Journal of Visualized Experiments

Predicting Amputation Using Local Circulating Mononuclear Progenitor Cells in Angioplasty-treated Patients with Critical Limb Ischemia

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61503R2
Full Title:	Predicting Amputation Using Local Circulating Mononuclear Progenitor Cells in Angioplasty-treated Patients with Critical Limb Ischemia
Corresponding Author:	Juan Antonio Suárez-Cuenca Centro Medico Nacional 20 de Noviembre Mexico City, Mexico City MEXICO
Corresponding Author's Institution:	Centro Medico Nacional 20 de Noviembre
Corresponding Author E-Mail:	suarej05@gmail.com
Order of Authors:	Juan Antonio Suárez-Cuenca
	Eduardo Vera-Gómez
	Alejandro Hernández-Patricio
	Atzín Suá Ruíz-Hernández
	Juan Ariel Gutiérrez-Buendía
	Carlos Ramiro Zamora-Alemán
	Alberto Melchor-López
	Yasser Alberto Rizo-García
	Oscar Antonio Lomán-Zúñiga
	Ignacio Escotto-Sánchez
	Juan Miguel Rodríguez-Trejo
	Rebeca Pérez-Cabeza de Vaca
	Mario Antonio Téllez-González
	Paul Mondragón-Terán
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Mexico City, Mexico
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Medicine
Please provide any comments to the journal here.	Thank you for the opportunity to evaluate the revised manuscript.

TITLE:

Predicting Amputation Using Local Circulating Mononuclear Progenitor Cells in Angioplasty-treated Patients with Critical Limb Ischemia

AUTHORS AND AFFILIATIONS:

Juan Antonio Suárez-Cuenca[1*], Eduardo Vera-Gómez[1], Alejandro Hernández-Patricio[1], Atzín Suá Ruíz-Hernández[1], Juan Ariel Gutiérrez-Buendía[1], Carlos Ramiro Zamora-Alemán[1], Alberto Melchor-López[1], Yasser Alberto Rizo-García[2], Oscar Antonio Loman-Zúñiga[2], Ignacio Escotto-Sánchez[2], Juan Miguel Rodríguez-Trejo[2], Rebeca Pérez-Cabeza de Vaca[3], Mario Antonio Téllez-González[3], Paul Mondragón-Terán[3].

[1]Experimental Metabolism and Clinical Research Laboratory, Clinical Research Department, Division of Biomedical Research. Centro Médico Nacional "20 de Noviembre", ISSSTE. Mexico City, Mexico

[2]Vascular Surgery and Angiology Department from Centro Médico Nacional "20 de Noviembre", ISSSTE, Mexico City, Mexico

[3]Regenerative Medicine and Tissue Engineering Laboratory; Coordination of Research. Centro Médico Nacional "20 de Noviembre", ISSSTE. Mexico City, Mexico

Corresponding Author:

Juan Antonio Suárez-Cuenca (suarej05@gmail.com)

Email Addresses of Co-authors:

Eduardo Vera-Gómez (eduardovera20@gmail.com)
Alejandro Hernández-Patricio (alejansdrospa44@gmail.com)
Atzin Suá Ruíz-Hernández (atzinruizh@gmail.com)
Juan Ariel Buendía-Gutiérrez (ariel.gtz.q@gmail.com)
Carlos Ramiro Zamora-Alemán (carlos_zamora_alem@hotmail.com)
Alberto Melchor-López (dralbertomelchor@gmail.com)
Yasser Alberto Rizo-García (wawule@hotmail.com)
Oscar Antonio Loman-Zúñiga (oscarloman1@yahoo.com.mx)
Ignacio Escotto-Sánchez (iescott@hotmail.com)
Juan Miguel Rodríguez-Trejo (jrodt@hotmail.com)
Rebeca Pérez-Cabeza de Vaca (esderebk@gmail.com)
Mario Antonio Téllez-González (mario.atg91@gmail.com)
Paul Mondragón-Terán (p.mondragonteran@gmail.com)

KEYWORDS:

flow cytometry, MPCs, vascular block, critical limb ischemia, angioplasty, amputation prognosis

SUMMARY:

Lower limb amputation may occur even after angioplasty of obstructed vessels in Critical Limb Ischemia (CLI). Mononuclear Progenitor Cells (MPCs) reflect vascular repair. The present protocol describes the quantification of MPCs from circulation close to angioplasty, and its relationship

with endothelial dysfunction and prediction of lower limb amputation.

ABSTRACT:

Critical limb ischemia (CLI) represents an advanced stage of the peripheral arterial disease. Angioplasty improves the blood flow to the lower limb; however, some patients irreversibly progress to limb amputation. The extent of vascular damage and the mechanisms of vascular repair are factors affecting post-angioplasty outcome. Mononuclear Progenitor Cells (MPCs) are reactive to vascular damage and repair, with the ability to reflect vascular diseases. The present protocol describes quantification of MPCs obtained from blood circulation from vessel close to the angioplasty site, as well as its relationship with endothelial dysfunction and its predictive ability for limb amputation in the next 30 days after angioplasty in patients with CLI.

INTRODUCTION:

Peripheral Arterial Disease (PAD) is characterized by a chronic and progressive vascular obstruction with limitation of blood supply[1]. At a global scale, PAD of the lower limbs affects around 10% of the elderly population, while up to 7% of such cases are submitted to limb amputation[2,3].

Critical Limb Ischemia (CLI) represents the most serious presentation of PAD[1]. Patients usually experience pain at rest, ulcers, or gangrene attributable to occluded arteries; while clinical prognosis is unfavorable and marked by a 30% risk of limb amputation and mortality during 1 year[3-5].

Angioplasty is a minimally invasive endovascular procedure that can restore blood flow to the lower limb in patients with CLI; however, some patients will inevitably require major limb amputation, even after angioplasty therapy[1,5]. Early identification of unfavorable outcomes after angioplasty is quite valuable, due to the possibility of therapy enforcement.

Traditional risk factors may provide a limited predictive ability for major limb amputation in patients with CLI undergoing angioplasty[6]. Pathophysiology-oriented biomarkers represent novel methods with potential clinical applications, which may result specifically useful in diseases related to vascular injury[7]. Nowadays, the participation of cellular populations owning endothelial repair properties, at the site of the atherosclerotic plaque, has been increasingly recognized[8,9].

