


Macromolecular Crowding Supports the Generation of Basal Membrane-Rich Pericyte-Based Cell Sheets Useful for Cell Therapy of Diabetic Wounds

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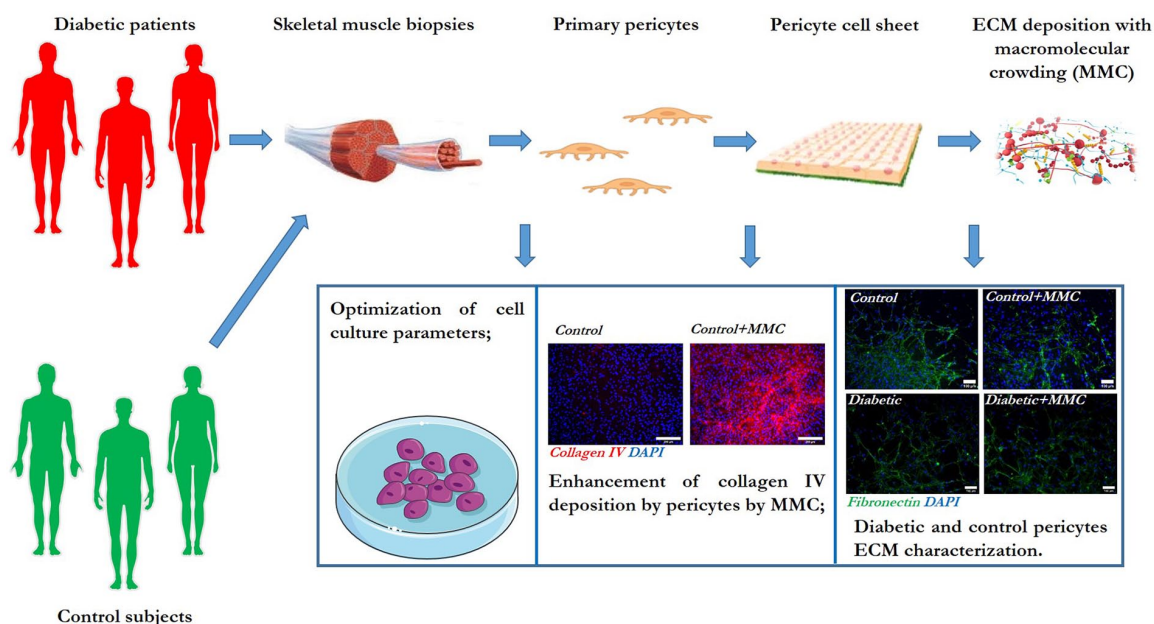
Abstract

Diabetic foot ulcers (DFUs) are associated with a high risk of amputations and a 50% 5-year survival rate due at least in part to the limited angiogenic and wound healing capacity of patients with diabetes. Cell therapy via intramuscular injection of peripheral blood mononuclear cells showed encouraging but limited results. Such limitations may arise from the limited ability of therapeutic cells to adhere to the target tissue. The development of a methodology able to support the targeted delivery of viable angiogenic cells would improve cell therapy outcomes in DFU. Here, we optimized a protocol for the production of autologous extracellular matrix (ECM)-rich pericyte-based cell sheets for cell delivery. Pericytes were isolated from skeletal muscle biopsies of DFU patients and non-diabetic controls and characterized by flow cytometry and immunofluorescence. Human umbilical vein endothelial cells used for the optimization of collagen IV deposition showed a positive correlation with seeding density and a negative one with sub-culture passaging ($P < 0.05$). Macromolecular crowding significantly increased collagen IV deposition both in human umbilical vein endothelial cells and in patient-derived pericytes ($P < 0.01$) without affecting proliferation ($P > 0.05$). Finally, DFU patient-derived pericytes effectively deposited ECM supporting their use for autologous cell sheet production.

Keywords

diabetic foot ulcer, pericytes, endothelial progenitor cells, cell sheets, macromolecular crowding

Graphical Abstract



Introduction

Wound healing and angiogenesis are impaired in diabetic patients. Wound healing is composed of four phases in which endothelial cells (ECs) and pericytes play a central role: homeostasis, inflammation, proliferation, and remodeling^{1,2}. Following vessel rupture, resident cells react to the reduced oxygen tension by synthesizing pro-angiogenic factors (e.g., vascular endothelial growth factor-A, VEGF-A), which in turn stimulate ECs proliferation and migration³. The hypoxic environment recruits leukocytes to the site of injury, with neutrophils being the first responders, followed by macrophages in the acute phase of inflammation⁴. At the end of the inflammatory phase, characterized by macrophage invasion, a disorganized network of newly formed capillaries is produced by the VEGF-driven proliferation of ECs⁵. The restored oxygen tension drives a switch from pro- to anti-angiogenic signals (e.g., Ang-2), which resolves in apoptosis of ECs in excess capillaries⁶. During this remodeling process, pericytes migrate after platelet-derived growth factor- β (PDGF- β) gradients toward the well-formed capillaries and prevent neighbor ECs from undergoing apoptosis⁷. As previously shown by our group and others, diabetes is associated with several dysfunctions affecting angiogenic potential⁸. Such impairments include an impaired amount of functionally compromised circulating pro-angiogenic CD34⁺ endothelial progenitor cells, a decreased CD34⁺ homing capacity at the wound site, a loss in pericytes number and function, and a misregulated production of angiogenic factors, contributing to peripheral artery disease and critical limb threatening ischemia (CLTI), which sets the basis for the development of diabetic foot ulcers (DFUs)^{9–12}. Current therapies failed to restore the angiogenic potential in diabetic patients; however, most recent approaches relying on cell delivery *in situ* with or without the support of a scaffold show promise¹³. Autologous CD34⁺ cell delivery, a matter of study in our hosting institute, showed positive results in the treatment of CLTI¹⁴. Therefore, the development of a biological, xenogenic-free platform for the delivery of autologous angiogenic cells is a pivotal step in the development of a strategy for the treatment of CLTI. A recent approach to tissue engineering is cell sheet technology (CST), which exploits the innate ability of cells to produce their own extracellular matrix (ECM), relying on thermoresponsive polymers for the growth and collection of the cell sheets¹⁵. CST has been applied so far

to the regeneration of several tissues, as well as to wound healing^{16,17}. However, the optimal cell type and cell culture conditions to produce an efficient and autologous cell sheet to stimulate angiogenesis in diabetic patients still need to be elucidated. Pericytes play a pivotal role in supporting angiogenesis *in vivo*, orchestrating the activation and migration of ECs and endothelial progenitor cells, besides retaining elevated plasticity and viability *in vitro*. Moreover, pericytes co-operate with ECs in basal lamina deposition *in vivo*, are easier than ECs to isolate and expand *in vitro*, and have mesenchymal origin which confers them a lower immunogenicity¹⁸. Therefore, we hypothesized that pericytes would represent the cell type of choice as a functional support for endothelial progenitor cells. A central player in the efficiency of a cell sheet is its ECM, which also plays a critical role in angiogenesis, as it provides fundamental mechanical and biochemical cues for the neovessels to sprout¹⁹: Macromolecular crowding (MMC) is emerging as a tool to sustain and enhance the innate capability of cells to deposit their own ECM, therefore providing a unique approach to produce autologous, self-assembled constructs for tissue engineering applications^{20,21}. MMC aims to improve cell culture conditions by reducing the gap between *in vivo* crowded and *in vitro* diluted environments^{21–23}. Its mechanism of action relies on the excluded volume effect (EVE), namely the reduction in the medium volume through which the cell-secreted molecules are free to diffuse²⁴. Coupled with the constant formation and disaggregation of transient, delimited micro-environments in the conditioned medium, EVE increases the reaction rate of the proteases involved in the processing of fibrillar procollagens (e.g., BMP-1, ADAMTS-2, MMPs) by increasing their effective concentrations and favoring the association with their substrates. Such general characteristics are influenced by the nature of the molecule chosen as a crowder. Negatively charged molecules such as carrageenans or dextran sulfate are more effective than neutral crowders like ficoll or polyvinylpyrrolidone (PVP) at lower concentrations on account of their larger hydrodynamic radius^{25,26}. Polydispersity index, generally larger in biological molecules such as hyaluronic acid (HA) or carrageenan, has also been shown to positively affect ECM deposition²⁷. By combining the innate ability of cells to deposit their matrix with the activity of MMC and the innovation of CST, we aim to produce an efficient and xenogeneic-free scaffold for the treatment of chronic DFU.

