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Coronary Progenitor Cells and Soluble Biomarkers in Cardiovascular Prognosis after Coronary Angioplasty

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TITLE:

Coronary Progenitor Cells and Soluble Biomarkers in Cardiovascular Prognosis after Coronary Angioplasty

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KEYWORDS:

coronary circulating MPCs, soluble biomarkers, sICAM-1, MMP-9, malondialdehyde, SOD, cardiovascular prognosis, MACEs

SUMMARY:

Development of major adverse cardiovascular events, which impact cardiovascular prognosis after coronary angioplasty, are influenced by the extent of coronary damage and vascular repair. The use of novel coronary cellular and soluble biomarkers, reactive to vascular damage and repair, are useful to predict the development of MACEs and prognosis.

ABSTRACT:

Major adverse cardiovascular events (MACEs) negatively impact the cardiovascular prognosis of patients undergoing coronary angioplasty due to coronary ischemic injury. The extent of coronary damage and the mechanisms of vascular repair are factors influencing the future development of MACEs. Intrinsic vascular features like the plaque characteristics and coronary artery complexity have demonstrated prognostic information for MACEs. However, the use of intracoronary circulating biomarkers has been postulated as a convenient method for the early identification and prognosis of MACEs, as they more closely reflect dynamic mechanisms involving coronary damage and repair. Determination of coronary circulating biomarkers during angioplasty, such as the number of subpopulations of mononuclear progenitor cells (MPCs) as well as the concentration of soluble molecules reflecting inflammation, cell adhesion, and repair, allows for assessment of future developments and the prognosis of MACEs 6 months post coronary angioplasty. This method is highlighted by its translational nature and better performance than peripheral blood circulating biomarkers regarding prediction of MACEs and its effect on the cardiovascular prognosis, which may be applied for risk stratification of patients with coronary artery disease undergoing angioplasty.

INTRODUCTION:

Coronary angioplasty and stenting represent a salvage procedure for patients with coronary artery disease (CAD). However, major adverse cardiovascular events (MACEs), including cardiovascular death, myocardial infarction, coronary restenosis, and episodes of angina or decompensate heart failure, may occur months after coronary intervention, prompting unscheduled visits to the hospital. MACEs are common worldwide and their morbi-mortality is high[1].

Coronary ischemic injury induces early vascular response and reparative mechanisms involving mobilization of MPCs due to their differentiation ability and/or angio-reparative potential, as well as the production of soluble molecules like intercellular adhesion molecules (ICAMs), matrix metalloproteinases (MMPs), and reactive oxygen species, reflecting cell adhesion, tissue remodeling, and oxidative stress. Although intrinsic vascular features like plaque characteristics and coronary artery complexity have been used to predict MACEs, some studies have suggested that biomarkers related to the mechanisms of injury and repair occurring in the coronary endothelium could be very useful for the early identification and prognosis of cardiovascular events in patients with CAD submitted to coronary angioplasty[2–5.]

Continuous interest in understanding the mechanisms underlying CAD injury and repair has motivated investigators to study intracoronary circulating biomarkers, because coronary sampling more closely reflects vascular damage and repair[6]. However, characterization of coronary biomarkers in human studies has been scarce[7–9]. Therefore, the purpose of the present study was to describe a method to determine the amount of coronary circulating MPCs and soluble molecules, reflecting both vascular injury and repair, and to show whether these biomarkers are associated with MACEs and the clinical prognosis of CAD patients that underwent coronary angioplasty. This method is based on the use of vascular-related, circulating MPCs and soluble molecules obtained by sampling locations closest to the vessel damage. It may also be useful for clinical studies for lower limb ischemia, stroke, vasculitis, venous thrombosis, and other

injuries involving vascular injury and repair.

PROTOCOL:

This protocol meets the institutional guidelines from the human research Ethics Committee.

1. Coronary angiography, ultrasound, and blood sampling

1.1. Request baseline clinical and demographic information before coronary intervention. Collect the individual's data: age, sex, current smoking status, body mass index (BMI), high blood pressure, dyslipidemia, diabetes mellitus, medications, and the indication for current coronary angiography.

1.2. Perform coronary angiography through heart catheterization using a radial approach. This procedure should be performed under a fluoroscopy guide in the hemodynamics room by expert cardiologists.

NOTE: Identify evaluable vessels. For the present study, evaluable vessels were defined as arteries with sections larger than 1.5 mm and lumen stenosis of more than 50%.

1.3. Advance the intravascular ultrasound catheter to the region of interest and record images. Use the appropriate software to locate and measure the smallest luminal area.

1.4. Use a coronary catheter to collect 10 mL of blood from the closest location to the plaque.

1.5. After patient discharge, schedule periodical medical evaluations to follow up study endpoints. If telephone contact is not possible or a physician visit is delayed for longer than 2 months, request an authorized person (previously designed) to verify the study endpoints.

