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Successful tissue engineering of competent allogeneic venous valves

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Objective: **The purpose of this study was to evaluate whether tissue-engineered human allogeneic vein valves have a normal closure time (competency) and tolerate reflux pressure in vitro.**
Methods: **Fifteen human allogeneic femoral vein segments containing valves were harvested from cadavers. Valve closure time and resistance to reflux pressure (100 mm Hg) were assessed in an in vitro model to verify competency of the vein valves. The segments were tissue engineered using the technology of decellularization (DC) and recellularization (RC). The decellularized and recellularized vein segments were characterized biochemically, immunohistochemically, and biomechanically.**
Results: **Four of 15 veins with valves were found to be incompetent immediately after harvest. In total, 2 of 4 segments with incompetent valves and 10 of 11 segments with competent valves were further decellularized using detergents and DNAse. DC resulted in significant decrease in host DNA compared with controls. DC scaffolds, however, retained major extracellular matrix proteins and mechanical integrity. RC resulted in successful repopulation of the lumen and valves of the scaffold with endothelial and smooth muscle cells. Valve mechanical parameters were similar to the native tissue even after DC. Eight of 10 veins with competent valves remained competent even after DC and RC, whereas the two incompetent valves remained incompetent even after DC and RC. The valve closure time to reflux pressure of the tissue-engineered veins was <0.5 second.**
Conclusions: **Tissue-engineered veins with valves provide a valid template for future preclinical studies and eventual clinical applications. This technique may enable replacement of diseased incompetent or damaged deep veins to treat axial reflux and thus reduce ambulatory venous hypertension. (J Vasc Surg: Venous and Lym Dis 2015;3:421-30.)**

Clinical Relevance: **The use of natural, human scaffolds to produce tissue-engineered venous segments containing functioning valves will revolutionize the surgical correction of deep venous reflux in patients with chronic venous insufficiency and leg ulcer. Reconstructive deep venous surgery in the form of valvuloplasty, transplantation, and neovalve construction has met limitations in the rare availability of valves to be repaired, lack of donor sites, and inadequate conditions to create new valves. This tissue-engineered procedure produces the functioning unit "valve-conduit," and surgery will be used only to implant it.**

Chronic venous insufficiency (CVI) describes a condition that affects the venous system of the lower extremities, in which persistent ambulatory venous hypertension is the main pathophysiologic factor leading to pain, edema, skin changes, and ulcerations.[1] The more serious consequences of CVI, such as venous ulcers, have an estimated prevalence of 0.1% to 1.0%.[2,3] The overall prognosis of venous ulcers is poor.[4] Risk factors found to be associated with CVI include age, sex, family history of varicose veins, obesity, pregnancy, and phlebitis.[5,6] The financial burden of venous ulcer disease on the health care system is an estimated $1 billion spent annually on treatment of chronic wounds in the United States or ≤2% of the total health care budget in Western countries.

The conventional treatment of CVI with compression stockings combined with superficial surgery seems to improve venous hemodynamics but achieves only a 65% ulcer healing rate after 24 weeks, with a recurrence rate of 12%/year.[7] Reconstructive deep venous surgery, such as valvuloplasty, autotransplantation, and neovalve construction, has proved to be an option, improving ulcer healing rates and providing an ulcer-free period in patients for

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Author conflict of interest: S.S.H. is a cofounder and board member of NovaHep AB, a company that has licensed the technology of tissue engineering of blood vessels. There is a patent pending related to the procedure of tissue engineering, for which A.R. and J.H. as scientists at the Oslo University Hospital are coinventors.

Additional material for this article may be found online at www.jvsvenous.org.

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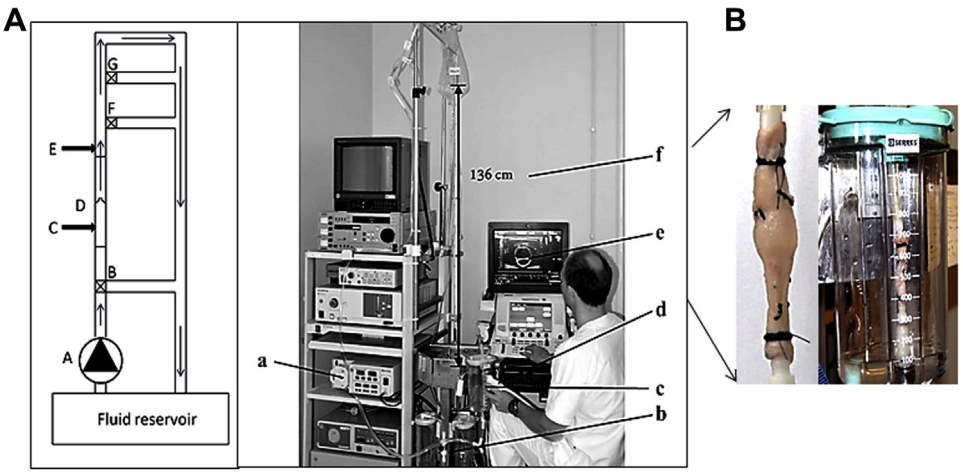

Fig 1. Picture of the setup and in vitro model for functional testing of veins. **A,** The system is circulated with room-temperature saline containing an ultrasound contrast agent for enhancement of the Doppler signals. A peristaltic pump (*a*) pumps the saline through the whole circuit; a mechanical valve (*b*) enables flow through the vein (*c*) during output from the pump. An ultrasound probe (*d*) is used for visualization of the vein (*e*) and evaluation of the flow through the vein valve. Reflux pressure at the valve site is adjusted by the height of the reservoir (*f*) above the vein. **B,** Enlarged picture of a valve-bearing venous segment mounted in the circuit and placed in a container filled with saline to facilitate visualization by ultrasound.

whom conventional treatment has failed. Axillary or saphenous transplantation to treat axial reflux in these patients has been reported to yield ulcer healing rates of up to 70%. However, the durability of these procedures remains an issue, taking into consideration that the average age of patients in reported materials is about 50 years, and only half of the transplants remained functional after 4 years.[8-11] In addition, the demanding surgical technique needed has limited the use of reconstructive deep venous surgery to very few centers, and although reports have been produced that show good results, no randomized clinical trials have yet been published. Experimental efforts to understand the venous valve function to be able to create mechanical and bioprosthetic valves also exist. The challenges encountered are to avoid immune reaction, thrombogenicity, and migration in the vein.[12-16]

Thus, given the prevalence and socioeconomic impact of CVI and the poor treatment modalities available for it, alternative, more effective therapeutic options need to be explored. Regenerative medicine may offer novel strategies to treat these patients. Tissue-engineered venous segments containing competent valves might represent a more durable solution for this young group of patients. In addition, the required surgical skills will not be as demanding (interposition of a vein segment), which might help spread this possibility of treating severe CVI cases.