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Table 1. Antibodies Used for Immunofluorescence and FACS Characterization of Pericytes.

Assay	Antigen	Conjugation	Dilution	Brand	Code
IF	CD146	None	1:50	Abcam	ab10816
	NG2	None	1:100	Abcam	ab83178
	PDGFR- β	None	1:50	Cell Signalling	3169s
	α -SMA	None	None	Dako	N1584
	CD10	None	None	Dako	M7308
FACS	CD31	VioBlue	1:50	Miltenyi	130-110-674
	CD45	VioBright 515	1:50	Miltenyi	130-110-640
	CD34	PE	1:50	Miltenyi	130-120-515
	CD90	PE-Vio770	1:50	Miltenyi	130-114-862
	CD133	APC	1:50	Miltenyi	130-110-963
	CD140b	APC-Vio770	1:1	Miltenyi	130-105-282
	CD146	PE-Vio615	1:50	Miltenyi	130-125-731

Based on these concepts, we hence hypothesized that the application of an optimized MMC preparation to the culture of patient-derived pericytes will lead to the increased deposition of an ECM capable of homing angiogenic cells, offering an important implementation to the autologous cell delivery-based approach in the treatment of CLTI. To select the most efficient combination of MMC to enhance the deposition of the peculiar ECM of pericytes, several molecules known to have crowding effects were tested. Finally, in view of a future clinical application on DFU patients, the ECM deposited by diabetic patient-derived pericytes was compared to the ECM deposited by non-diabetic counterparts in terms of expression and quantification of typical basal membrane molecules.

Materials and Methods

Donor Patients

After approval by the ethics committee of MultiMedica (protocol number 452.2020, approval report no. CE-119.2020 of December 16, 2020), and in conformity with the Declaration of Helsinki, 27 patients (mean age 68.23 ± 12.61 years, 27.27% females) attending the diabetic foot unit and 28 patients (mean age 45.68 ± 13.69 years, 53.57% females) from the hand surgery and rehabilitation units at the MultiMedica hospital in Sesto San Giovanni (Milan) were enrolled upon signing of the informed consent. From each patient, a skeletal muscle biopsy was sent to the research laboratory from which pericytes were isolated.

Isolation of Vascular Cells From Muscle Tissue

Muscle tissue was weighed and then minced with the help of a scalpel before being incubated with a cocktail of bacterial collagenase and dispase (Merck, #11097113001) at a concentration of 1 mg/ml for 2 h at 37°C with shaking. The resulting digested tissue was then filtered with a cell strainer with a pore size of 70 μ m and washed in phosphate-buffered saline (PBS). Cells contained in the pellet were selected with anti-CD31 magnetic beads (Miltenyi, #130-091-935), to eliminate ECs.

CD31⁻ cells were further selected with anti-CD146 magnetic beads (Miltenyi, #130-093-596). The resulting CD31⁻CD146⁺ cells were seeded as pericyte candidates. Cells were grown with a culture medium for vascular cells (ATCC, #PCS-100-030) supplemented (ATCC, #PCS-100-041) with VEGF, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), basic fibroblasts growth factor (bFGF), heparan sulfate, L-glutamine, hydrocortisone, ascorbic acid, penicillin/streptomycin (P/S) (Euroclone, #ECB3001), and 20% fetal bovine serum (FBS) (Gibco, #A5256701).

Characterization of the Isolated Cell Populations by Immunofluorescence Assay

Upon reaching the right level of confluence (70%–80%), the cell cultures specifically grown in chamber slides for immunofluorescence were washed with PBS and fixed by incubating for 20 min in 4% paraformaldehyde (Sigma, #P6148) in PBS. After three washes in PBS, they were permeabilized with a 0.1% Triton X-100 (Sigma, #T8787) solution in PBS for 10 min, washed three times in PBS, incubated with 10% goat serum (abcam, # ab7481) solution for 30 min to cover the non-specific binding sites for the antibodies, and subsequently incubated with the immunofluorescence antibodies listed in Table 1 at the relevant dilutions. After O/N incubation, cells were washed three times in PBS and incubated for 45 min with secondary antibodies (Thermo Fisher #A-21428, #A-11001) diluted 1:500 in PBS. At the end of the incubation with secondary antibodies, cells were washed three times in PBS and the chambers were disassembled and coverslipped with Vectashield mountant with DAPI (Vector laboratories, #H-1200-10) and examined with an inverted fluorescence microscope (Leica, DM IL Fluo).

Characterization of the Isolated Cells by Flow Cytometry

Magnetic bead-sorted cells were seeded in culture-treated dishes and grown until reaching approximately 80% confluence before characterization. Cells were then harvested by