Mononuclear Progenitor Cells (MPCs) are derived from the bone marrow and own structural and functional characteristics of stem cells with vascular regenerative abilities. Due to MPC's ability to proliferate, migrate and show vascular adherence; these cells have become good candidates to reflect endothelial repair in response to ischemia[10-12]. In addition, continuous interest in mechanisms underlying vascular injury has motivated exploring the prognostic role of local occurring biomarkers, since they are considered to reflect vascular damage and repair[7,13,14].

The purpose of the present study is to describe how to determine the amount of MPCs that circulate close to the vascular obstruction in patients with CLI undergoing angioplasty; and how

to evaluate the relation between MPCs with indicators of endothelial dysfunction and limb amputation.

Compared to the prognosis based on comorbidities and intrinsic vascular features, the amount of local MPCs show specific ability to predict clinical outcome regarding endothelial dysfunction and limb amputation. Consistently, some studies have described the prognostic role of similar biomarkers during the evaluation of patients with PAD[15,16].

Based on previous results[7], the method described here may be useful for an early identification of population at risk of adverse vascular outcomes in several clinical settings, such as lower limb and coronary ischemia, stroke, vasculitis, venous thrombosis and others involving vascular injury and repair.

PROTOCOL:

The institutional research ethics committee from Centro Médico Nacional "20 de Noviembre" ISSSTE approved this prospective protocol, all enrolled patients provided written informed consent.

1. Evaluation of vascular block of lower limb, blood sampling and balloon angioplasty

NOTE: The study sample used for this experiment comprised of 20 diabetic patients, aged 68 years old and 10 out of 20 were males. Half of the sample were smokers and most prevalent co-morbidities were type 2 diabetes mellitus, systemic arterial hypertension and/or dyslipidemia. The sample was intended to be standardized for age-, sex- and co-morbidities. Possible bias due to clinical-demographic influence on the relation between MPCs and CLI could not be ruled out.

1.1. Evaluate clinical severity of limb ischemia according to the Rutherford classification[13] (see **Supplementary Table 1**).

1.2. Perform lower limb angiography, blood sampling and balloon angioplasty.

1.2.1. Use anticoagulant and anesthetic drugs before surgery.

1.2.2. Place an 18 G needle into the blood vessel at the groin site selected.

1.2.3. Place an introducer and advance a flexible guide wire. Then, the initial guide is further changed for a 6 Fr introducer.

1.2.4. Use periodic injection of contrast media or CO_2 under fluoroscopic guide to identify artery trajectory and vascular blocked sites (**Figure 1**).

NOTE: Use contrast media 40 cc, diluted 1:1 in 0.9% saline, or CO_2 at 10 to 20 mL per shot at a pressure of 12 psi.

1.2.5. Introduce two 0.014 Fr navigation guide wires and two 0.014 Fr support guide wires into the vessel and advance them up to the blocked site.

NOTE: Two 0.014 Fr guide wires (260 cm long) are used for navigation and two 0.014 Fr guide wires (260 cm long) are used for support, during a single procedure.

1.2.6. Introduce 5 Fr and 3 Fr catheters sequentially and collect 10 mL of blood from the closest site to the vascular obstruction. Maintain blood samples on ice.

NOTE: Catheters sizes may be 5 Fr and 3 Fr, and they are changed during the procedure.

1.2.7. Advance a guide wire again. Then, introduce an angioplasty balloon catheter, which contains an inflatable balloon located at the end of the catheter. Advance the angioplasty balloon catheter and place the balloon right at the site of lesion. Perform the angioplasty by inflating the balloon against the blocking plaque located at the vascular wall. Verify blood flow restoration.

NOTE: A stent may be placed in the blocked area to help keep the artery open after the procedure.

1.2.8. Introduce a catheter and advance up to the closest site to the vascular block. Collect 10 mL of blood at 30 min time interval after angioplasty. Maintain blood samples on ice.

NOTE. Collection of blood samples before and after the angioplasty is recommended to further evaluate the influence of the angioplasty on the number of MPCs.

1.2.9. Remove all the wires under fluoroscopic guidance.

1.2.10. Provide post-operative care procedures, including anti-coagulation therapy using enoxaparin at 1 mg/kg subcutaneous every 12 h, aspirin 100 mg, statin, and analgesia. Compress at the site of vascular puncture during 24 h.

2. Quantification of circulating mononuclear progenitor cells (MPCs) (Figure 2)

2.1. To a fresh 15 mL conical tube, transfer 6 mL of the collected blood and dilute 1:1 (v/v) with PBS.

NOTE: Process the blood within 1 h from collection.

2.2. Prepare for density gradient separation, by adding 2 mL of the density gradient medium to 3 test tubes each. Then, add 3 equal volume aliquots of the diluted blood into each test tube.

NOTE: Do not exceed three-fourths of the test tube maximal capacity.

2.3. Centrifuge at 1,800 *x g* for 30 min at 4 °C. Collect the interface layer present as a ring using a

pipette, and transfer into a new tube. Add 2 mL of PBS and spin at 1,800 *x g* for 6 min at 4 °C. Save the pellet as this will contain MPCs.

2.4. Wash the pellet containing MPCs with PBS by centrifugation as described in step 2.3. Use fresh PBS for each wash and spin at 1,800 *x g* for 2 min at 4 °C. Repeat the process for 6 times.

2.5. After the last wash, use 1 mL of PBS to resuspend the cell pellet. Dilute 20 µL of the cell suspension with 20 µL of 0.4% trypan blue, 1:1 (v/v). Use 10 µL of this cell suspension for cell counting using hemocytometer and a light microscope.

2.6. Aliquot 1 x 10^6 MPCs in previously labeled 5 mL flow cytometry tubes.

NOTE: Prepare corresponding isotype-matched control antibodies.

2.7. Centrifuge the tubes at 1,800 *x g* for 6 min at 4 °C. Aspirate and discard the supernatant.

2.8. Dilute primary antibody in 100 µL of antibody incubation solution [1x PBS, pH 7.4, EDTA 2 mM, BSA 0.05%] and add to the tube. Resuspend for 10 s and incubate for 20 min at 4 °C, in the dark.

NOTE: Final concentrations of primary antibodies used in the present protocol were CD45 1:50, CD34 1:20, KDR 1:50, CD184 1:20, CD133 1:50. Protocol may be stopped at this step by fixing lymphocytes in 4% paraformaldehyde in PBS and storing samples up to 24 h at 4 °C.

