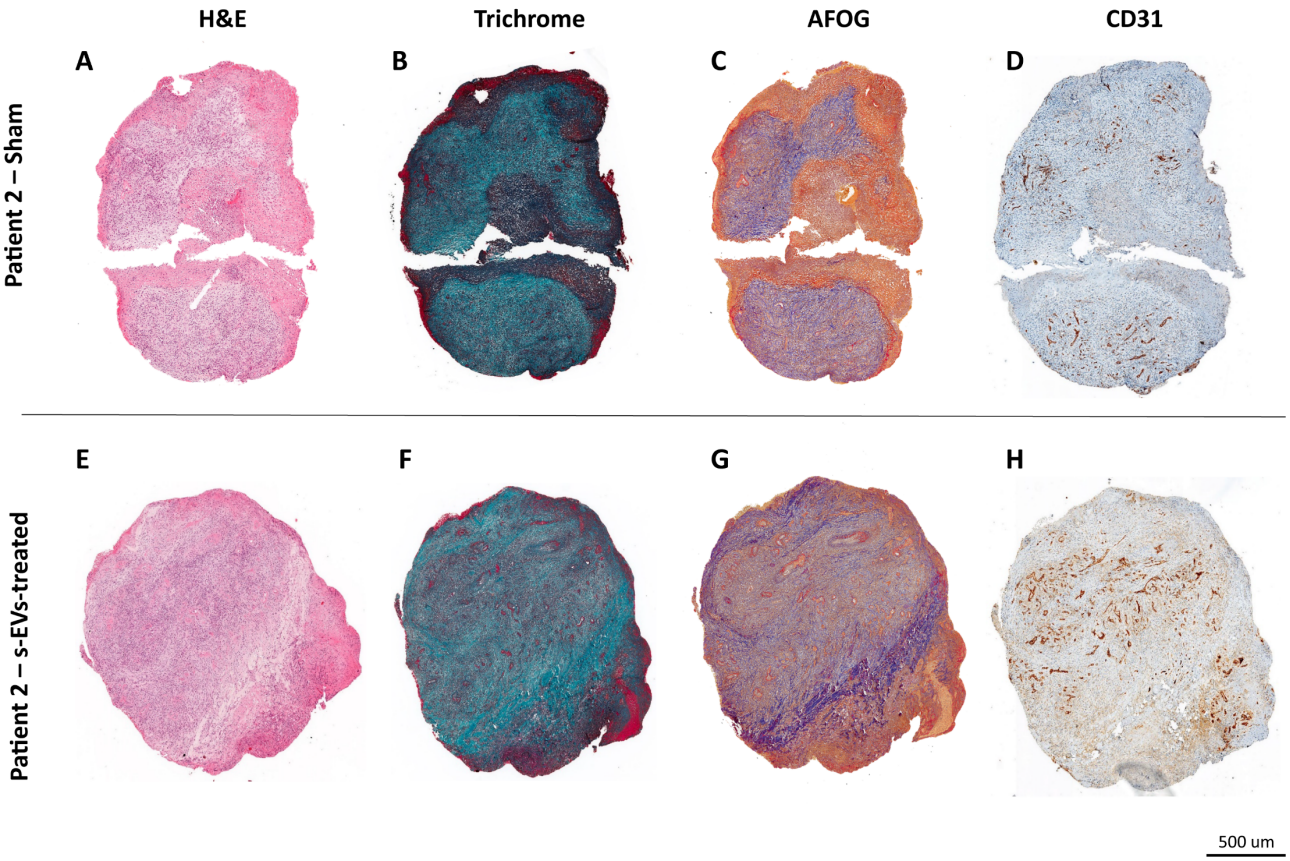