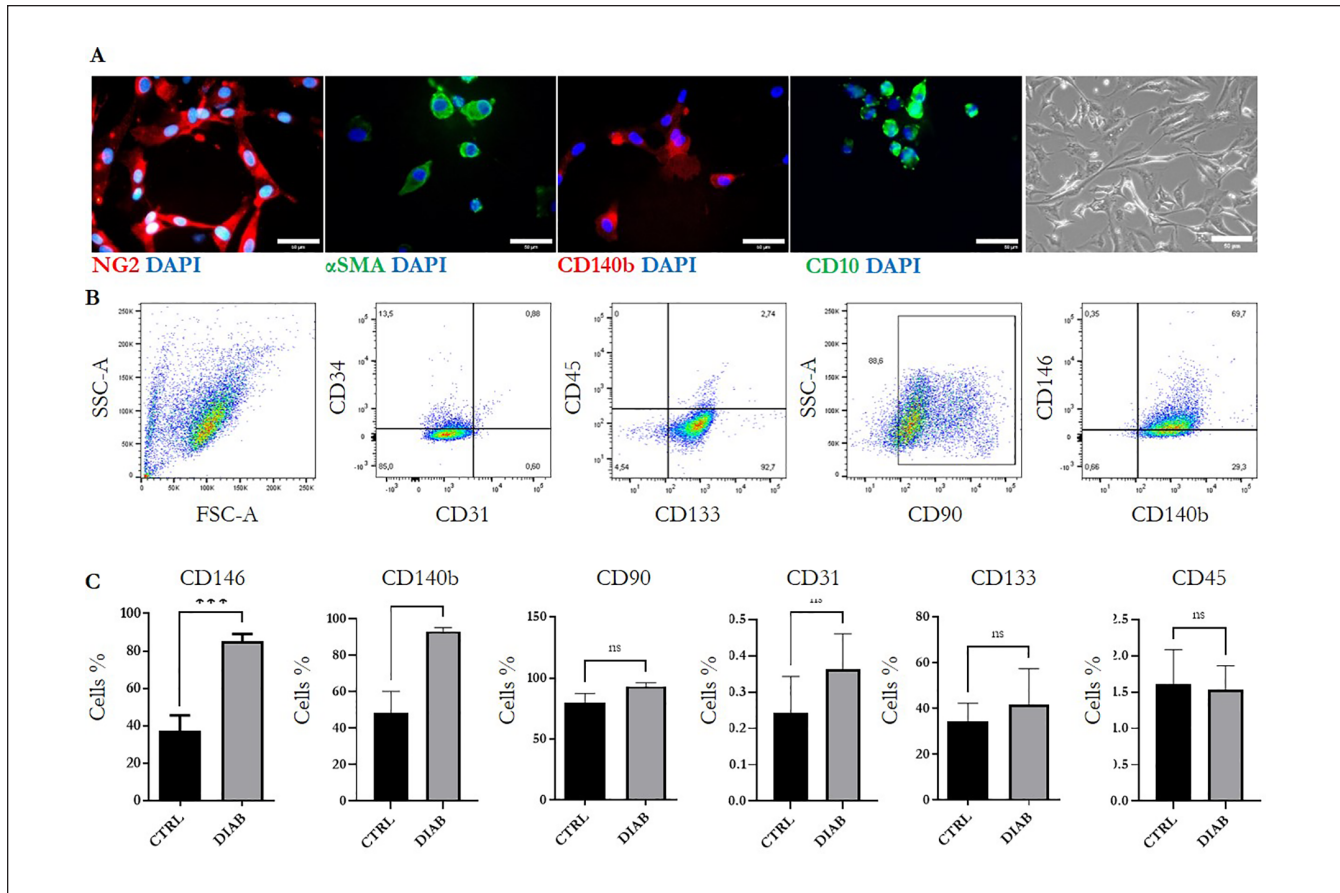


Figure 1. Pericytes characterization by FACS and immunofluorescence. Pericytes isolated from human skeletal muscle biopsies by CD146⁺ magnetic beads sorting express typical pericyte/mesenchymal markers by immunofluorescence (A) and FACS (B) characterization; (C) comparison of pericyte markers expression between control and diabetic patients. Ns: nonstatistically significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $N = 10$ controls, $N = 9$ diabetic patients.

Table 2. Descriptive of the Macromolecular Crowders Tested.

Crowder	Molecular weight	Tested concentrations		Hydrodynamic radius (nm)	
Ficoll mixture	70 kDa	37.5 mg/ml		4	
	400 kDa	25 mg/ml		8	
Polyvinylpyrrolidone	40 kDa	40 mg/ml	60 mg/ml	5.1	
	360 kDa	4 mg/ml	12 mg/ml	19	
Dextran	40 kDa	50 mg/ml	100 mg/ml	200 mg/ml	4.78
Dextran sulfate	500 kDa	10 μ g/ml	100 μ g/ml	200 μ g/ml	46
κ -Carrageenan	70 kDa	30 μ g/ml	60 μ g/ml	90 μ g/ml	29.7

incubation with trypsin-EDTA (Sigma, #T2601) at 37°C, washed in PBS, and incubated with the fluorescence activated cell sorting (FACS) antibodies listed in Table 1 for 10 min at 4–8°C. Visualization and recording were then performed with the BD LSRFortessa™ X-20 Cell Analyzer using at least 150,000 cells per sample and comparing the samples to the antibody-free negative control. The gating strategy of Fig. 1 shows the selection of a morphologically defined cell population free of doublets.

Treatment With Macromolecular Crowders

Cells were seeded on day 0 of the experiment between passages 2 and 4 (pericytes) or 4 and 8 for human umbilical vein endothelial cells (HUVECs) at a density of 20,000 per cm² in chamber slides (Thermo Fischer, # 16250681) with medium containing 2% (HUVECs) or 10% (HUVECs, pericytes) FBS. On day 1, the medium was changed to medium containing the MMC (see Table 2 for a complete list of crowders

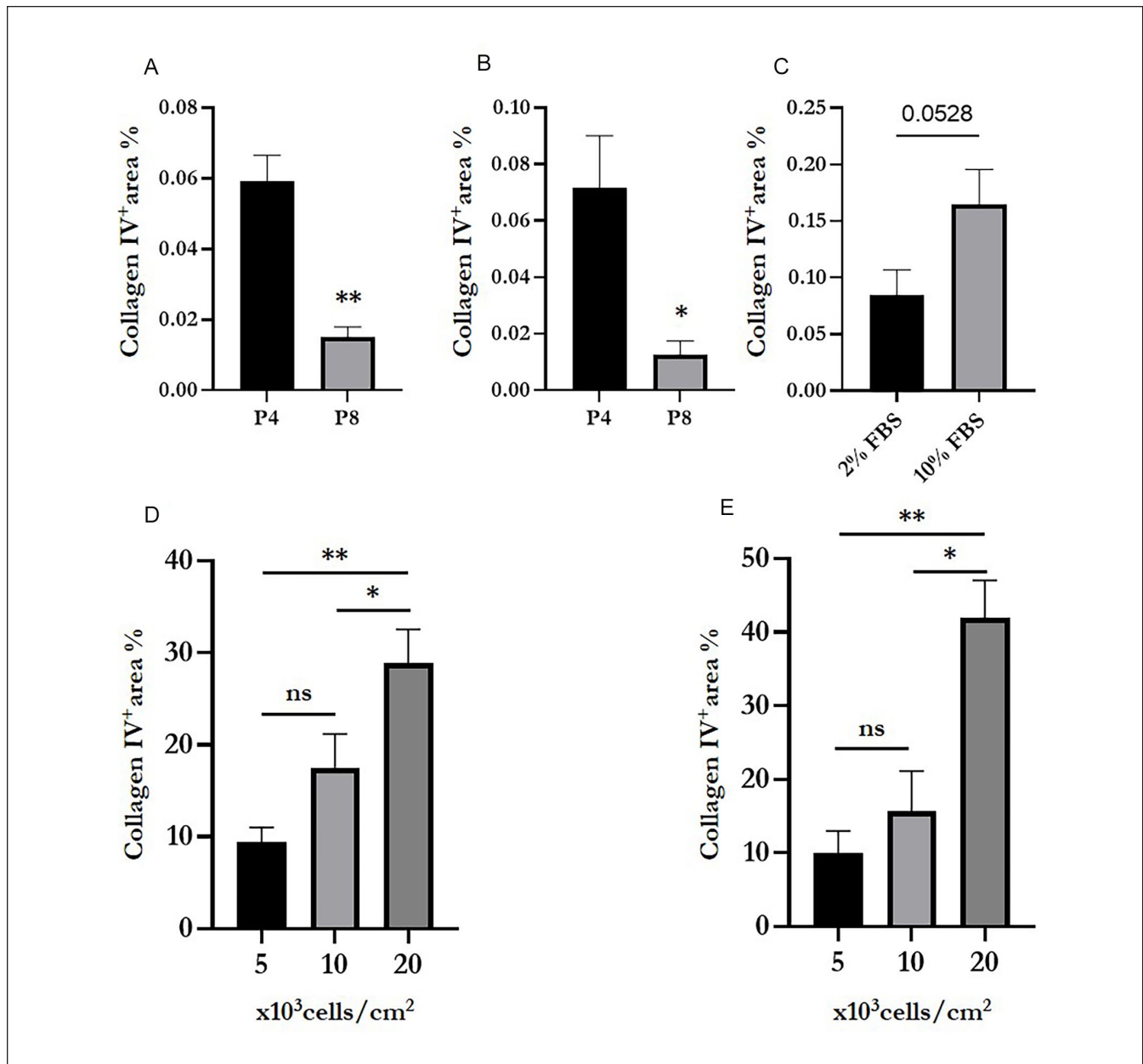


Figure 2. Effect of culture conditions on collagen IV deposition by HUVECs. Subculture passing effect on collagen deposition by HUVECs with 10% (A) and 2% (B) FBS supplementation; effect of serum concentration (C) and cell seeding density in the presence of 10% (D) and 2% (E) FBS on collagen deposition. Ns: nonstatistically significant, * $P < 0.05$, ** $P < 0.01$, $N = 3$.

and concentrations tested). For optimization experiments with HUVECs, time points were set on days 3 and 6. Based on observations made on HUVECs, day 3 was identified as the optimal time point for the following experiments.