Perfusion decellularization (DC) and recellularization (RC) of tissues and organs is believed to be a successful platform technology for creating scaffolding materials for tissue engineering and regenerative medicine.[17,18] DC is the process of removal of all host cells and nuclear material by physical, chemical, and enzymatic methods.[19] Thus, whole organ acellular matrices provide an attractive scaffold for the repopulation with cells for an engineered tissue or organ because of the physiologic resemblance to the original tissue, including intact three-dimensional anatomic architecture, preserved spatial array of extracellular matrix (ECM) components, vascular network, and biomechanical properties. Creating regenerated donor organs by repopulating organ scaffolds with patient-specific mature or stem cell-derived populations would allow us to personalize transplantation medicine and reduce the need for long-term immunosuppressive therapy. Using this approach, we recently tissue engineered human veins using autologous stem cells from the patients and successfully transplanted three pediatric patients with portal vein thrombosis.[20,21] In the present study, we have further expanded the application of this technology to tissue engineer human veins containing valves. We hypothesized that vein segments with valves tissue engineered by the technology of DC and RC with stem cells would yield a blood vessel with physiologic properties resembling the original tissue and that the valves would retain their competency, making them resistant to reflux.

METHODS

The Swedish and Norwegian Institutional Review Boards and Ethics Committees approved all research protocols.

Harvesting of valve-bearing veins. All veins (N = 15) used in this study were harvested at Oslo University Hospital, Norway. Vein segments about 8 cm (range, 5.5-10 cm), including the common femoral vein, profunda femoral vein, and femoral vein, were harvested from adult human cadavers by using vascular surgical technique and carefully ligating all side branches. Valves were identified, and 15 segments were cut with a margin of 3 to 4 cm on each end of the valves. Sterile saline solution was injected

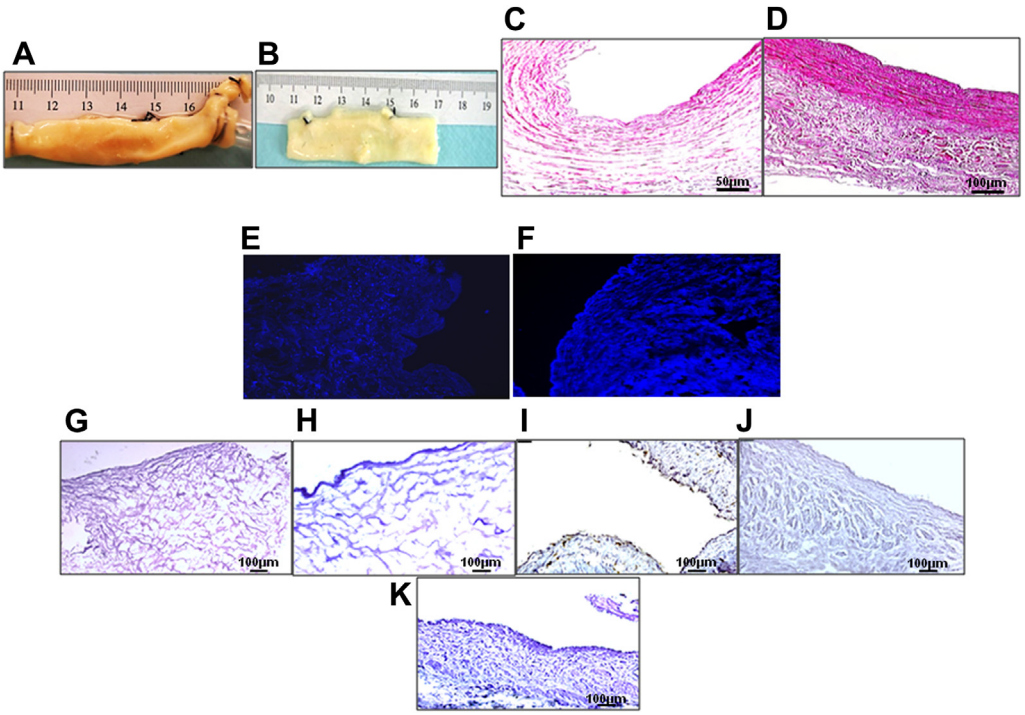

Fig 2. Gross morphology and microscopic view of the decellularized valve-containing vein segments. Gross morphology of **(A)** native vein and **(B)** decellularized vein after 14 cycles. Hematoxylin and eosin (HE) staining of **(C)** decellularized vein after 14 cycles showing preserved tissue architecture and absence of blue-black nuclei and **(D)** a native vein (positive control) showing presence of nuclei. DAPI staining of native vein showed several nuclei **(E)**, but not in **(F)** decellularized vein. Immunohistochemical staining of decellularized veins showed absence of HLA class I **(G)** and class II **(H)** antigen expression. Normal vein (positive control) stained positive for HLA class I (**I,** *brown*) but not HLA class II **(J)**. **K,** Negative control.

manually, and functioning valves were identified by the retainment of solution above the valves. It was also possible to see the valve leaflets closing. We had approximately 8-cm segments of the veins with a valve in the middle. Four of 15 veins with valves were found to be incompetent immediately after harvest. All vein segments were thoroughly rinsed in phosphate-buffered saline (PBS) containing 0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B and preserved at 4°C. The samples were transported within 1 week on ice to the Laboratory for Transplantation and Regenerative Medicine at the Sahlgrenska University Hospital, Gothenburg, Sweden. In total, 2 incompetent veins and 1 competent vein with valves were used for the biomechanical tests, whereas 10 competent and 2 incompetent veins with valves were used for DC and RC.