2.9. Centrifuge at 1,800 *x g* for 2 min at 4 °C and discard the supernatant. Resuspend in 500 µL of 1x PBS, pH 7.4, EDTA 2 mM.

2.10. Perform flow cytometry analysis.

2.10.1. Set up the background with isotype-matched control antibodies. Then, at the FSC/SSC plot select lymphocytes spread, trying to exclude cellular debris, residual granulocytes, and other particles. Such distribution is considered as 100%.

NOTE: Lymphocytes usually spread in the lower-left region of the plot.

2.10.2. Use a gate with common immunophenotype containing high number of cells $CD45^+$ and $CD34^+$. Then, select for double positive immunophenotypes using gate which previously identified $CD45^+$, $CD34^+$, and adding either KDR $(VEGFR\text{-}2)^+$, $CD133^+$ or $CD184^+$. Identify MPCs subpopulations by their specific cell surface markers. Report as the percentage of gated events.

2.11. Identify main subpopulations of MPCs. In the present study the immunophenotypes analyzed were $CD45^+CD34^+CD133^+$; $CD45^+CD34^+CD184^+$; $CD45^+CD34^+CD133^+CD184^+$; $CD45^+CD34^+KDR^+$; $CD45^+CD34^+KDR^+CD133^+$ and $CD45^+CD34^+KDR^+CD184$[7,17].

NOTE: These cell surface markers were used for the study: CD45 (lymphocytes), CD34 (endothelial and/or vascular cells), KDR (VEGFR-2) (membrane marker of endothelial cells), CD133 (endothelial progenitor cells) and CD184 (hematopoietic stem cells and endothelial cells).

3. Relation of MPCs with modification of endothelial function and hemodynamic test (FMD)

3.1. Determine flow-mediated dilation (FMD), pre- and post-angioplasty.

3.1.1. Use a vascular linear transducer to measure the diameter of the brachial artery.

3.1.2. Place the cuff of the sphygmomanometer above the measurement site in the forearm and insufflate at 50 mmHg above the systolic blood pressure for 5 min and deflate.

3.2.3. Determine again the diameter of the brachial artery within the next 60 s. Use the equation below to estimate FMD.

NOTE: Calculate the degree of dilatation using the equation (%) = (maximum diameter after transient ischemia - basal diameter) × 100 / basal diameter.

3.2. Correlate the number of MPCs with the baseline FMD value and post-angioplasty delta of FMD.

4. Prognostic ability of MPCs for limb amputation

4.1. Schedule periodic medical appointments after balloon angioplasty and patient discharge, to evaluate the quality of blood flow to the lower limb.

4.2. Evaluate clinical severity of limb ischemia at 2 weeks after angioplasty. Evaluate resolving rest pain, lower ischemia, and preservation of a functional foot, according to the Rutherford classification[13].

4.3. Compare clinical severity of limb ischemia, at baseline versus follow up. Identify those cases requiring major amputation due to unfavorable outcome.

4.4. Correlate the number of MPCs with the proportion of patients requiring major amputation of the lower limb.

REPRESENTATIVE RESULTS:

Blood samples from blocked arteries, at the site addressed for angioplasty, were collected from 20 diabetic patients, aged 68 years old and 10 out of 20 were males. Half of sample population were smokers. Vascular lesions were mainly scored as Rutherford class VI; whereas patients showed a higher prevalence of type 2 Diabetes Mellitus (100%), hypertension (70%) and dyslipidemia (55%).

A 30 days clinical follow up after lower limb angioplasty was carried out. The percentage of MPC subpopulations at baseline or dynamics after angioplasty were correlated (Spearman analysis) with the degree of endothelial dysfunction, as evaluated by FMD; and the baseline number of MPCs were compared between patients undergoing, or not, limb amputation after angioplasty (U-Mann Whitney). The study showed that baseline MPCs subpopulation $CD45^+CD34^+KDR^+$ negatively correlated with FMD (**Figure 3A**, left), whereas the change of MPCs $CD45^+CD34^+CD133^+CD184^+$ after angioplasty significantly correlated with FMD improvement (**Figure 3B**, right). Furthermore, increased baseline number of MPCs subpopulation $CD45^+CD34^+KDR^+$ (**Figure 4A,B**, left) were observed in those patients who evolved to limb amputation; as well as post-angioplasty reduction of MPCs subpopulation $CD45^+CD34^+CD133^+CD184^+$ (**Figure 4A,C**, right).

FIGURE LEGENDS:

Figure 1: Lower limb angiography and blood collection. (**A**) Vascular trajectory evidenced by contrast media under fluoroscopy. (**B**) Vascular obstruction before angioplasty. (**C**) Vascular obstruction after angioplasty. (**D**) Vascular surgeon uses a catheter to collect blood from the closest location to the vascular obstruction and atheroma plaque, and Lab researcher is ready to obtain the blood sample. Arrows indicate the site of vascular obstructions.

Figure 2: Blood sample preparation and mononuclear progenitor cells (**MPCs) determination.** (**A**) Density gradient preparation. (**B**) Lymphocytes ring separation after blood centrifugation. (**C**) Collection of the lymphocyte phase. (**D**) Centrifugation. (**E**) Pellet formation at the bottom of the test tube. (**F**) Cell suspension count. (**G**) Preparation of lymphocytes for flow cytometry. (**H**) Determination of cell subpopulations by flow cytometry.

Figure 3: Relation of MPCs with hemodynamic indicators. (**A**) Position of the ultrasound to acquire FMD and representative results. (**B**) Relationship between baseline %MPCs subpopulations (left, $CD45^+CD34^+KDR^+$; right, $CD45^+CD34^+CD133^+CD184^+$) and baseline FMD values; as well as the relation of (**C**) %MPCs after angioplasty with FMD improvement after angioplasty. Abbreviatures: MPCs, Mononuclear Progenitor Cells; FMD, Flow Mediated Dilation.

Figure 4: MPCs and prognosis of lower limb amputation after angioplasty. (**A**) Representative flow cytometry images of MPCs subpopulations. (**B**) The association of baseline %MPCs subpopulations (left, $CD45^+CD34^+KDR^+$; right, $CD45^+CD34^+CD133^+CD184^+$), or (**C**) %MPCs after angioplasty, with lower limb amputation after angioplasty, during a 30-days follow up. Abbreviatures: MPCs, Mononuclear Progenitor Cells.

Supplementary File 1: Rutherford's classification of severity of limb ischemia.