Evaluation of the Expression of Individual Matrix Components

Pericytes were seeded at a density of 20,000 cells/cm² in 8-well chamber slides with complete vascular cell culture

medium with supplements, P/S, and 10% FBS. The following day (day 1) the medium was replaced with a control medium without crowders (in controls) or a medium containing 25 mg/ml ficoll 400 kDa and 37.5 mg/ml ficoll 70 kDa as MMC mix (in treated samples).

Statistical Analysis

Variables are summarized by frequency and percentage or mean and standard error of means for categorical and

continuous variables, respectively. Differences between the groups were tested by Student *t*-test or non-parametric Wilcoxon's test. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. No statistical significance was defined as $P \geq 0.05$. Statistical analysis was conducted using GraphPad Prism 9 software (Prism 9.0.0).

Results

Isolation and Characterization of Pericytes

In order to evaluate the matrix deposition by human pericytes, we set up a protocol for isolating these cells from muscle tissue samples of diabetic patients affected by CLTI and non-diabetic patients by sorting the total cells with magnetic beads recognizing CD146, a typical antigen expressed by pericytes. Pericytes were then characterized by FACS and immunofluorescence using the specific antibodies listed in Table 1. Flow cytometric analysis revealed that pericytes expressed on average $59.36\% \pm 30.01\%$ CD146, $85.88\% \pm 16.71\%$ CD90, and finally $66.44\% \pm 31.52\%$ CD140b, typical markers of the pericyte phenotype, while they showed as expected a lower expression of antigens characteristic of ECs ($0.48\% \pm 0.72\%$ CD31, $33.32\% \pm 25.76\%$ CD34) or leukocytes ($5.06\% \pm 12.61\%$ CD45). Figure 1 shows the morphological gating strategy and a representative image of the results of the flow cytometric analyses, as well as the characterization by immunofluorescence, which confirms the positivity for the pericyte markers CD140b, NG2, and α SMA.

Seeding Density and Sub-Culture Passaging Affect Collagen Type IV Deposition by HUVECs

While optimizing the protocol for pericyte isolation, to fine-tune the ideal MMC condition for increasing collagen IV deposition, HUVECs were used. ECs were chosen as they secrete collagen IV as a matrix component, similar to pericytes. To characterize our study model, we first verified the effect of the number of sub-culture passages on the intrinsic capacity of HUVECs to deposit ECM, observing that this is an influential variable: both in the presence of 2% and 10% fetal bovine serum, collagen IV depositions, measured as % of the area covered by collagen IV/number of cells, decreases significantly at passage 8 compared to passage 4 (0.071 ± 0.03 vs 0.012 ± 0.009 with 2% serum and 0.059 ± 0.01 vs 0.015 ± 0.004 with 10% serum) (Fig. 2A, B). Second, we evaluated the seeding density and serum concentration in order to maximize collagen IV deposition: in terms of absolute values, the seeding density of 20,000 cells per cm^2 proved to be significantly more efficient than the others in both serum concentrations (41.96 ± 8.87 vs 9.93 ± 5.31 at 5000 cells/ cm^2) (Fig. 2D, E), while no significant differences were found in the use of 2% or 10% fetal bovine serum in HUVEC ($P = 0.0528$, Fig. 2C). For these reasons,

in the following experiments, we used cells at a passage in culture not exceeding 4 and seeded at a density not lower than 15,000/ cm^2 .

Macromolecular Crowding Enhances Collagen Type IV Deposition by HUVECs

Having set the basic culture conditions for collagen IV synthesis by HUVECs, we ventured into assessing the effect of several MMCs on HUVECs in terms of collagen type IV deposition. When collagen IV positive area % was normalized on cell number, carrageenan (day 3, 90 $\mu\text{g}/\text{ml}$, $P < 0.05$), dextran-40 kDa (day 3, 200 mg/ml , $P < 0.01$), and PVP-40 kDa (day 3, 100 mg/ml , $P < 0.01$) significantly increased collagen type IV deposition compared to the untreated controls in the presence of 2% FBS supplementation (Fig. 3). In the presence of 10% FBS supplementation, ficoll mix (day 6, $P < 0.05$), dextran-40 kDa (day 3, 200 mg/ml , $P < 0.01$), and PVP-40 kDa (day 3, 200 mg/ml , $P < 0.05$) significantly enhanced collagen IV deposition. However, dextran-40 kDa (day 3, 200 mg/ml , $P < 0.01$) and PVP-360 kDa (2% FBS, day 3, 200 mg/ml , $P < 0.05$ and 10% FBS, day 6, 200 mg/ml , $P < 0.01$) significantly affected cell proliferation, PVP-40 also reduced cell density though not significantly, while carrageenan (2% FBS, 90 $\mu\text{g}/\text{ml}$, $P < 0.05$) and ficoll did not affect cell density in the presence of 10% FBS and were therefore selected to be tested on primary pericytes (Fig. 4). Figure 5 shows that only carrageenan (2% FBS, day 3, 90 $\mu\text{g}/\text{ml}$ and 10% FBS, day 6, 30 $\mu\text{g}/\text{ml}$) and ficoll (day 6, 10% FBS) medium supplementation significantly increase collagen type IV deposition independently of cell density.

Macromolecular Crowding Enhances Collagen Type IV Deposition by Patient-Derived Pericytes

Following the observations made on HUVECs and consequently to the optimization of the protocol for the isolation of pericytes, we proceeded to apply the pre-selected MMCs on pericytes isolated from the skeletal muscle of non-diabetic patients. Ficoll proved to be effective on pericytes in enhancing ECM deposition, unlike carrageenan. Densitometric analysis data shown in Fig. 6 demonstrate a significant increase in collagen IV deposition following the addition of the MMC ficoll ($0.08 \pm 0.05 \mu\text{m}^2/\text{cell}$ with ficoll vs $0.01 \pm 0.01 \mu\text{m}^2/\text{cell}$ in the negative control, $P < 0.05$), regardless of cell density (Fig. 6).

Characterization of the Composition of the Extracellular Matrix Deposited by Pericytes

After assessing that MMC enhances collagen type IV deposition by patient-derived pericytes, we compared diabetic and non-diabetic patient-derived pericytes on their ability to