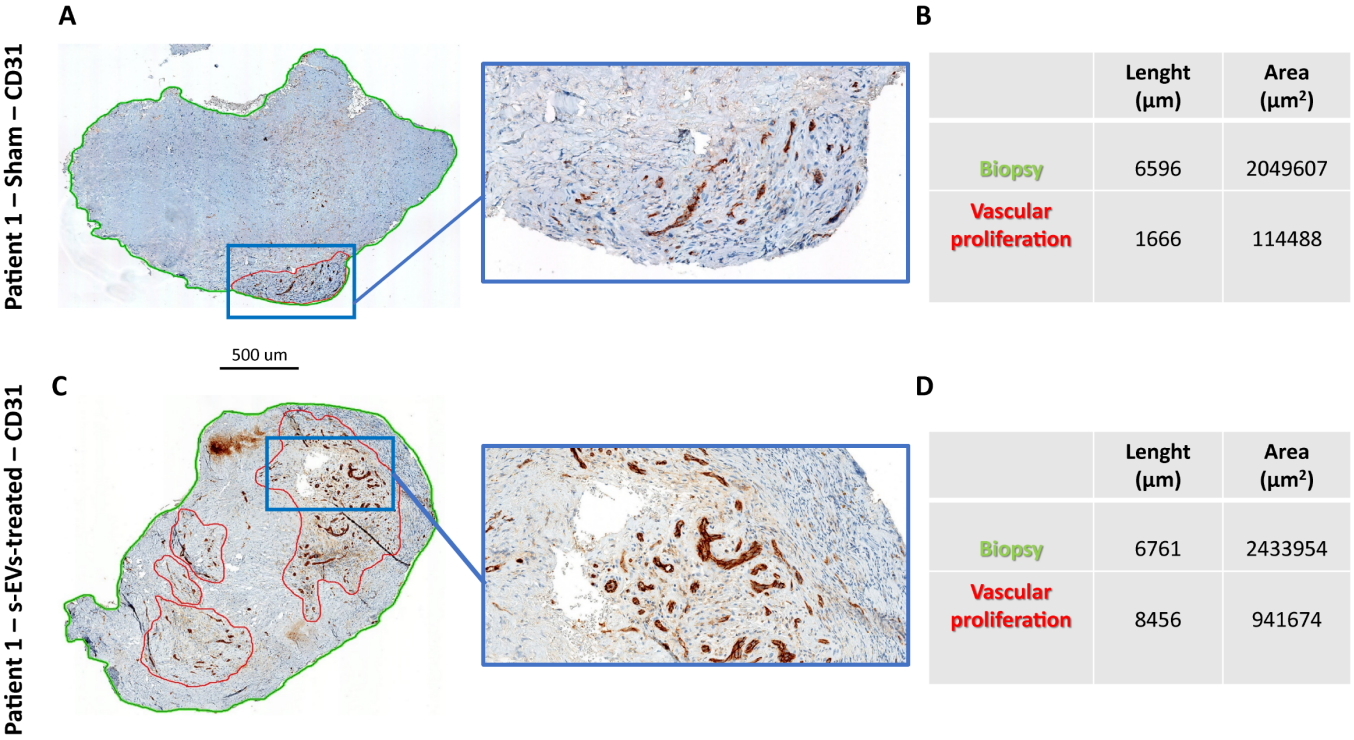