Functional in vitro testing of valve-bearing veins. This test was performed at the Oslo Vascular Centre, Oslo University Hospital, Norway. A custom-made test setup was used to assess the functionality of the veins before and after RC (Fig 1, *A*). The in vitro test setup used in the present study for evaluating the functionality is a modification of the one used by Geselschap et al.[22] The aim of our setup was basically to have a model that would allow us to assess venous valve function only at a determined pressure.

The vein was mounted vertically in the in vitro flow circuit and perfused with room-temperature saline. The vein was connected to the fluid circuit by conic connectors, secured with sutures at both ends (Fig 1, *B*). A commercial peristaltic pump (Model No. 700044; Baxter Healthcare Corp, Deerfield, Ill) delivered intermittent flow to the circuit.

A mechanical valve was used to regulate the flow direction through the circuit during outflow from the pump, and when the same valve was switched to open position, it achieved backflow in the vein until the valve closed. Reflux pressure in the vein was adjusted by the height of the column of fluid above the valve. The choice of reverse flow pressure level at 100 mm Hg was based on the estimated distance between the heart and the popliteal fossa and supported by the mathematical model published by Fragomeni et al.[23] Ultrasound Doppler technique was used to detect potential reflux at the site of the venous valve (9 MHz linear probe, Vivid E9; GE Healthcare, Boston, Mass). To optimize the visualization with ultrasound, the vein segment was submerged in a plastic container filled with saline. A contrast agent (SonoVue; Bracco Diagnostics, Cranbury, NJ) was administered in the saline solution to enhance the echogenicity and to enable recordings of the flow and flow direction in the circuit through the

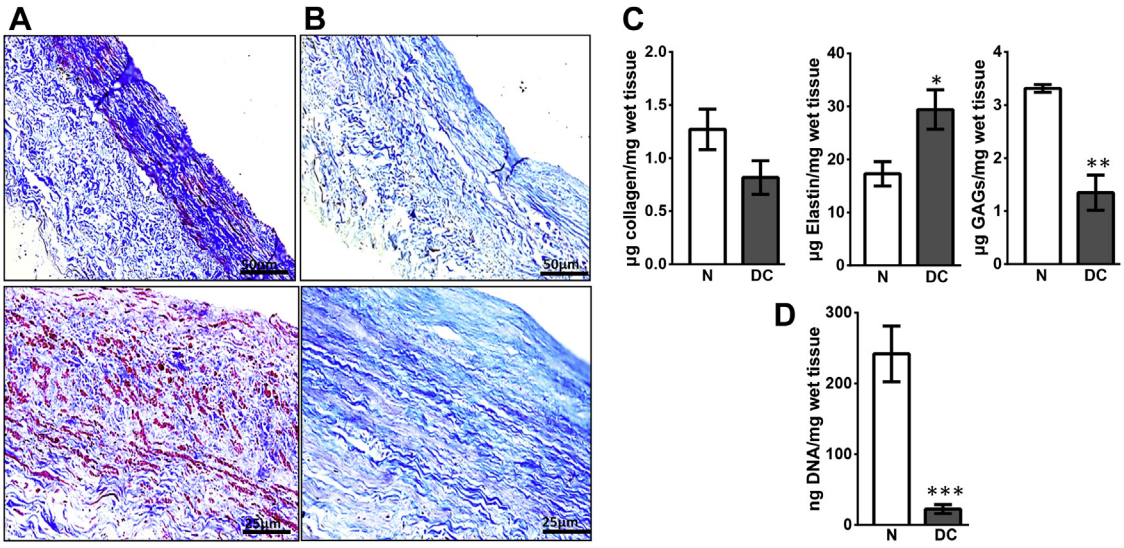

Fig 3. Extracellular matrix (ECM) in decellularized veins. **A,** Masson trichrome (MT) staining of normal vein (positive control) showing presence of nuclei (*black*), cytoplasm (*red/pink*), and collagen (*blue*). **B,** In the decellularized vein, no nuclei were found, indicating lack of endothelial and smooth muscle cells, but with abundant collagen still present. **C,** Graphs showing decrease in amount of collagen (n = 5), significant increase in elastin (n = 5; $P = .03$), and significant decrease in glycosaminoglycans (*GAGs*; n = 5; $P = .007$), respectively, after 14 decellularization (*DC*) cycles in comparison to native (*N*). **D,** Graph showing significant decrease in amount of DNA after DC ($P = .0002$). *Scale bar* **A** and **B**, *upper panel* = 50 μm, and *lower panel* = 25 μm.

vein valve. The time from flow reversal until cessation of flow was used as a measure of valve closure time. In addition, the vein diameter was measured at the site of the leaflet at a reflux pressure at 100 mm Hg.

DC and characterization of veins. The processes of DC, RC, and associated analysis were carried out in Gothenburg, Sweden. DC was carried out as described by us earlier using 1% Triton, 1% tri-*n*-butyl phosphate (TnBP), and 4 mg/L DNAse.[20] In brief, the veins were washed for 72 hours in distilled water. One end of the vein was sealed with 4-0 suture, and the vein was filled with the decellularizing solution; the other end was clamped. The vein was placed in a container with the same solution and agitated on a shaker for 4 hours at 170 rpm and 37°C. All the solutions used for DC contained 0.02% sodium azide (71290; Sigma-Aldrich, Seelze, Germany) and 0.18% EDTA (ED2SS; Sigma-Aldrich). Triton (X-100; Sigma-Aldrich) and TnBP (A16084; Alfa Aesar, Karlsruhe, Germany) solutions were prepared in distilled water; DNase was prepared in PBS containing calcium and magnesium (D8662; Sigma-Aldrich). After every detergent, the veins were washed with distilled water for 10 minutes. The procedure from Triton to DNase is termed one cycle. The decellularized vein segments were evaluated for residual DNA content, staining with hematoxylin and eosin (HE) and Masson trichrome (MT) by standard procedures for acellularity and quantification of various ECM proteins. Immunohistochemistry for detection of HLA class I and class II antigens was performed by standard procedure.