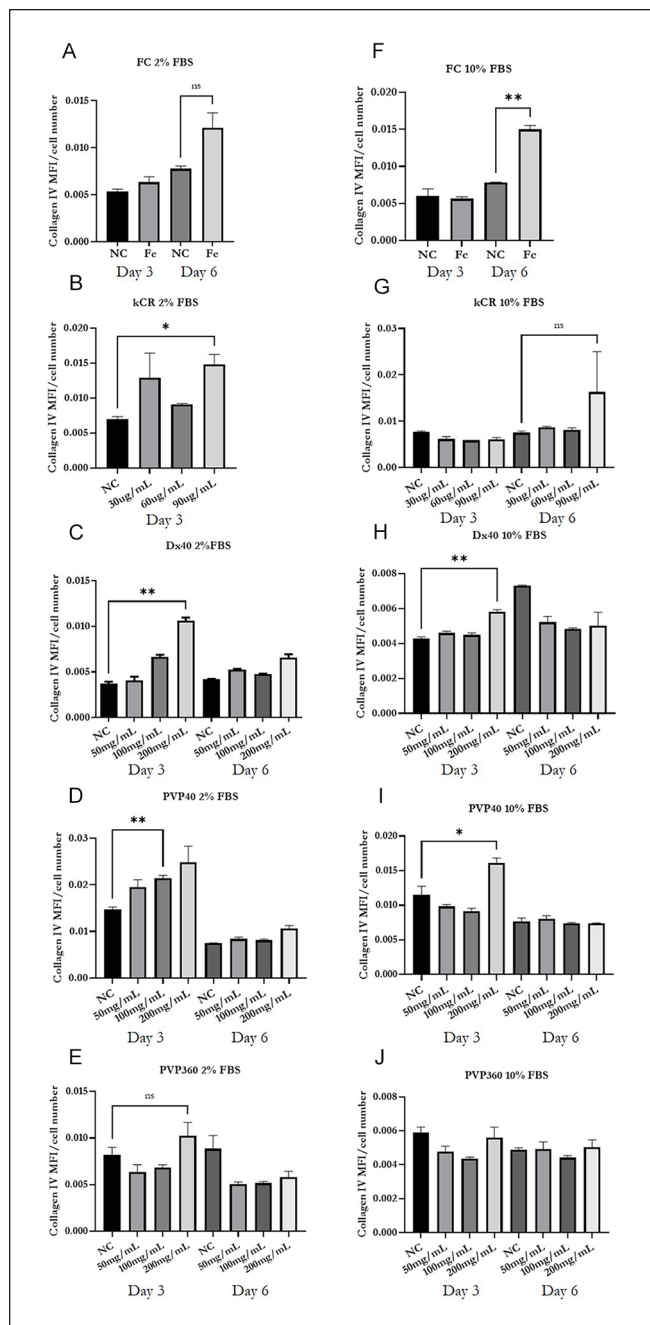


Figure 3. Effect of macromolecular crowding on collagen IV deposition by HUVECs normalized on cell density. Mean collagen IV covered area % normalized on HUVECs density after treatment with ficoll mixture (A, F), k-carrageenan (B, G), dextran-40 kDa (C, H), polyvinylpyrrolidone-40 (D, I) and polyvinylpyrrolidone-360 kDa (E, J) in the presence of 2% (A-E) or 10% (F-J) FBS. Fc: ficoll; kCR: k-carrageenan; Dx: dextran; PVP: polyvinylpyrrolidone; Ns: nonstatistically significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $N = 3$.

deposit an ECM complete with all the components of a typical basal lamina: collagen type IV, laminin, nidogen, perlecan, and fibronectin. In the presence of MMC, diabetic patient-derived pericytes showed higher levels of both

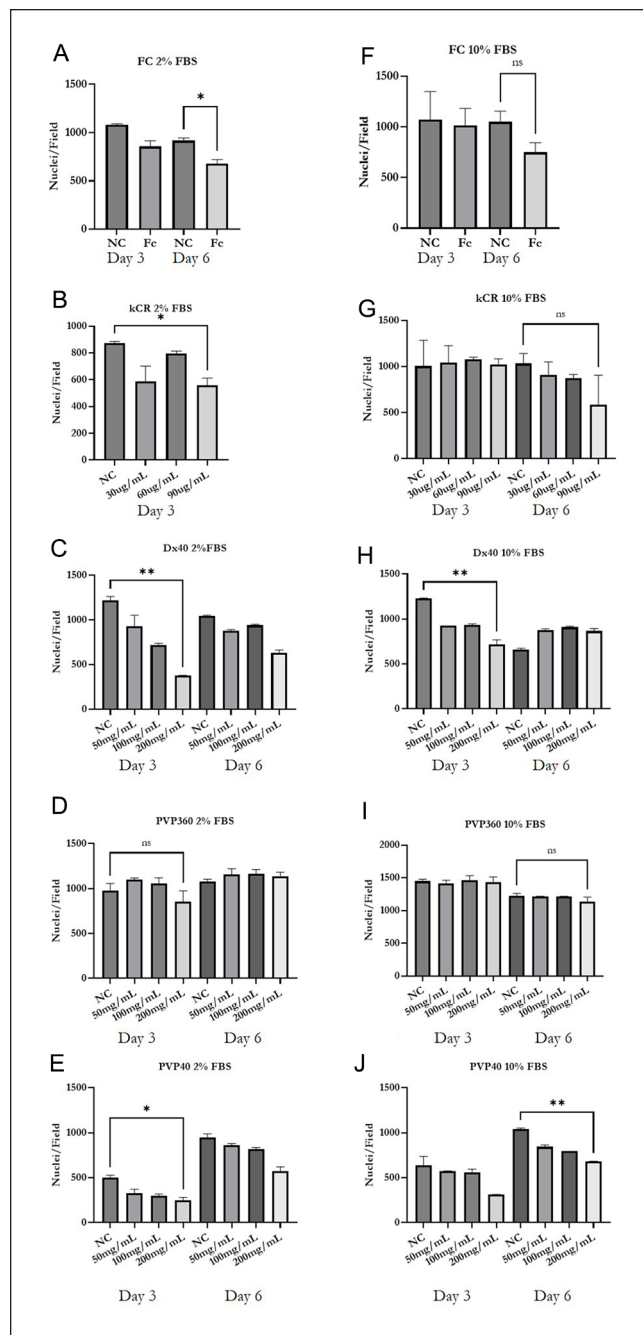


Figure 4. Effect of macromolecular crowding on HUVECs cell density. Mean cell number per field after HUVECs treatment with ficoll mixture (A, F), k-carrageenan (B, G), dextran-40 kDa (C, H), polyvinylpyrrolidone-40 (D, I) and polyvinylpyrrolidone-360 kDa (E, J) in the presence of 2% (A-E) or 10% (F-J) FBS. Fc: ficoll; kCR: k-carrageenan; Dx: dextran; PVP: polyvinylpyrrolidone; Ns: nonstatistically significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $N = 3$.

fibronectin (% of covered area/cell density = 18.07 ± 0.005 in the non-diabetic and 60.903 ± 0.017 in the diabetic, $P < 0.05$) and laminin (0.35 ± 0.092 in the non-diabetic and 10.433 ± 3.327 in the diabetic, $P < 0.01$) (Fig. 7). No statistically significant difference was observed between