DISCUSSION:

Blood collection at the precise site of the vascular block may show technical difficulties; therefore, we performed blood collection in the proximity to vascular block. Likewise, the amount of MPCs close to the vascular plaque seems to be highly dynamic and may originate

variations before and after angioplasty. According to our observations, it is recommended to evaluate baseline- and 30min-post-angioplasty changes in the number of MPCs, since they may reflect several pathophysiological processes occurring within vascular damage and repair.

Blood sample processing for MPCs determination is recommended to be performed within the first 3 h; therefore, adequate organization plan, and even a previous simulation practice, may be established between angiology-vascular surgery team and lab researchers. MPCs isolation should be carefully performed, particularly deposing blood sample into density gradient and washes of the pellet containing MPCs. Our group use to transfer cells into cytometry tube, add primary antibodies, fix, and store cells overnight at 4 °C; due to time-administration convenience, and flow cytometry reading would be performed the day after.

Regarding the role of circulating MPCs as a useful clinical biomarker of vascular damage and repair, important efforts have been reported to standardize immunophenotypes between progenitor cells[17]. A comprehensive characterization should include subpopulations of circulating progenitor cells participating in the different clinical scenarios within vascular diseases. Using the methods described here, we found that post-angioplasty reduction of MPCs subpopulation $CD45^+CD34^+CD133^+CD184^+$ is predictive for major amputation. This finding supports the notion that inflammatory response during vascular injury or angioplasty stimulate homing signals for MPCs, promoting local tissue repair[18,19].

Likewise, the observation is consistent with reported effect of reduced number of $CD45^+CD34^+CD133^+$ and $CD45^+CD34^+CD133^+184^+$ subpopulations of MPCs as a predictor of adverse cardiovascular outcomes after coronary angioplasty[7], which may be explained by the increased ability of less differentiated phenotypes of endothelial progenitors to adhere to extracellular matrix, to proliferate and to respond to vasculogenic stimulus [18].

Furthermore, we observed that an increased number of $CD45^+CD34^+KDR^+$ subpopulation of MPCs after angioplasty was related with limb amputation although they have been considered to contribute to vascular repair. This controversy may be explained due to: 1) variations in the study design, since other studies have compared the number of $CD45^+CD34^+KDR^+$ MPCs between patients with CLI and healthy controls[19]; 2) variations in the methods for blood sampling, including the site and the time regarding the angioplasty; and 3) the type of artery blocked and vascularized.

Prognostic characterization of novel biomarkers, based on mechanisms responsible for vascular repair in several diseases, has received significant attention in translational research. This is the first description of a method to explore the role of MPCs, locally determined at a site close to the vascular block, in the prognosis of limb amputation after angioplasty in cases with CLI. One limitation is the lack of MPCs determination at more time points after angioplasty. However, we believe that our findings enrich the field of vascular research by characterizing the translational role of MPCs during vascular damage, repair, and prognostic potential for major amputation in patients with CLI. Particularly, we consider that the method described here may be useful in the prediction of adverse vascular outcomes in clinical settings involving a vascular injury and repair

mechanisms, such as lower limb and coronary ischemia, stroke, vasculitis, and/or venous thrombosis.

ACKNOWLEDGMENTS:

The authors thank the support of Institutional Program E015 for the project ID 356.2015.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Serrano-Hernando, F. J., Martín-Conejero, A. Peripheral artery disease: Pathophysiology, diagnosis and treatment. *Revista Española de Cardiología.* **60** (9), 969-982 (2007).
2. Agarwal, S., et al. Burden of re-admissions among patients with critical limb ischemia. *Journal of the American College of Cardiology.* **69** (15), 1897-1908 (2017).
3. Kolte, D., et al. Thirty-day re-admissions after endovascular or surgical therapy for critical limb ischemia: Analysis of the 2013 to 2014 nationwide re-admissions databases. *Circulation.* **136** (2), 167-176 (2017).
4. Rowlands, T. E., Donnelly, R. Medical therapy for intermittent claudication. *European Journal of Vascular and Endovascular Surgery.* **34**, 314-321 (2007).
5. Cronewett, J. L. *Acute limb ischemia and lower extremity chronic arterial disease: Rutherford's vascular surgery (8th ed.)*. Saunders Elsevier. Philadelphia, PA. (2014).
6. Dick, F., et al. Surgical or endovascular revascularization in patients with critical limb ischemia: influence of diabetes mellitus on clinical outcome. *Journal of Vascular Surgery*. **45** (4), 751-761 (2007).
7. Suárez-Cuenca, J. A., et al. Coronary circulating mononuclear progenitor cells and soluble biomarkers in the cardiovascular prognosis after coronary angioplasty. *Journal of Cellular and Molecular Medicine*. **23** (7), 4844-4849 (2019).
8. Franz, R., et al. Use of autologous bone marrow mononuclear cell implantation therapy as a limb salvage procedure in patients with severe peripheral arterial disease. *Journal of Vascular Surgery.* **50** (6), 1378-1390 (2009).
9. Benoit, E., O´Donnell, T. F., Patel, A. N. Safety and efficacy of autologous cell therapy in critical limb ischemia: A systematic review. *Cellular Transplantation.* **22** (3), 545-562 (2013).
10. Hill, J. M., et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *New England Journal of Medicine.* **348** (7), 593-600 (2003).
11. Schmidt-Lucke, C., et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation.* **111** (22), 2981-2987 (2005).
12. Smadja, D. M., et. al. Early endothelial progenitor cells in bone marrow are a biomarker of cell therapy success in patients with critical limb ischemia. *Cytotherapy.* **14** (2), 232-239 (2012).
13. Kremastinos, D. T. et al. Intracoronary cyclic-GMP and cyclic-AMP during percutaneous transluminal coronary angioplasty. *International Journal of Cardiology.* **53** (3), 227-232 (1996).
14. Truong, Q. A., Januzzi, J. L., Szymonifka, J., Thai, W. E., Wai, B., Lavender, Z. Coronary

sinus biomarker sampling compared to peripheral venous blood for predicting outcomes in patients with severe heart failure undergoing cardiac resynchronization therapy: the BIOCRT study. *Heart Rhythm.* **11** (12), 2167-2175 (2014).
15. Ding, N. et al. Fibrosis and inflammatory markers and long-term risk of peripheral artery disease: The ARIC study. *Arteriosclerosis, Thrombosis and Vascular Biology.* **40** (9), 2322-2331 (2020).
16. Potier, L. *et al.* Plasma copeptin and risk of lower-extremity amputation in Type 1 and Type 2 diabetes. *Diabetes Care.* **40** (12), 2290-2297 (2019).
17. Schmidt-Lucke, C., et al. Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. *PLoS One*. **5**(1), e13790 (2010).
18. Marboeuf, P., et al. Inflammation triggers colony forming endothelial cell mobilization after angioplasty in chronic lower limb ischemia. *Journal of Thrombosis and Haemostasis.* **6** (1), 195-197 (2008).
19. Regueiro, A., et al. Mobilization of endothelial progenitor cells in acute cardiovascular events in the PROCELL study: Time-course after acute myocardial infarction and stroke. *Journal of Molecular and Cellular Cardiology.* **80**, 146-155 (2015).