See the Supplementary Methods (online only) for details of DNA quantification, ECM staining and quantification, acid- and pepsin-soluble collagen quantification, sulfated glycosaminoglycan (GAG) quantification, and soluble elastin quantification.

RC of veins. On the day of RC, 20 to 25 mL of peripheral venous blood was collected from healthy donors (age group, 25-35 years) in sterile heparin-coated Vacutainer tubes and transported to the laboratory as soon as possible (within 2 hours). Healthy donors were individuals and personnel with no obvious diseases or disorders and with no venous reflux disease. The volume of blood required was determined by the length of the vessel and of the pipes used in the bioreactor.

The entire RC process was performed under sterile conditions, and all perfusions were carried out in an incubator at 37°C supplied with 5% CO_2. Before RC, the veins were perfused with heparin (387107; LEO Pharma, Malmö, Sweden) at a concentration of 50 IU/mL PBS for 2 hours. The heparin was drained off, and whole blood was immediately perfused for 48 hours at 2 mL/min speed. The blood was then drained off, and the vein was rinsed with PBS containing 1% penicillin-streptomycin-amphotericin until blood was completely removed. The vein was subsequently perfused 4 days with endothelial and 4 days with smooth muscle media. The complete endothelial medium was prepared with MCDB131 (10372; Life Technologies, Stockholm, Sweden) basal medium supplemented with 10% heat-inactivated human AB serum (34005100; Life Technologies), 1% glutamine (25030; Lonza, Copenhagen, Denmark), 1% penicillin-streptomycin-amphotericin, and EGM-2 SingleQuot kit (CC4176; Lonza) that contained ascorbic acid, hydrocortisone, transferrin, insulin, recombinant human vascular

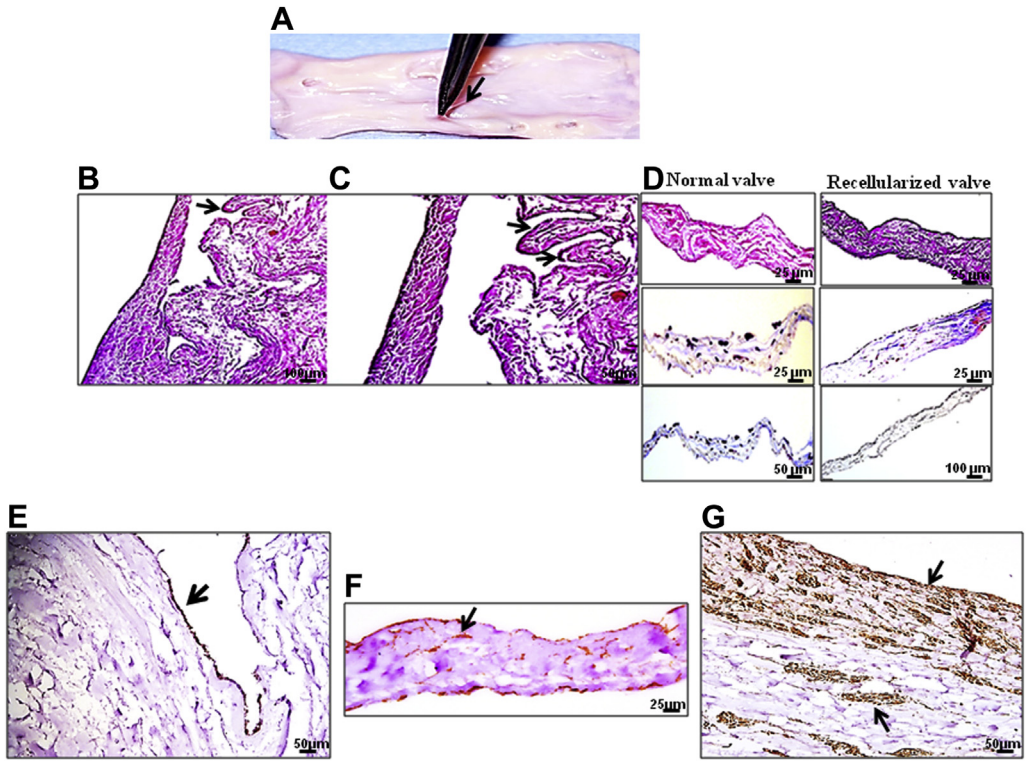

Fig 4. Characterization of recellularized valve containing vein segments. **A,** Gross picture of a recellularized vein showing valves. **B** and **C,** Hematoxylin and eosin (HE)-stained microscopic pictures of a recellularized vein and valve at low and high magnification, respectively. The pictures show presence of continuous cells at endothelial lining on both vein and valve (*arrow*). **D,** HE and Masson trichrome (MT) staining of normal (positive control) and recellularized valves showing presence of nuclei in all. **E,** CD31 staining of a recellularized vein showing continuous endothelial lining. **F,** Alpha smooth muscle actin staining of valve showing presence of smooth muscle cells in the valve. **G,** Alpha smooth muscle actin staining showing smooth muscle cells in media of a recellularized vein.

endothelial growth factor (VEGF), human fibroblast growth factor, human epithelial growth factor, heparin, and gentamicin sulfate. The complete smooth muscle medium was prepared using 500 mL Medium 231 (M231; Life Technologies) supplied with 10% heat-inactivated human AB serum, 1% penicillin-streptomycin-amphotericin, and 20 mL of smooth muscle growth supplement (S00725; Life Technologies). Vein scaffolds were recellularized for a total of 10 days.[21]

See the Supplementary Methods (online only) for details of characterization of recellularized veins, biomechanical analysis, sterility control test, and statistics.