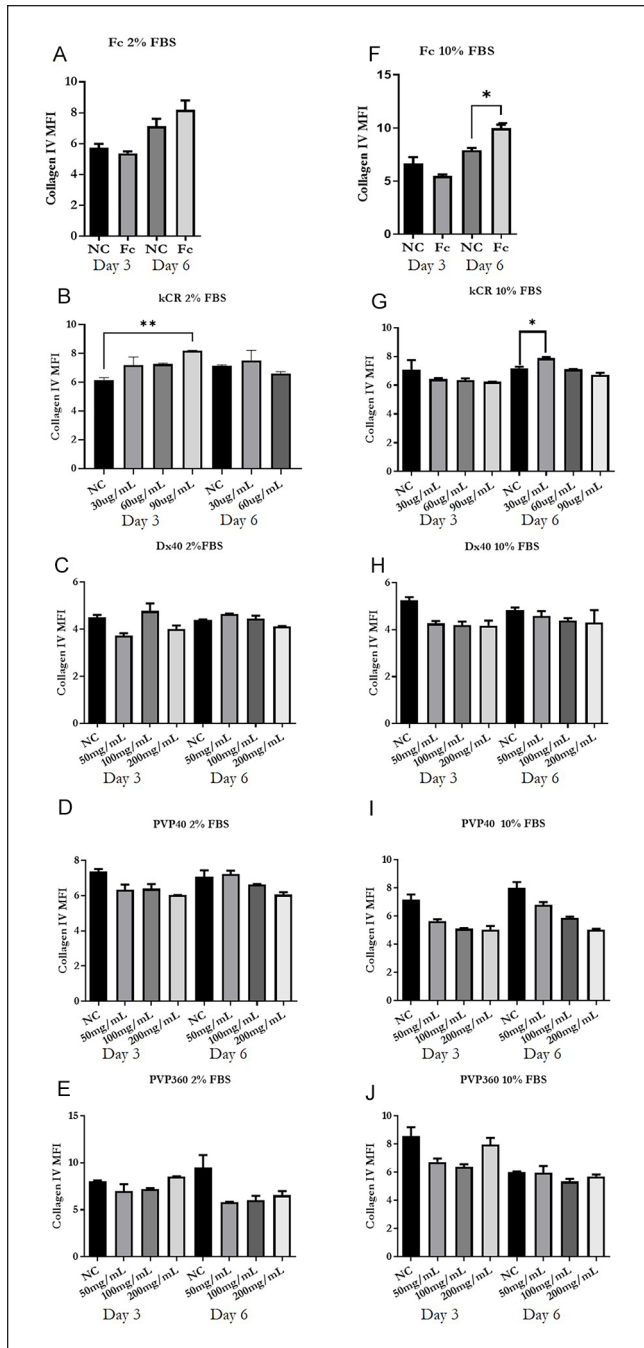


Figure 5. Effect of macromolecular crowding on collagen IV deposition by HUVECs. Mean collagen IV covered area % after treatment of HUVECs with ficoll mixture (A, F), k-carrageenan (B, G), dextran-40 kDa (C, H), polyvinylpyrrolidone-40 (D, I), and polyvinylpyrrolidone-360 kDa (E, J) in the presence of 2% (A-E) or 10% (F-J) FBS. Fc: ficoll; kCR: k-carrageenan; Dx: dextran; PVP: polyvinylpyrrolidone; Ns: nonstatistically significant; * $P < 0.05$, ** $P < 0.01$, $N = 3$.

pericytes isolated from diabetic and non-diabetic patients with regard to the deposition of collagen IV, perlecan, and nidogen in the presence of MMC, although for all the molecules examined a tendency toward an increase was

observed in favor of pericytes from diabetic donors. The deposition of nidogen in the absence of MMC was significantly increased in pericytes from diabetic patients compared to controls ($P < 0.05$) (Fig. 7).

Discussion

The importance of pericytes as a cell type of choice in order to support ECs and endothelial progenitor cells in their angiogenic function is being backed by a growing number of studies²⁸. In mice, the implantation of fibronectin/collagen I-based scaffolds seeded with HUVECs and MSCs, which are able to differentiate toward the pericytic fate, lead to the formation of a functional vascular network²⁹. In bone tissue engineering, integration of an inner angiogenic layer seeded with pericytes and ECs between two nanofiber-based osteo-inductive layers allowed for the subcutaneous integration of a bone-like structure in mice which was integrated with the host vascular network³⁰. Finally, pericytes were applied in combination with ECs and MSCs to the generation of cell sheets-based bone constructs, corroborating the importance of using pericytes to achieve the formation of stable vascular networks. Our work so far has led us to lay the foundations for the generation of pro-angiogenic cell sheets from DFU patient-derived pericytes. Exploratory studies on HUVEC have allowed us to select the most effective MMCs to increase collagen IV deposition, namely ficoll and carrageenan, as well as to highlight the importance of some parameters such as the number of passages in culture and the seeding density to produce a confluent sheet capable of producing a homogeneous matrix. The data collected on pericytes through FACS and immunofluorescence characterization confirm the setup of a protocol to efficiently isolate and culture *in vitro* human skeletal muscle cells as supported by the high expression of the pericyte markers CD146, NG2, CD90, and CD140b in the populations sorted with microbeads for CD146. The analysis of the deposited matrix shows a significant effect of the MMC via ficoll on the deposition of type IV collagen: this would allow the production of a sheet of cells rich in ECM ready to home angiogenic cells to be administered to the patient a few days after the biopsy from which autologous pericytes are isolated. One limit of this study is represented by the preliminary nature of the results with respect to clinical translation. First applying the new pericyte cell sheet will be possible only after testing the capability of the cell sheet to support seeded cell function in terms of adhesion and viability. In addition, the support will need to undergo all the required testing for approval to be use in humans via the appropriate *in vitro* and *in vivo* steps. More proof-of-concept studies will be needed in the immediate future: therefore, we also plan to evaluate the pro-angiogenic effect of the cell sheets using 2D and 3D *in vitro* approaches as well as *in vivo* in appropriate animal models of DFU, and to determine the ideal administration route (injection or deposition). In doing so, also the role and added value of both the cellular and ECM components will need to be evaluated. It is important to note that the

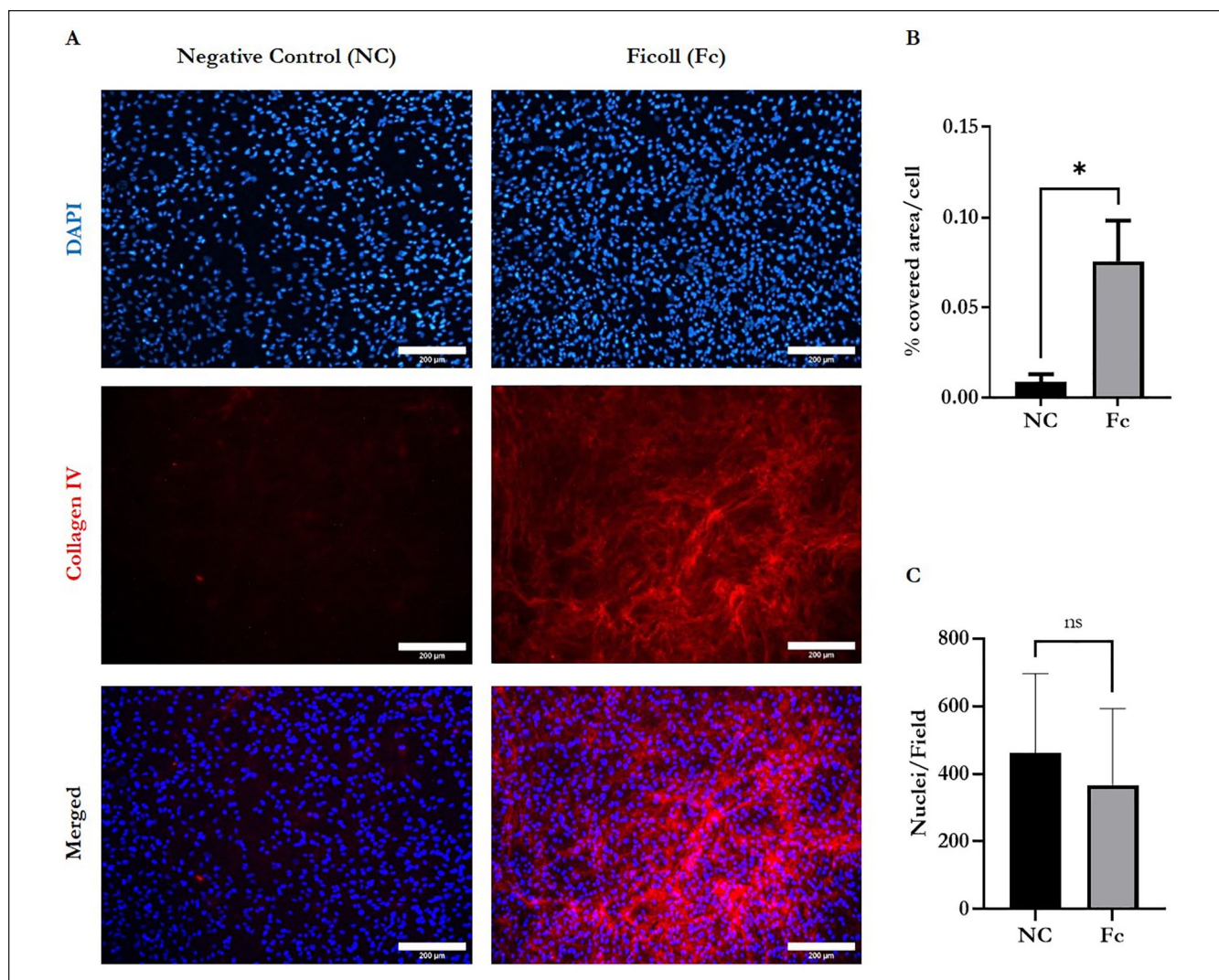


Figure 6. Ficolin treatment induced an increase in collagen IV deposition by pericytes. Ficolin treatment significantly increased collagen IV deposition by muscle pericytes (A, B) without significantly altering cell proliferation (C). “*” indicates a $P < 0.05$ for the mean percentage of collagen IV positive area versus negative control, $N = 5$.