Figure 1

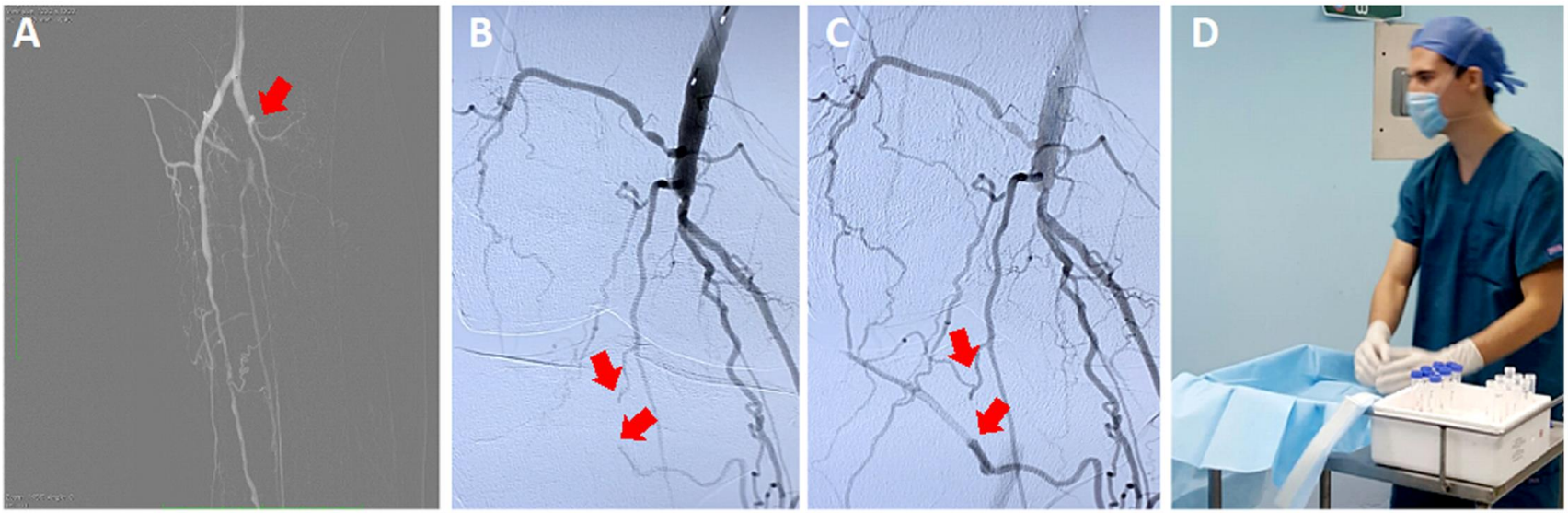


Figure 2

Click here to access/download;Figure;Fig 2-HD@2.tif

Figure 3

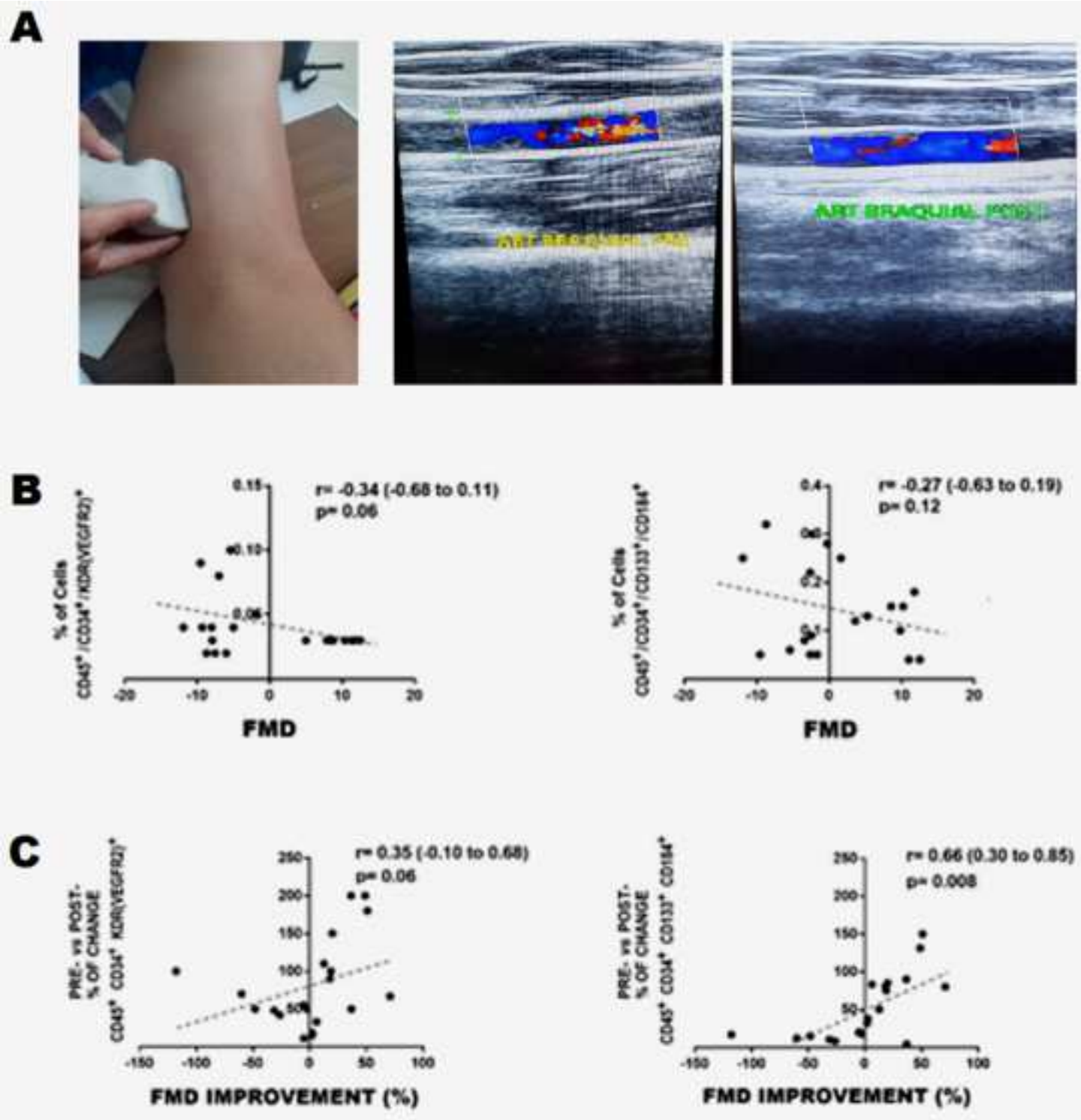
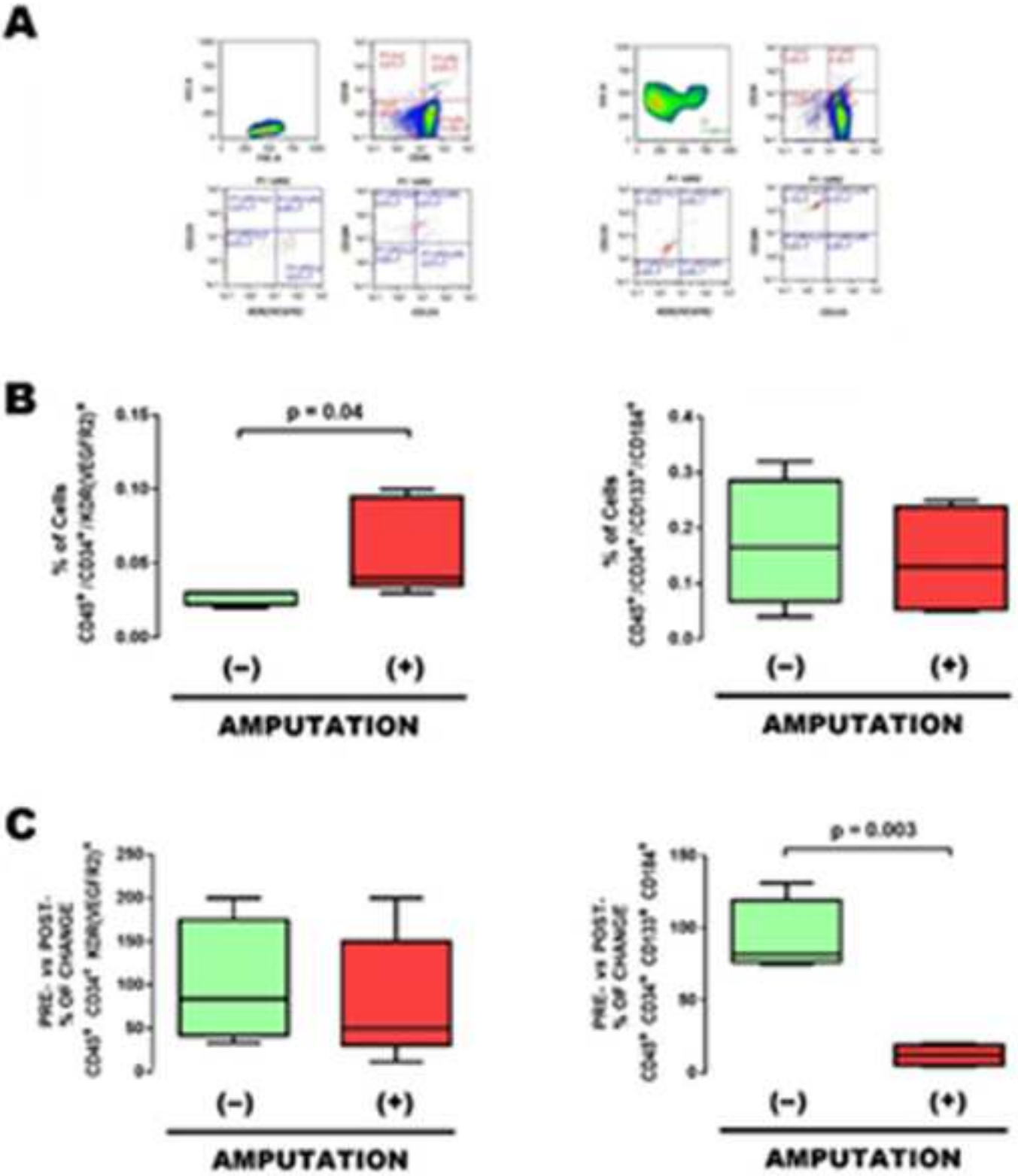


Figure 4



Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
BSA	Roche	10735086001	Bovine Serum Albumin (BSA) as a buffering agent, stabilizer, standard and for blending.
Calibration Beads	Miltenyi Biotec / MACS	#130-093-607	MACQuant calibration beads are supplied in aqueous solution containing 0.05% sodium azide. 3.5 ml for up to 100 tests
CD133/1 (AC133)-PE	Milteny Biotec / MACS	#130-080-801	Antibody conjugated to R-Phycoerythrin in PBS/EDTA buffer
CD184 (CXCR4)-PE-VIO770	Miltenyi Biotec / MACS	#130-103-798	Monoclonal, Isotype recombinant human IgG1, conjugated
CD309 (VEGFR-2/KDR)-APC	Miltenyi Biotec / MACS	#130-093-601	Antibody conjugated to R-Phycoerythrin in PBS/EDTA buffer
CD34-FITC	Miltenyi Biotec / MACS	#130-081-001	The monoclonal antibody clone AC136 detecs a class III epitope of the CD34