RESULTS

Functional in vitro testing of cadaveric valve-bearing veins. A total of 15 femoral vein specimens were harvested from human cadavers. The median time between death and harvest was 3 days (2-6) and between death and testing, 6 days (5-7). Four of 15 veins with valves were incompetent immediately after harvest. In general, the median diameter of the vein specimens was 9.8 mm (7.5-14) and 9.8 mm (8-14.2) after RC. The 11 vein segments with competent valves (normal closure time ≤0.5 second at a pressure of 100 mm Hg)[24] and the 4 vein segments with incompetent valves were then transported to Sweden. In Sweden, further examination of the four incompetent valves showed presence of holes in the valves of two veins, mechanical damage in one, and no apparent problems with the fourth. This was examined by turning the veins inside-out. Two of the four incompetent veins with valves were used for biomechanical testing; the other two were used for DC and RC. Further, one vein with competent valves was used for biomechanical testing, whereas 10 of 11 were used for DC and RC. Thus, in total, 3 of 15 veins were used for biomechanical studies, whereas 12 of 15 were used for DC and RC.

We found that 8 of 10 segments with competent valves retained their competency after the RC process, whereas 2 showed reflux already after DC. In both cases, the valves in the segments were damaged mechanically while the specimens were handled during DC. In addition, the two segments with incompetent valves already at the start were also found to be incompetent even after DC and RC. Thus, RC did not repair the damaged valves.

DC, characterization, and functional in vitro testing of decellularized valve-bearing veins. Treatment with 1% Triton and 1% TnBP successfully decellularized the

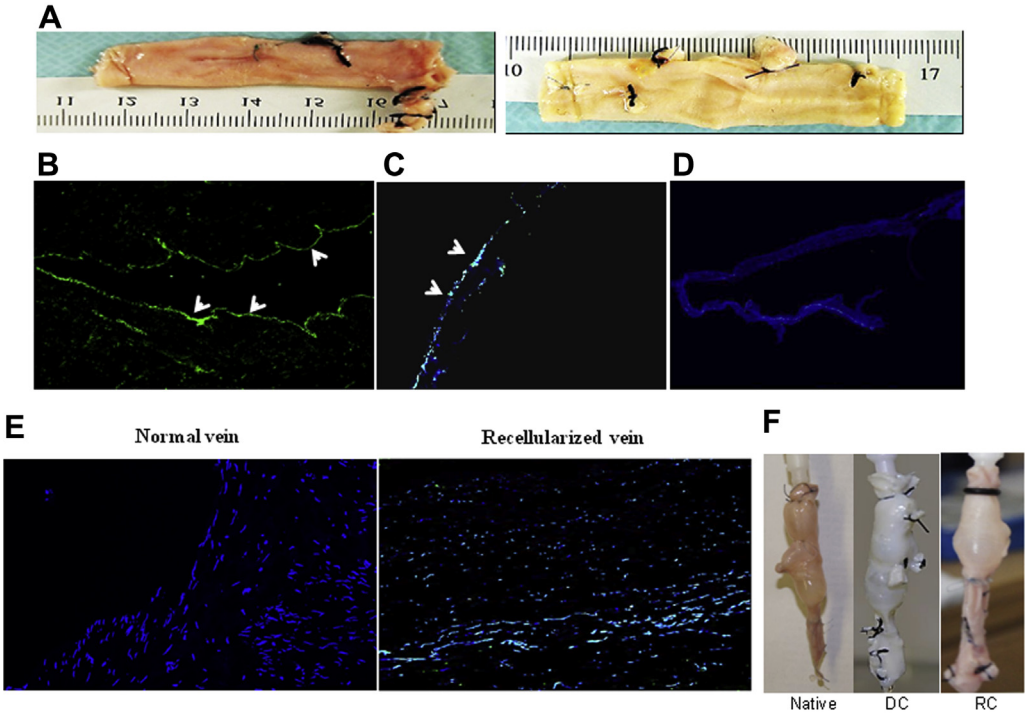

Fig 5. Macroscopic and microscopic views of the bioengineered vein grafts with autologous whole peripheral blood. **A,** Gross morphology of recellularized vein appearing pinkish in color. **B** and **C,** Immunofluorescence staining of tissue-engineered vein **(B)** and valve **(C)** showing presence of endothelial cells (*green*) in the lumen when stained with antibodies to von Willebrand factor. **D,** Negative control. **E,** DAPI staining showing abundant nuclei in normal vein and vein recellularized with peripheral whole blood. **F,** The picture shows functioning valves when a solution is flushed through native, decellularized (*DC*), and recellularized (*RC*) veins. **B-D,** Magnification ×400. **E,** Magnification ×100.

veins in 14 cycles. The gross morphology of a harvested vein (Fig 2, *A*) and a decellularized vein (Fig 2, *B*) appeared pale and translucent. There was absence of nuclei in the veins (Fig 2, *C*) of all specimens tested after 14 cycles as detected by HE staining compared with native (Fig 2, *D*). This was further confirmed by DAPI staining in native and DC veins (Fig 2, *E* and *F*). In addition, no positive staining for HLA class I (Fig 2, *G*) or class II antigens (Fig 2, *H*) was detected compared with native veins (Fig 2, *I* and *J*). Note that normal endothelial cells and smooth muscle cells do not constitutively express HLA class II (Fig 2, *J*). Fig 2, *K* shows no staining in the negative control. MT staining showed the presence of an abundant amount of collagen (*blue color*) but no nuclei (*black*) after DC (Fig 3, *A* and *B*). The quantification of collagen, elastin, and GAGs with Sircol, Fastin, and Blyscan assays (Biocolor, County Antrim, United Kingdom), respectively (Fig 3, *C*), showed significant loss of GAGs ($P = .007$) and significant increase in elastin ($P = .03$) but not collagen ($P = .09$) compared with native veins. The DC protocol also led to significant decrease in DNA amount from 241.95 ± 39.44 ng/mg of tissue in normal veins to 22.44 ± 6.29 ng/mg in decellularized veins (Fig 3, *D*) ($P = .0002$). All decellularized veins were tested for functionality by flushing a fluid retrograde through the valves. Two of 10 competent valves showed reflux, demonstrating loss of their function during DC; 8 valves did not show reflux, indicating no effect of DC on function. Four decellularized veins were tested for valve closure time using in vitro setup, and the valve closure time was <0.5 second.