comparison with matrices deposited by pericytes of non-diabetic patients shows that also the matrices from pericytes of diabetic patients contain fundamental elements of the basal lamina such as laminin 5, nidogen, perlecan, and fibronectin and tend to deposit them in higher amounts. These data extend to pericytes some observations present in the literature, as it is known that fibronectin is highly expressed by ECs of diabetic patients *in vivo*, or in culture following exposure to high levels of glucose³¹. The use of autologous pericytes, while advantageous in terms of immunogenicity, might also represent a limitation to the clinical application of pro-angiogenic cell sheets for it requires technical time for its development in culture, meaning that a patient should either be hospitalized for longer periods of time, or receive pre-hospitalization care in order to donate the pericytes needed for cell sheet production in advance with respect to the surgical intervention. To overcome these potential

hurdles, the efficiency of cryopreserved allogenic pericyte sheets, as well as decellularized pericyte-derived ECM, is currently under evaluation in our laboratory.

Conclusion

These results suggest that pericytes from diabetic patients are competent in the deposition of ECM when treated in culture with MMCs, making it possible to produce autologous cell sheets for the treatment of CLTI in diabetic patients. CLTI represents a major cause of DFU manifestation and recurrence, therefore the refinement of cell sheets supporting cell therapy for CLTI-dependent DFU will open the path to the clinical application of pro-angiogenic cell therapy to diabetic patients. The autologous nature of pericyte and endothelial progenitor cell-based angiogenic cell sheets should provide a novel healthcare product with very low immunogenicity.

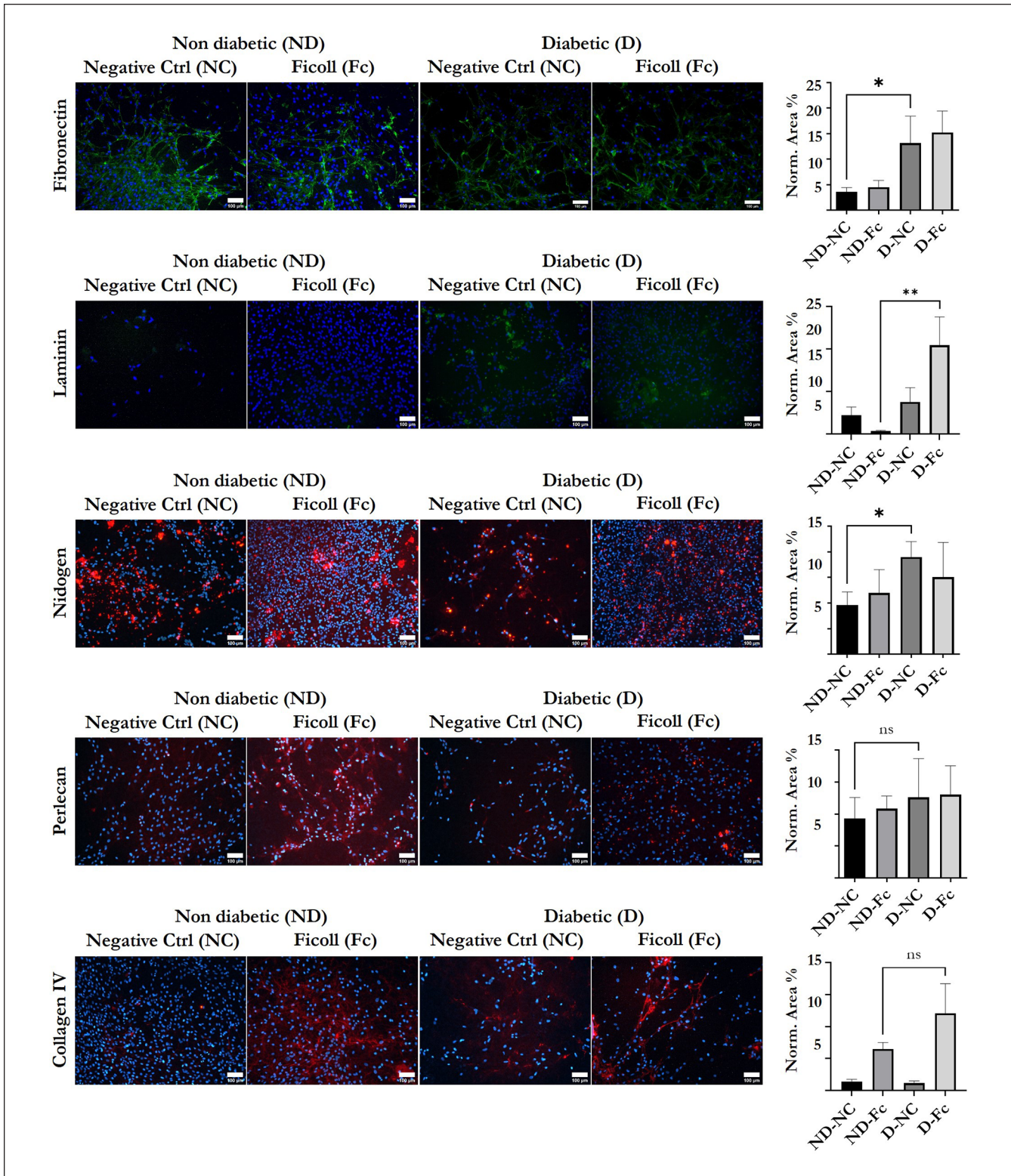


Figure 7. Composition of the matrix deposited by pericytes from diabetic and non-diabetic patients in response to ficoll treatment (Fc). Representative images and graphs showing the mean percentage + SEM of positive area for laminin 5, fibronectin, nidogen, perlecan, and collagen IV in pericyte cultures from patients with or without diabetes and in the presence or absence of ficoll treatment. In the graphs, the percentage of positive area is normalized on the number of nuclei multiplied *1,000 per field. “*” and “**” represent $P < 0.05$ and $P < 0.01$, respectively, compared to the non-diabetic control. ND = non-diabetic; D = diabetic; CN = negative control without MMCs.

However, the use of autologous pericytes might result in longer hospitalization; besides, pericyte immunogenicity is very low. Therefore, development of allogenic cell sheets readily implantable in patients upon isolation and seeding of autologous peripheral blood-derived endothelial progenitors might represent a valuable alternative. Moreover, decellularized pericyte-derived extracellular matrices seeded with endothelial progenitors could also represent a valuable choice. Future efforts will therefore focus on evaluating the angiogenic and therapeutic potential of these alternative choices.

Acknowledgments

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Author Contributions

Andrea Rampin carried out all the experiments and wrote the first draft of the paper, Carlo Maria Ferdinando Caravaggi selected and enrolled diabetic patients with DFU from the diabetic foot unit, harvested, and sent the skeletal muscle biopsies to the laboratory to be studied, Luigi Troisi selected and enrolled non-diabetic patients from the reconstructive surgery unit, harvested, and provided the skeletal muscle biopsies, and Gaia Spinetti provided mentorship and corrected the first draft of the paper.