CD45- VioBlue	Miltenyi Biotec / MACS	#130-092-880	Monoclonal CD45 Antibody, human conjugated
Conical Tubes	Thermo SCIENTIFIC	#339651	15ml conical centrifuge tubes
Cytometry Tubes	FALCON Corning Brand	#352052	5 mL Polystyrene Round-Bottom Tube. 12x75 style. Sterile.
EDTA	BIO-RAD	#161-0729	Heavy metals, (as Pb) <10ppm, Fe<0.01%, As<1ppm, Insolubles<0.005%
Improved Neubauer	Without brand	Without catalog number	Hemocytometer for cell counting. (range 0.1000mm, 0.0025mm^2)
K2 EDTA Blood Collection Tubes	BD Vacutainer	#367863	Lilac plastic vacutainer tube (K2E) 10.8mg, 6 mL.
Lymphoprep	Stemcell Technologies	01-63-12-002-A	Sterile and checked on the presence of endotoxins. Density: 1.077±0.001g/mL
Paraformaldehyde	SIGMA-ALDRICH	#SZBF0920V	Fixation of biological samples, (powder, 95%)
Pipette Transfer 1,3mL	CRM Globe	PF1016, PF1015	The transfer pipette is a tool that facilitates liquid transfer with greater accuracy.