RC, characterization, and functional in vitro testing of recellularized valve-bearing veins. All 12 decellularized veins were recellularized in a custom-designed bioreactor with peripheral blood. Fig 4, *A* shows the gross morphology of recellularized veins containing valves. HE staining showed the presence of several nuclei (*blue*) in the vein (Fig 4, *B*) and the valves (Fig 4, *C*). HE and MT staining of recellularized vein valves also showed abundant nuclei (*blue* and *black*, respectively; Fig 4, *D*). In addition, immunohistochemistry staining with endothelial cell marker CD31 showed a continuous endothelial cell layer in veins (Fig 4, *E*); staining with smooth muscle actin confirmed the presence of spindle-shaped smooth muscle cells in the valves (Fig 4, *F*) and tunica adventitia of the veins (Fig 4, *G*). The gross morphology of recellularized veins appeared pinkish with good tunica externa (Fig 5, *A*). Immunofluorescence staining with antibodies to another endothelial cell marker, von Willebrand factor (*green*), confirmed the presence of endothelial cells in the lumen (Fig 5, *B*) and the valves (Fig 5, *C*) compared with the negative control (Fig 5, *D*). Fig 5, *E* depicts the DAPI staining of a native and

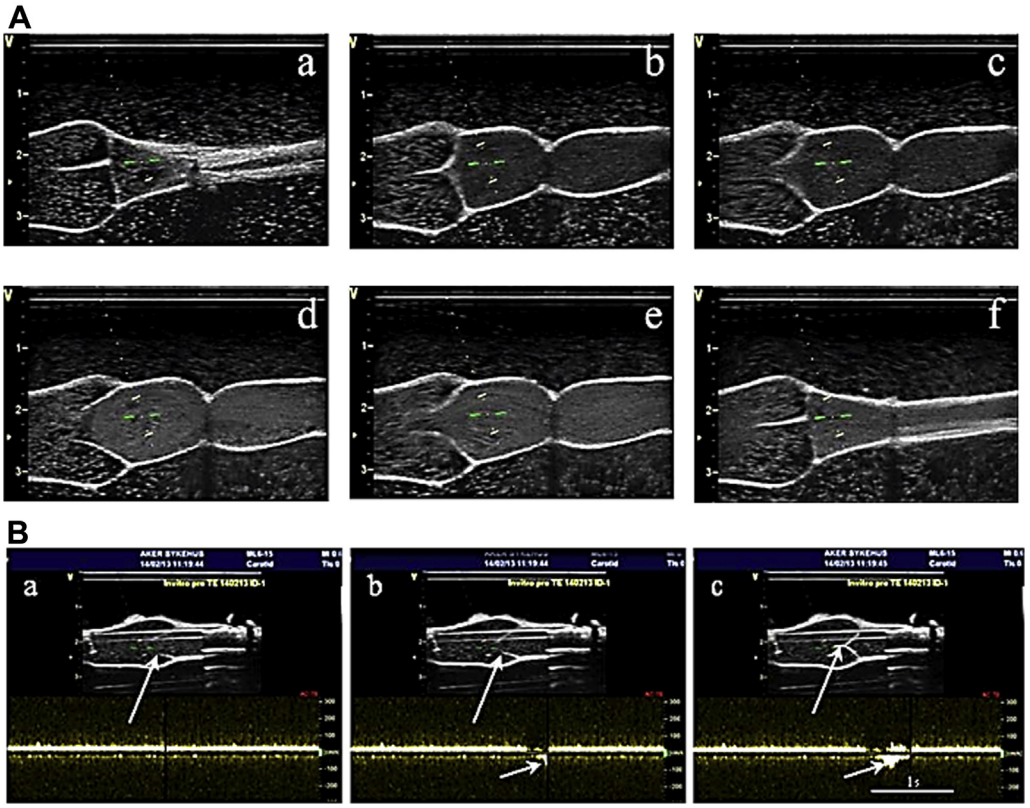

Fig 6. Functional testing of tissue-engineered valve-containing veins. **A,** Two-dimensional pictures from ultrasound show a longitudinal projection of the vein segment: a closed valve (*a*); the gradual opening of the valve during antegrade flow (*b-d*); and the closing of the valve with retrograde flow (*e* and *f*). **B,** The *yellow* Doppler scale gives the valve closure time in seconds.

recellularized vein showing the presence of blue nuclei in both sections. Testing of valve function showed that freshly harvested, decellularized and recellularized veins distend when flushed with fluid through a syringe (Fig 5, *F*). Ultrasound testing demonstrated the gradual opening of the valve during antegrade flow and closing of the valve with retrograde flow (Fig 6, *A*), and the Doppler scale revealed the valve closure time in seconds (Fig 6, *B*; Video, online only). Only 8 of 12 recellularized veins were functional. The veins that lost function while decellularized and the veins nonfunctional from the beginning did not show function.

Biomechanical testing of the valves. In total, six recellularized veins (n = 12 valves) and three normal veins (n = 6 valves) were tested. The vein valves were gradually torn apart starting from the suture. The tear progressed horizontally, giving rise to peaks when the fibers of the valve broke. Eventually one of the ends of the valve was torn from the vein wall, as indicated by the last peak in the force/elongation diagram (Fig 7, *A*). Forces at first break (peak) were above 0.8 N for all vein valves that proved to function in the functional in vitro test (Fig 7, *B*). The median force was not significantly different between normal and RC functional valves (Fig 7, *C*).

Sterility control test. In both culture media of all the veins, no marked increase in absorbance was recorded at 14 days of culture. The optical density at 600 nm measured in the spectrophotometer for fresh media (0.006) was similar to that of perfused media (0.010), which was the same as the negative control (0.010). The optical density of the positive control was high (0.135). No colonies of bacteria or fungal growth were seen on the tryptone soya agar plates, confirming sterility of the vein segments.