Ethical Approval

The study was authorized by the independent ethics committee of MultiMedica, a section of the Central Ethics Committee IRCCS Lombardy (protocol number 452.2020, approval report no. CE-119.2020 of December 16, 2020).

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Consent to Participate

Patients participating in the study signed a written informed consent before donating their biopsies.

Consent for Publication

N/A.

Data Availability

Raw data of this project are available upon request to the authors at the Zenodo online repository: <https://zenodo.org/records/12821092>.

Declaration of Conflicting Interests

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References

1. Fernández-Guarino M, Hernández-Bule ML, Bacci S. Cellular and molecular processes in wound healing. *Biomedicines*. 2023;11(9):2526. doi:10.3390/biomedicines11092526.
2. Guo S, Dipietro LA. Factors affecting wound healing. *J Dent Res*. 2010;89(3):219–29.
3. Tandara AA, Mustoe TA. Oxygen in wound healing—more than a nutrient. *World J Surg*. 2004;28(3):294–300.
4. Turabelidze A, Dipietro LA. Inflammation and wound healing. *Endodontic Topics*. 2012;24(1):26–38.
5. Fukumura D, Jain RK. Imaging angiogenesis and the microenvironment. *APMIS*. 2008;116(7–8):695–715.
6. Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. *Circ Res*. 2000;87(6):434–39.
7. Chen RR, Silva EA, Yuen WW, Mooney DJ. Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation. *Pharm Res*. 2007;24(2):258–64.
8. Okonkwo UA, DiPietro LA. Diabetes and wound angiogenesis. *Int J Mol Sci*. 2017;18(7):1419. doi:10.3390/ijms18071419.
9. Elsayed S, Clavijo LC. Critical limb ischemia. *Cardiol Clin*. 2015;33(1):37–47.
10. Vono R, Fuoco C, Testa S, Pirro S, Maselli D, Ferland McCollough D, Sangalli E, Pintus G, Giordo R, Finzi G, et al. Activation of the pro-oxidant pkbetaii-p66shc signaling pathway contributes to pericyte dysfunction in skeletal muscles of patients with diabetes with critical limb ischemia. *Diabetes*. 2016;65(12):3691–704.
11. Spinetti G, Cordella D, Fortunato O, Sangalli E, Losa S, Gotti A, Carnelli F, Rosa F, Riboldi S, Sessa F, Avolio E, et al. Global remodeling of the vascular stem cell niche in bone marrow of diabetic patients: implication of the microrna-155/foxo3a signaling pathway. *Circ Res*. 2013;112(3):510–22.
12. Besnier M, Gasparino S, Vono R, Sangalli E, Facoetti A, Bollati V, Cantone L, Zaccagnini G, Maimone B, Fuschi P, Da Silva D, et al. Mir-210 enhances the therapeutic potential of bone-marrow-derived circulating proangiogenic cells in the setting of limb ischemia. *Mol Ther*. 2018;26(7):1694–1705.
13. Rigato M, Monami M, Fadini GP. Autologous cell therapy for peripheral arterial disease: systematic review and meta-analysis of randomized, nonrandomized, and noncontrolled studies. *Circ Res*. 2017;120(8):1326–40.
14. Panunzi A, Madotto F, Sangalli E, Riccio F, Sganzeroli AB, Galenda P, Bertulesi A, Barmina MF, Ludovico O, Fortunato O, Setacci F, et al. Results of a prospective observational study of autologous peripheral blood mononuclear cell therapy for no-option critical limb-threatening ischemia and severe diabetic foot ulcers. *Cardiovasc Diabetol*. 2022;21(1):196.

15. Zurina IM, Presniakova VS, Butnaru DV, Timashev PS, Rochev YA, Liang X-J. Towards clinical translation of the cell sheet engineering: technological aspects. *Smart Materials in Medicine*. 2023;4:146–59.
16. Guo R, Wan F, Morimatsu M, Xu Q, Feng T, Yang H, Gong Y, Ma S, Chang Y, Zhang S, Jiang Y, et al. Cell sheet formation enhances the therapeutic effects of human umbilical cord mesenchymal stem cells on myocardial infarction as a bioactive material. *Bioact Mater*. 2021;6(9):2999–3012.
17. Kato Y, Iwata T, Morikawa S, Yamato M, Okano T, Uchigata Y. Allogeneic transplantation of an adipose-derived stem cell sheet combined with artificial skin accelerates wound healing in a rat wound model of type 2 diabetes and obesity. *Diabetes*. 2015;64(8):2723–34.
18. Maier CL, Pober JS. Human placental pericytes poorly stimulate and actively regulate allogeneic CD4 T cell responses. *Arterioscler Thromb Vasc Biol*. 2011;31(1):183–89.
19. Senger DR, Davis GE. Angiogenesis. *Cold Spring Harb Perspect Biol*. 2011;3(8):a005090.
20. Kumar P, Satyam A, Fan X, Collin E, Rochev Y, Rodriguez BJ, Gorelov A, Dillon S, Joshi L, Raghunath M, Pandit A, et al. Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. *Sci Rep*. 2015;5(1):8729.
21. Rampin A, Skoufos I, Raghunath M, Tzora A, Diakakis N, Prassinos N, Zeugolis DI. Allogeneic serum and macromolecular crowding maintain native equine tenocyte function in culture. *Cells*. 2022;11(9). doi:10.3390/cells11091562.
22. Rampin A, Rossoni A, Chaniotaki L, Gkiatas IS, Tzora A, Skoufos I, Diakakis N, Prassinos N, Zeugolis DI. Xenogeneic versus allogeneic serum and macromolecular crowding in human tenocyte cultures. *Eur J Cell Biol*. 2024;103(3):151445.
23. Garnica-Galvez S, Skoufos I, Tzora A, Diakakis N, Prassinos N, Zeugolis DI. Macromolecular crowding in equine bone marrow mesenchymal stromal cell cultures using single and double hyaluronic acid macromolecules. *Acta Biomater*. 2023;170:111–23.
24. Suematsu K. Concentration dependence of excluded volume effects polymer solution as inhomogeneous system. *Colloid and Polymer Science*. 2012;290(6):481–90.
25. Rashid R, Lim NS, Chee SM, Png SN, Wohland T, Raghunath M. Novel use for polyvinylpyrrolidone as a macromolecular crowder for enhanced extracellular matrix deposition and cell proliferation. *Tissue Eng Part C Methods*. 2014;20(12):994–1002.
26. Satyam A, Kumar P, Fan X, Gorelov A, Rochev Y, Joshi L, Peinado H, Lyden D, Thomas B, Rodriguez B, Raghunath M, et al. Macromolecular crowding meets tissue engineering by self-assembly: a paradigm shift in regenerative medicine. *Adv Mater*. 2014;26(19):3024–34.
27. Garnica-Galvez S, Korntner SH, Skoufos I, Tzora A, Diakakis N, Prassinos N, Zeugolis DI. Hyaluronic acid as macromolecular crowder in equine adipose-derived stem cell cultures. *Cells*. 2021;10(4):859. doi:10.3390/cells10040859.
28. Avolio E, Alvino VV, Ghorbel MT, Campagnolo P. Perivascular cells and tissue engineering: current applications and untapped potential. *Pharmacol Ther*. 2017;171:83–92.
29. Koike N, Fukumura D, Gralla O, Au P, Schechner JS, Jain RK. Tissue engineering: creation of long-lasting blood vessels. *Nature*. 2004;428(6979):138–39.
30. Sathy BN, Mony U, Menon D, Baskaran VK, Mikos AG, Nair S. Bone tissue engineering with multilayered scaffolds-Part I: an approach for vascularizing engineered constructs *in vivo*. *Tissue Eng Part A*. 2015;21(19-20):2480–94.
31. Roy S, Sala R, Cagliero E, Lorenzi M. Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proc Natl Acad Sci USA*. 1990;87(1):404–8.