Test Tubes	KIMBLE CHASE	45060 13100	Heat-resistant test tubes. SIZE/CAP 13 x 100 mm

ANSWER TO REVIEWERS	
Editorial comment. The editor has formatted the manuscript to match the journal's style. Please retain.	The authors appreciate the comment. The manuscript format was retained.
Editorial comment. Employ professional copyediting services.	The authors appreciate the comment. The manuscript was submitted to proofreading.
Editorial comment. Please address all specific comments marked in the manuscript.	The authors appreciate the comment. Title proposal is acceptable: "*Predicting Amputation Using Local Circulating Mononuclear Progenitor Cells in Angioplasty-treated Patients with Critical Limb Ischemia*"
Editorial comment. The manuscript needs a thorough proofreading, please use professional copyediting services.	The authors appreciate the comment. The manuscript was submitted to proofreading.
Editorial comment. Please ensure that summary is no more than 50 word limit. Also please ensure that summary clearly states the goal of the protocol: "*This protocol presents…"*	The authors appreciate the comment. The summary was modified to express clearer ideas and fit word number limit, as follows: *"Lower limb amputation may occur even after angioplasty of obstructed vessels in Critical Limb Ischemia (CLI). Mononuclear Progenitor Cells (MPCs) reflect vascular repair. The present protocol describes the quantification of MPCs from circulation close to angioplasty, and its relation with endothelial dysfunction and prediction of lower limb amputation".*
Editorial comment. Please ensure that the Abstract is within 150-300-word limit and clearly states the goal of the protocol.	The authors appreciate the comment. The abstract was modified to express clearer ideas (word number limit was correct), as follows: *"…Mononuclear Progenitor Cells (MPCs) are reactive to vascular damage and repair, with ability to reflect vascular diseases. The present protocol describes quantification of MPCs obtained from blood circulation from vessel close to the angioplasty site, as well as its relation with endothelial dysfunction and its predictive ability for limb amputation in the next 30 days after angioplasty in patients with CLI."*
Editorial comment. Please revise the Introduction to include all of the following: a) A clear statement of the overall goal of this method	The authors appreciate the comment. The introduction was modified to clearly express: a) the goal of this method: *"The purpose of the present study is to describe how to determine the amount of MPCs circulating close to the vascular obstruction in patients with CLI undergoing angioplasty; and how to evaluate the prognostic relation between MPCs with indicators of endothelial dysfunction and limb amputation."*

b) The rationale behind the development and/or use of this technique	b) the underlying rationale: *"Due to MPC's ability to proliferate, migrate and vascular adherence; these cells have become good candidates to reflect endothelial repair in response to ischemia; while the local determination of MPC's has been considered useful to reflect clinical outcome."*
c) The advantages over alternative techniques with applicable references to previous studies	c) the advantages over alternative techniques: *"Compared with the prognosis based on comorbidities and intrinsic vascular features, the amount of local MPCs show specific ability to predict clinical outcome regarding endothelial dysfunction and limb amputation. Consistently, some studies have described the prognostic role of similar biomarkers during the evaluation of patients with PAD".*
d) A description of the context of the technique in the wider body of literature	d) the context of the technique: *"In addition, continuous interest in mechanisms underlying vascular injury has motivated exploring the prognostic role of local occurring biomarkers, since they are considered to closer reflect vascular damage and repair".*
e) Information to help readers to determine whether the method is appropriate for their application	e) information to help readers: *"Based on previous results, the method described here may be useful for an early identification of population at risk of adverse vascular outcomes in several clinical settings, such as lower limb and coronary ischemia, stroke, vasculitis, venous thrombosis and others involving vascular injury and repair".* <u>Accordingly, some references were added:</u> Ding, N. et al. Fibrosis and Inflammatory Markers and Long-Term Risk of Peripheral Artery Disease: The ARIC Study. Arterioscler Thromb Vasc Biol. 40(9), 2322-2331 (2020). Potier, L. et al. Plasma Copeptin and Risk of Lower-Extremity Amputation in Type 1 and Type 2 Diabetes. Diabetes Care. 40(12), 2290-2297 (2019). Kremastinos, D.T., Iliodromitis, E.K., Markianos, M., Apostolou, T.S., Kyriakides, Z.S., Karavolias, G.K. Intracoronary cyclic-GMP and cyclic-AMP during percutaneous transluminal coronary angioplasty. *International Journal of Cardiology.* 53(3), 227-232 (1996). Truong, Q.A., Januzzi, J.L., Szymonifka, J., Thai, W.E., Wai, B., Lavender, Z. Coronary sinus biomarker sampling compared to peripheral venous blood for predicting outcomes in patients with severe heart failure undergoing cardiac resynchronization therapy: the BIOCRT study. *Heart Rhythm.* 11(12), 2167-2175 (2014).
Specific comment: Please reword to bring out clarity.	The authors appreciate the comment. The introduction was modified to: *"…..; while clinical prognosis is unfavorable, and marked by a 30% risk of limb amputation and mortality during 1 year".*