Figure 5. Histology of untreated (A-D) and s-EVs-treated lesions (E-H) in patient 2 (corresponding to patient 5). Although the **s**ham lesion showed fibrosis and vascular proliferation in the center of the sample with only marginal fibrinoid necrosis (**A**: H&E, **B**: Masson's trichrome; **C**: AFOG; **D**: CD31), in **s**-EVs-treated lesion neo-angiogenesis and collagen deposition resulted more evident (**E**: H&E, **F**: Masson's trichrome, **G**: AFOG; **H**: CD31).

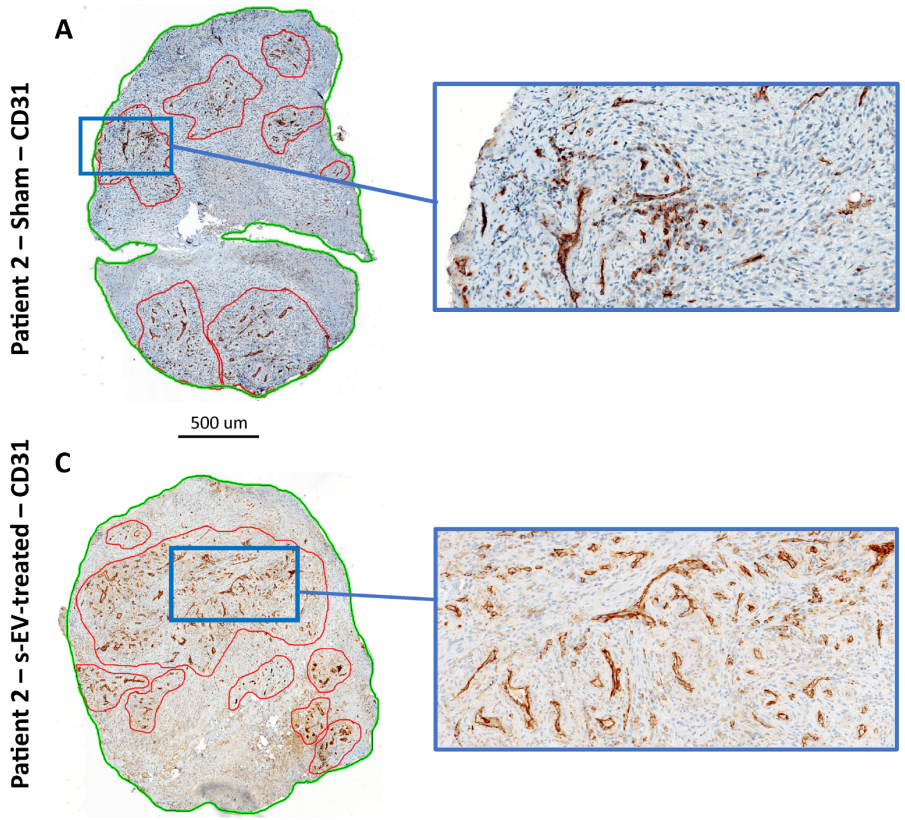


	Lenght (µm)	Area (µm²)
Biopsy	6596	2049607
Vascular proliferation	1666	114488

	Lenght (µm)	Area (µm²)
Biopsy	6761	2433954
Vascular proliferation	8456	941674

Figure 6. Evaluation of microvascular proliferation in Sham (A-B) compared to s-EVs-treated lesions (C-D) using CD31 immunohistochemistry in patient 1 (corresponding to patient 4). In panel **A** and **C**, green lines surround the perimeter of the biopsy whereas red lines the perimeter of the neo-angiogenesis foci.

The length (µm) and the area (µm^2) of the total surface of the biopsy and of the vascular proliferation foci in Sham (**B**) and s-EVs-treated lesion (**D**) are reported.



B

	Lenght (µm)	Area (µm^2)
Biopsy	11281	3932843
Vascular proliferation	10438	1384486

D

	Lenght (µm)	Area (µm^2)
Biopsy	8223	4488296
Vascular proliferation	14719	2062873

Figure 7. Evaluation of microvascular proliferation in Sham (A-C) compared to s-EVs-treated lesions (C-D) using CD31 immunohistochemistry in patient 2 (corresponding to patient 5). In panel **A** and **C**, green lines surround the perimeter of the biopsy whereas red lines the perimeter of the neo-angiogenetic foci. The length (µm) and the area (µm^2) of the total surface of the biopsy and of the vascular proliferation foci in Sham (**B**) and s-EVs-treated lesion (**D**) are reported.

DISCUSSION

This is the first pilot case-control interventional study aimed to investigate the effectiveness of autologous s-EVs to improve the healing process of CVUs. We selected patients unresponsive to conventional treatment and without any tendency to heal in the former 3 months. s-EVs were characterized by TEM, western blot, and FACS analyses and evaluated *in-vitro* for their functional activity before CVU treatment. We demonstrated that, compared to standard of care medicament, s-EVs were effective in healing the lesion at the end of treatment, but more importantly at day 30 follow-up. Overall, this study first demonstrated the healing properties of autologous s-EVs in patients to whom currents approaches failed.