DISCUSSION

For the first time, we demonstrate the successful preservation of valve function of tissue-engineered human valve-bearing vein segments in an in vitro setting. The main aim of the study was to demonstrate that the technology of DC and RC generates a graft with a competent valve, provided the native vein already has a functioning valve. The present study also demonstrates the successful re-endothelialization of decellularized human vein segments and vein valves with a simple peripheral blood sample that enabled significantly the formation of extracellular monolayers. Although transplant donors would provide fresher tissue, we for the first time explored the use of cadavers as a source of the tissue. Interestingly, the majority of the vein segments taken from human cadavers 4 to

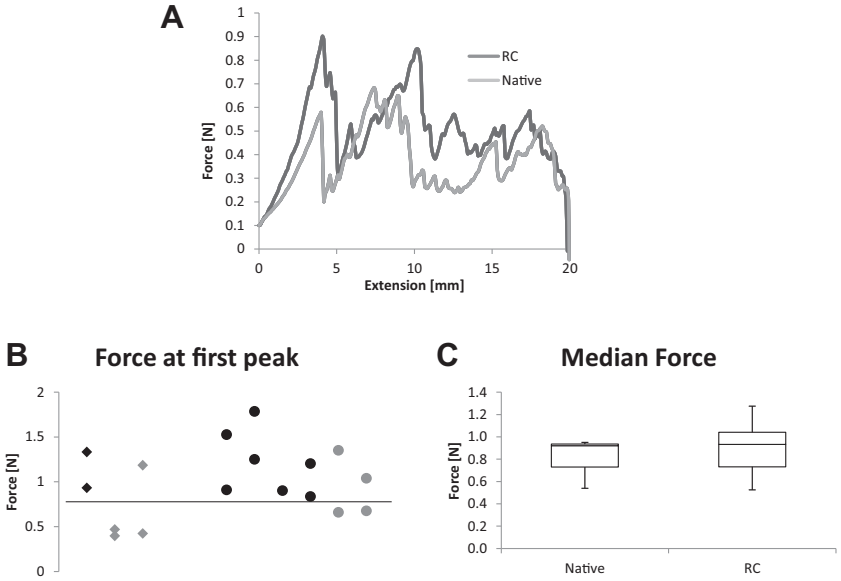

Fig 7. Mechanical analyses of the recellularized (*RC*) valves. **A,** Representative diagram of the deformation behavior for native and RC vein valves. The valves were gradually torn apart horizontally, resulting in a series of peaks. **B,** Force at first peak for each pair of valves from the same vein. Native vein valves are *square marked*, while RC valves are *circle shaped*. *Black color* marks functioning veins, while grey colored do not function. The *black line* represents a cutoff for how high force at first peak a vein needs to have to function. **C,** Box plots of median force of working valves indicating no significant differences.

6 days after demise still had functioning grafts and could be used for tissue engineering. This promising observation can help increase the pool of blood vessel donors. Because of the lack of transplant donors and limited access to these types of donors, we are currently developing a program for using cadaver veins. The ability of the decellularizing process to leave "footprints" for RC may open the possibility for brain-dead, heart-beating transplant donors and xenograft tissue.

We found it important for the specimen to be harvested with precise vascular surgical technique, and the distance between the actual vein valve and the ends should be at least 4 cm. In a future clinical study, we intend to test the specimens after each of the following procedures: (1) harvesting, (2) DC, and (3) RC. We believe that the in vitro model can be further simplified by connecting the vein segment directly to a fluid reservoir that is placed at 1.36 meters (100 mm Hg) high to assess the competence of the veins.

Mechanical analysis of the valves showed that in most of the functioning valves, the force at first peak was above 0.8 N for both vein valves in a pair, indicating that 0.8 N may be the cutoff criterion required to define strength of functional valves. However, larger numbers of samples will have to be tested to confirm this interesting finding, and such studies are ongoing in our laboratory.

Interestingly and importantly, the vein specimen's structure and function do not seem to be affected by a window of time of up to 7 days from death to harvest and testing. Thus, the pool of cadaver donors for blood vessels can be increased enormously. The histologic results showed that DC with Triton/TnBP/DNase was complete, as adjudged by DNA quantification, which was <50 ng/mg weight. It is reported that >50 ng/mg dry weight is normally required to evoke a potential immunologic activity.[25]

We found that perfusion of peripheral whole blood results in the formation of a clear extracellular monolayer and presence of smooth muscle cells in the media. The use of a simple peripheral blood sample to recellularize the vein segments is a clear advantage over the more tedious approaches, such as isolation and expansion of mature cells or stem cells from bone marrow or peripheral blood. In our recent study,[21] we reported that the major cell types in circulating peripheral blood that contribute to re-endothelialization and smooth muscle cell repopulation are $VEGFR2^{+}/CD45^{+}$ cells and a small fraction of $VEGFR2^{+}/CD14^{+}$ cells. Furthermore, function and strength were also preserved in the recellularized vein valves as indicated by results obtained using the in vitro test model and biomechanical test. Thus, the present results are further supported by our recently published clinical proof-of-concept study, in which we successfully transplanted three pediatric patients with tissue-engineered veins using autologous peripheral whole blood. Interestingly, none of the four incompetent veins with valves regained their competency after RC, indicating that cells and elastin on the valves are not critical factors for valve functionality. However, a greater number of incompetent valves need to be tissue

engineered to fully understand the problems involved. According to our observations, maintenance of the mechanical property may be one of the most important factors for competency.

Patients with CVI who prove refractory to conventional treatment may benefit from reconstructive deep valve reconstruction. However, this is a treatment that is not widespread and readily available because of complex workup and demanding surgical technique. Both venous valve transfer and valve construction have a durability that could be improved because the average age of these patients is 50years.[8-11] Venous valvuloplasty has been reported to provide competency in ≈60% and ulcer-free recurrence in ≈60% at 30 months.[26] Other procedures for reconstruction of nonfunctioning venous valves resulting from post-thrombotic valve destruction include transposition, transplantation, cryopreserved vein valve allografts, and neovalve construction. Cryopreserved vein valve allografts have also been used but are limited because of frequent complications, such as early thrombosis and poor patency and competency.[27] Thus, reconstructive deep venous surgery in the form of valvuloplasty, transplantation, and neovalve construction has its limitations. However, the use of natural, human scaffolds to produce tissue-engineered venous segments containing functioning valves may revolutionize the surgical correction of deep venous reflux in patients with CVI and leg ulcer.

Although the results are promising, this is an in vitro study and cannot predict the outcome for future human implantations. Other limitations are the small number of patients studied and the limited number of biomechanical studies performed on decellularized valves. It would also be good in future studies to include tests such as burst pressure and compliance. Tissue-engineered valve-containing vein segments may be a therapeutic option in selected patients in whom deep venous reflux and venous hypertension are the main pathophysiologic features leading to recurrent leg ulcer. We believe that the results of the present study are encouraging and constitute the basis required to start a clinical pilot study.