Specific comment: Please include the name of the institute.	The authors appreciate the comment. The following sentence in the section of PROTOCOL was modified to:*"…committee of the Centro Médico Nacional "20 de Noviembre", ISSSTE."*
Specific comment: Any age or sex specific bias of the patient undergoing surgery? Please include details about the patient cohort. This is important to bring out the link between MPC and CLI as the relationship might differ in different category of patients.	The authors appreciate the comment. The following note was added: *"Our study sample was constituted by 20 diabetic patients, aged 68 years old and 10 out of 20 were males. Half of the sample were smokers and most prevalent co-morbidities were type 2 diabetes mellitus, systemic arterial hypertension and/or dyslipidemia. The sample was intended to be standardized for age-, sex- and co-morbidities. Therefore, possible bias due to clinical-demographic influence on the relation between MPCs and CLI could not be ruled out".*
Specific comment: Please include how each step is performed.	The authors appreciate the comment. A detailed description of the steps was added.
Specific comment: This (regarding Rutherford's classification) can be moved to a table in .xlsx format and uploaded separately to your editorial manager account.	The authors appreciate the comment. Rutherford's Classification was described in an excel file, and it was included as additional material.
Specific comments:	The authors appreciate the comment. The information requested was provided, and included in the text.
Size of the needle?	Gauge size was 18g
Diameter and length?	The initial guide is further changed for a 6 Fr introducer.
Amount (contrast media) / concentration introduced (CO_2)?	The amount of contrast media used is 40 cc, diluted 1:1 in 0.9% saline. CO_2 is used at 10 to 20 mL per shot at a pressure of 12 psi.
Please label the individual panels as A, B, C, D. Please use an arrow to show the blocked site/s	Individual panels were labelled as A, B, C, D, and arrows were used to show blocked site/s. Figure legend was also modified.
Is this wire same as 1.2.3? Size?	No. Two 0.014 Fr wires (260 cm long) are used for navigation and two 0.014 Fr wires (260 cm long) are used for support, during a single procedure.
Catheter size?	Catheters sizes are 5 Fr and 3 Fr, and they are changed during the procedure.
How is this done (catheterism)? Impacting how? Please reword for clarity.	The text was modified to increase clarity: *"Advance a guide wire again. Then, introduce an angioplasty balloon catheter, which contains an inflatable balloon located at the end of the catheter. Advance the angioplasty balloon catheter and place the balloon right at the site of lesion. Perform the angioplasty by inflating the balloon against the blocking plaque located at the vascular wall. Verify blood flow restoration."*

Do you store the blood on ice or room temp?	Yes. We maintain on ice blood samples. This specification was added.
Please include post-operative procedures.	Provide post-operative care procedures, including anti-coagulation therapy using enoxaparin at 1mg/kg subcutaneous every 12 hours; aspirin 100 mg, statin and analgesia. Compress at the site of vascular puncture during 24 hours.
Please use micro symbol throughout. 10 microliters	The text was modified as suggested. The text was modified as suggested
Please include all the button clicks.	The description of the spread of lymphocytes was added as a NOTE.
Citation to show that these phenotype are actually associated with MPCs. Calculation steps cannot be filmed.	References were added. Calculation were re-redacted as a NOTE.
How is this done (clinical endpoints)?	The text was modified to increase clarity: *"Step 4.2. Evaluate clinical severity of limb ischemia at 2 weeks after angioplasty. Evaluate resolving rest pain, lower ischemia and preservation of a functional foot, according to the Rutherford classification [13]. Step 4.3. Compare clinical severity of limb ischemia, at baseline vs follow up. Identify those cases requiring major amputation because of unfavorable outcome."*
So which means 10 out of 20 were males? Please bring out clarity.	The text was modified to increase clarity: *"…were collected from 20 patients, aged 68 years old and 10 out of 20 were males. Half of sample population were smokers".*
Please upload high resolution figures.	Figures were uploaded at higher resolution.
Please ensure that the Discussion explicitly cover all of the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol b) Any modifications and troubleshooting of the technique c) Any limitations of the technique d) The significance with respect to existing methods e) Any future applications of the technique	The aspects recommended were covered.

Editorial comment. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.	The authors appreciate the comment. The references were re-formatted as recommended.
Editorial comment 4. Please include patient details as asked by the reviewer.	The authors appreciate the comment. The following note was added: *"Our study sample was constituted by 20 diabetic patients, aged 68 years old and 10 out of 20 were males. Half of the sample were smokers and most prevalent co-morbidities were type 2 diabetes mellitus, systemic arterial hypertension and/or dyslipidemia. The sample was intended to be standardized for age-, sex- and co-morbidities. Therefore, possible bias due to clinical-demographic influence on the relation between MPCs and CLI could not be ruled out"*.
Editorial comment 5. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.	The authors appreciate the comment. The highlight text is no more than 2.75 pages including headings and spacings.

Mexico City, September 9th, 2020.

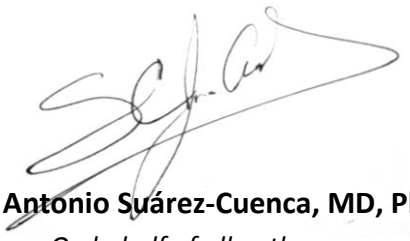
Dear JoVE

By this letter, the authors of the REVISED manuscript entitled**: "Predicting Amputation Using Local Circulating Mononuclear Progenitor Cells in Angioplasty-treated Patients with Critical Limb Ischemia"** appreciate the opportunity of its evaluation and consideration of publication in your renowned Journal JoVE.

The authors state that there are not actual or potential conflict of interests derived from relationships with pharmaceutical companies, biomedical device manufacturers or other corporation whose products or services may be related to the subject matter of the article or who have sponsored the study.

The authors certify that each author participated sufficiently in the study conception or design, data analysis or interpretation, and drafting of revision of the manuscript. Each author had approved the final version of the manuscript. This manuscript has not been published or is being considered for publication in other journal.

Sincerely,



Juan Antonio Suárez-Cuenca, MD, PhD

On behalf of all authors

RUTHERFORD'S CLASSIFICATION OF SEVERITY OF LIMB ISCHEMIA		
Category	**Characteristic**	**Definition**
0	asymptomatic	Normal treadmill
1	mild claudication	Ankle pressure (AP) after exercise >50 mmHg, but at least 20 mmHg lower than resting value
2	moderate claudication	Between categories 1 and 3
3	severe claudication	Cannot complete standard treadmill exercise and AP after exercise <50 mmHg
4	ischemic rest pain	Resting AP <40 mmHg, flat or barely pulsatile ankle or metatarsal pulse volume recording (PVR), toe pressure (TP) <30 mmHg
5	minor tissue loss	Resting AP <60 mmHg, flat or barely pulsatile ankle or metatarsal PVR, TP <40 mmHg
6	major tissue loss	Same as category 5, extending above transmetatarsal level, functional foot no longer salvageable