The impact of EVs as therapeutics has gained particular interest in the last decades[21]. EVs from different sources have provided evidence of effectiveness in several preclinical models of disease[15-18]. However, their clinical application has been delayed by the lack of standard isolation procedures providing adequate yields and scalability[19]. In the present pilot case-control interventional study, we demonstrated that the charge-based precipitation protocol offers a great s-EV yield and scalability and is suitable for application in a Blood Bank. In an autologous contest the limitation for blood obtainable from patients obliged us to exclude alternative purification methods known to produce highly purified EVs but with insufficient yield. Although the precipitation protocol applied to s-EVs is known to co-precipitate contaminants such as lipoproteins[20], we tested their functional impact after removing contaminants (Sephadex-100 column, and floating density separation)[18,20] without modifications in their proangiogenic activity. The same results were obtained when proteomic and transcriptomic profiling were evaluated[15]. Moreover, a positive s-EV safety and benefit profile can be provided by their autologous origin, already experienced in clinical settings for diverse blood derivatives. Finally, the enrichment of s-EVs in the autologous product used in this study was supported by the expression the exosome markers and by TEM analysis. Consistent with data obtained in high cardiovascular risk patients[15,17], we found that s-EVs do not substantially differ in their cell of origin among patients. Indeed, they highly expressed endothelial and platelet markers, features supported by the inflammatory environment commonly connoting CVU patients.

This is the first study evaluating the regenerative potential of s-EVs in humans, and in CVU patients. Since the absence of underlying arterial blood malperfusion is mandatory to guarantee a correct tissue repair[12], we started by selecting patients with CVUs, instead of those with ulcers generated by arterial disease. However, to minimize potential biases connected with systemic and local factors affecting the healing process, we enrolled only patients with multiple lesions in the same limb, confident that this would increase the robustness of the results obtained, despites the drawback of patient selection/recruitment. We did not perform randomization, while favored the “worst case scenario” thereby treating the lesions with the higher area of damage. We were confident that this approach would also minimize the effects of other not deemed local factors affecting the healing process thus favoring the control group. Comparing the e-EVs to the Sham controls, we obtained significant and promising results allowing for the surface wound reduction and the type of tissue distinguishing the lesions. Indeed, we proved that s-EVs are instrumental for the healing processes and that their therapeutic properties persist beyond the end of treatment. Both clinical and histopathological analysis revealed that s-EVs promote angiogenesis at ulcer’s bed with a progressively reduction of fibrous-fibrinous content replaced by granulation and epithelized tissue. We have previously reported

that s-EV TGFβ1 content predicts their proangiogenic activity in high cardiovascular risk patients.[17] The impact of TGFβ1 in the angiogenic switch and in tissue regeneration have been proven in different models of disease[22]. Moreover, the contribution of TGFβ1 s-EV cargo in boosting angiogenesis and in preventing muscle damage was also reported in a mouse model of hind limb ischemia[18]. The histological analysis demonstrating an increased vessel density and collagen deposition upon s-EV treatment, sustains the renewing impact of TGFβ1 that we found enriched in patients derived s-EVs.

Overall, this study provides the first proof-of-concept for s-EV effectiveness in the treatment of broad CVUs unresponsive to current therapeutics. Moreover, the lack of local or systemic adverse events that we recorded during the treatment and at follow-up strongly supports the excellent s-EV safety profile.

Strengths, limitations, and future perspectives

Strengths: *i.* This study represents the first s-EV application in CVU patients, confirming the efficacy provided by preclinical studies; *ii.* it provides the rational for the feasibility in clinic based on the potency test, s-EV yield, and hasty approval in health centers equipped with a blood transfusion service; *iii.* the ready availability of autologous products locally triggering regenerative process; *iv.* the EV enriched autologous precipitate shows an excellent safe profile.