CONCLUSIONS

In the future, personalized tissue-engineered veins with competent valves may enable the replacement of incompetent or destroyed deep vein valves in patients with CVI.

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AUTHOR CONTRIBUTIONS

Conception and design: AR, JH, SH, JJ
Analysis and interpretation: VK, AR, JH, SH, JJ
Data collection: VK, AR, JH, EO, JS, HB
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Final approval of the article: VK, AR, JH, EO, JS, HB, SH, JJ
Statistical analysis: VK, HB
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SUPPLEMENTARY METHODS (online only).

DNA quantification. DNA was extracted from seven native and nine decellularized veins by use of a commercially available kit; 20 mg of tissue was collected, and DNA extraction was carried out according to the manufacturer's instructions (69506; Qiagen, Sollentuna, Sweden). Extracted DNA was quantified at 260-nm wavelength by use of a NanoDrop (ND-1000; Thermo Fisher Scientific, Wilmington, Del).

Extracellular matrix (ECM) staining and quantification. Briefly, to demonstrate collagen and the connective tissues, the formalin-fixed vein tissue sections were refixed overnight at room temperature in Bouin fixative, followed by staining with the Masson trichrome staining kit (No. 25088; Polysciences Inc, Warrington, Pa). The dyes employed during the staining procedure stained the collagen fibers blue, the nuclei black, and the cytoplasm and muscle fibers red.

Acid- and pepsin-soluble collagen quantification. The acid- and pepsin-soluble collagen content in the ECM was measured by a Sircol soluble collagen assay kit (S1000; Biocolor, County Antrim, United Kingdom). To extract acid- and pepsin-soluble collagen, the 25 mg of native and decellularized tissue (n = 5) was digested with 0.5 M acetic acid containing 0.1 mg/mL pepsin (P7012; Sigma-Aldrich, Seelze, Germany) for 48 hours at 4°C. The soluble collagen was incubated with 1 mL of Sircol dye reagent for 30 minutes at room temperature. The collagen-dye complex was precipitated by centrifugation at 10,000 *g* for 10 minutes, and the supernatant was removed. The pellets were dissolved in 1 mL of alkali reagent, and the relative absorbance was measured in a 96-well plate at 555 nm by a microplate reader (PowerWave XS; BioTek Instruments, Winooski, Vt).

Sulfated glycosaminoglycan (GAG) quantification. The sulfated GAG content in the ECM was measured with a Blyscan sulfated GAG assay kit (B1000; Biocolor). To extract sulfated GAGs, 25 mg of native and decellularized tissue (n = 5) was digested with a 0.2 M sodium phosphate buffer (pH, 6.8) containing 250 μg/mL of papain (P3125; Sigma-Aldrich), 5 mM cysteine hydrochloride (C3290000; Sigma-Aldrich), and 10 mM EDTA (ED2SS; Sigma-Aldrich) for 4 to 6 hours (until the tissue was completely dissolved) at 65°C. The suspension was centrifuged at 10,000 *g* for 10 minutes. The extracted sulfated GAGs (100 μL) were mixed with 1 mL of Blyscan dye and shaken for 30 minutes. The precipitate was collected by centrifugation for 10 minutes and then dissolved in 0.5 mL of dissociation reagent. The absorbance was measured in a 96-well plate at 656 nm by a microplate reader.

Soluble elastin quantification. The soluble elastin content in the ECM was measured by a Fastin elastin assay kit (F2000; Biocolor). To extract soluble elastin, 20 mg of native and decellularized tissue (n = 5) was hydrolyzed with 0.25 M oxalic acid at 100°C for 4 to 5 hours (until the tissue was completely dissolved). The insoluble residues were separated by centrifugation. The supernatant was collected, and the sediment underwent an additional extraction under the same conditions. The extracted soluble elastin was mixed with 1 mL of Fastin dye and shaken for 90 minutes. The precipitate was collected by centrifugation for 10 minutes and then dissolved in 250 μL of dissociation reagent. The absorbance was measured in a 96-well plate at 513 nm by a microplate reader.

Characterization of recellularized veins. To visualize the presence of endothelial cells, antibodies to CD31 (1:100, ab9498; Abcam, Cambridge, UK) and von Willebrand factor (1:100, SC73268; Santa Cruz Biotechnology, Heidelberg, Germany) were selected and stained by immunohistochemistry and immunofluorescence; smooth muscle actin (1:50, ab7817; Abcam) was stained by immunohistochemistry to visualize smooth muscle cells.

Biomechanical analysis. All biomechanical analyses were performed at the SP Technical Research Institute, Borås, Sweden. After the veins were cut open with surgical scissors, the mechanical properties of the vein valve were evaluated by tearing the valve in the horizontal direction with the help of a 4-0 nonabsorbable monofilament suture made of polypropylene attached to the grips of an Instron 5566 (Instron, Norwood, Mass). A preload of 0.1 N with a test speed of 20 mm/min was used. The accuracy of the tensile tester is 0.5% in force and 0.5% in elongation, based on calibrations performed regularly according to ISO 7500-1:2004 and ISO 9513:1999. Force at first peak was measured, and median force was calculated for each sample.

Sterility control test. Sterility during recellularization was evaluated by collecting 1 mL of perfused endothelial and smooth muscle media collected after every 2 days during culture and tested for microbial contaminants. About 500 μL of collected media was added to fluid thioglycollate broth, plated on tryptone soya agar plates, and incubated at 37°C for 14 days. The medium exposed to outside air was used as the positive control, and only the medium was used as the negative control. The growth of fungi and aerobic and anaerobic bacteria was visualized and also measured in a spectrophotometer for absorbance at 600 nm. Differences in absorbance were noted.

Statistics. Results are presented as median and range. The error bars represent the standard error mean for original value. Mann-Whitney *U* tests were performed to compare the effects of decellularization and recellularization on the ECM and valves. $P \leq .05$ was considered to be a significant difference.