Limitations and perspectives: The number of patients can be considered the most relevant limitation of this study. However, this drawback has been balanced by the stringent inclusion criteria we adopted for the recruitment and the allocation of patients. The time consuming for both patients and clinicians also emerges as a limitation of our proposed protocol. However, since treatment efficacy was still evident at day 30 follow up different s-EV administration schedules (i.e., once a week for 6 weeks) can be proposed to improve patient compliance and managing in outpatient setting. Additionally, the development of transdermal s-EV enriched patches can be pursued to improve the feasibility in clinical settings. Finally, we cannot exclude that serum derived contaminants may contribute to the beneficial effects of s-EV enriched preparations obtained by the charge-based precipitation technique.

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[1] Division of Vascular Surgery, Department of Surgical Sciences, University of Turin, Turin, Italy

[2] Politecnico of Turin, Turin, Italy, PhD programme.

[3] Blood Bank, Molinette Hospital, Città Della Salute e Della Scienza of Turin, Turin, Italy

[4] Pathology Unit, Department of Oncology, Città Della Salute e Della Scienza of Turin, University of Turin, Turin, Italy

[5] Department of Medical Sciences, University of Turin, Turin, Italy

[6] Dermatologic Clinic, Department of Medical Sciences, University of Turin, Turin, Italy

LG: contributed study conception and design, recruited patients, recovered medical records, performed patient treatment and data analysis; SDA supervised quality requirements of the blood products and contributed to the study design; MS prepared extracellular vesicles for clinical application; RS: performed the histological analyses; MACP: performed the test of potency; GO: performed the histological analyses; AS: isolated s-EV for characterization including Western Blot analysis; TL: performed TGFβ1 ELISA assay; PQ: contributed to data interpretation; ML: contributed to data interpretation; FV: contributed to data interpretation; GC performed TEM and contributed to the study conception, design, and data interpretation; MFB performed study conception and design, and supervised manuscript redaction with input from all authors. All authors revised the manuscript.

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DISCLOSURES

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ABBREVIATIONS

AFOG: acid fuchsine orange G

CVU: chronic venous ulcer

EDQM: European Directorate for the Quality of Medicines

EVs: extracellular vesicles

GPGs: good practice guidelines

GMP: good manufacturing practice

H&E: Hematoxylin and Eosin

IQR: interquartile range

PAD: peripheral artery disease

PEG: polyethylene glycol

RPM: revolution per minute

s-EVs: serum-derived extracellular vesicles

TGFβ1: transforming growth factor-β

VEGF: vascular endothelial growth factor

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Overall PhD Activities

- teaching assistant and examiner to the Medicine and Surgery degree course of “Clinica e Patologia del Torace”
- teaching assistant to the Vascular Surgery Master’s degree courses.

Publications

1. **Gibello L**, Verzini F, Spalla F, Frola E, Porro L, Peluttiero I, Ripepi M, Boero M, Varetto G. Long-term outcomes of open and Endovascular Abdominal aortic repair in younger patients. Ann Vasc Surg 2022; 85:323-330
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Training

Hard skills (already submitted and approved in the Google form):

Course Name	**Validated Hours**
Segmentazione, riconoscimento e ricostruzione di immagini mediche in applicazioni chirurgiche	20
European Vascular Course 2021	20
Terapie avanzate (nanomedicina, terapia genica e cellulare)	20
Principi, materiali ed applicazioni della robotica nella biomedicina	20
Data mining in healthcare and biomedicine	20

Additive manufacturing in Bioengineering and Surgery	20

Hard skills hours: 120

Soft Skills (already submitted and approved in the Google form):

Course Name	Validated Hours
SCUDO Research integrity	5
SCUDO Responsible research and innovation, the impact of social challenges	5
SCUDO Public speaking	5
SCUDO Navigating the hiring process: CV, tests, interview	5
Open Science and FAIR Data	8
SCUDO Personal branding	5
SCUDO Time management	5
SCUDO Thinking out of the box	5
SCUDO Entrepreneurial finance	5
SCUDO Communication	5

Soft skills hours: 53