NOTE: Consider any of the following a MACE: 1) cardiovascular death, 2) new myocardial infarction, 3) unstable angina prompting an unscheduled medical visit within 24 h, 4) stent restenosis as demonstrated by coronary angiography, 5) episodes of decompensated heart failure requiring clinical attention.

2. Determination of circulating MPCs (Figure 2)

2.1. Process the blood within 1 h from collection. Transfer 6 mL of the collected blood to a 15 mL conical tube and dilute 1:1 (v/v) with 1x phosphate buffered saline (PBS), pH = 7.4.

2.2. Add 2 mL of density gradient medium to three test tubes. Carefully transfer three equal volume aliquots of diluted blood into each test tube containing the density gradient medium.

NOTE: The total volume of density gradient medium and diluted blood should not exceed three-fourths of the test tube maximal capacity.

2.3. Centrifuge at 1,800 x *g*, 4 °C for 30 min. Transfer the band at the interface between the layers into a new tube. Add 2 mL of PBS and centrifuge at 1,800 x *g,* 4 °C for 6 min. The pellet will contain the MPCs.

2.4. Wash the pellet several times. Aspirate off the previous solution and gently resuspend the cell pellet in fresh PBS. For subsequent washes, centrifuge at 1,800 x *g*, 4 °C for 2 min. Repeat the process 6x.

2.5. Resuspend the cell pellet in 1 mL of PBS. Mix 20 µL of the cell suspension with 0.4% trypan blue, diluted 1:1 (v/v). Apply a drop to a hemocytometer and count the unstained cells under a light microscope.

2.6. Proceed to MPCs determination. Label 5 mL flow cytometry tubes and aliquot out 1 x 10^6 cells per tube. Prepare the corresponding isotype-matched control antibodies. Centrifuge at 1,800 x *g*, 4 °C for 6 min and discard the supernatant.

2.7. Add the primary antibody diluted in 100 µL of an antibody incubation solution consisting of 1x PBS (pH = 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% bovine serum albumin (BSA). Resuspend for 10 s and incubate for 20 min at 4° C, light-protected. The protocol may be paused at this step by fixing the lymphocytes in 4% paraformaldehyde in PBS and storing samples up to 24 h at 4 °C.

NOTE: The final concentrations of the primary antibodies used in the present protocol were CD45 1:50, CD34 1:20, KDR 1:50, CD184 1:20, CD133 1:50.

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

2.9. Perform flow cytometry analysis. Use isotype-matched control antibodies to set up the background staining. Then, select lymphocytes spread at the FSC/SSC plot, trying to exclude residual granulocytes, cellular debris, and other particles, which are usually located in the lower, left-distributed in the plot. Such distribution is considered as 100%.

2.10. Use a gate containing a high number of cells with common immunophenotype $CD45^+$ and $CD34^+$. For double positive immunophenotypes, use a gate previously identifying $CD45^+$, $CD34^+$, with the addition of either KDR $(VEGFR\text{-}2)^+$, $CD133^+$, or $CD184^+$. Identify the MPC subpopulations by their specific cell surface markers. Report as the percentage of gated events.

2.11. Identify the main subpopulations of MPCs. In the present study the main immunophenotypes were $CD45^+CD34^+CD133^+$, $CD45^+CD34^+CD184^+$, $CD45^+CD34^+CD133^+CD184^+$, $CD45^+CD34^+KDR^+$, $CD45^+CD34^+KDR^+CD133^+$, and $CD45^+CD34^+KDR^+CD184$.

NOTE: The cell surface markers used were 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. Determination of plasma soluble biomarkers

3.1. Use an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of SICAM-1 and MMP-9 (**Figure 3**, upper row).

3.1.1. Centrifuge the blood samples at 3,000 x *g*, room temperature for 5 min and collect the plasma.

3.1.2. Label the standards, sample tubes, and control tubes. Equilibrate the pre-coated wells in the assay plate by washing 2x with the washing buffer provided in the ELISA kit.

3.1.3. Transfer the standards, samples, and controls to the wells. Seal and incubate at 37 °C for 90 min.

NOTE: Do not let the wells dry completely.

3.1.4. Discard the contents and add the biotin-detection antibody. Seal and incubate at 37 °C for 60 min.

3.1.5. Discard the contents and wash 3x. Seal the plaque and incubate sequentially with streptavidine working solution followed by tetramethylbenzidine substrate at 37 °C for 30 min, light-protected. Wash 3x between incubations. When the color develops, add the stop solution, and read the optical density absorbance in a microplate ELISA reader.

3.2. Use an immuno-magnetic multiplexing assay to determine the concentration of tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) (**Figure 3**, lower row).

3.2.1. Label the standards, sample tubes, and control tubes.

3.2.2. Vortex the magnetic beads vials for 30 s. Transfer the bead suspension to appropriately sized tubes, and then to the wells in the multiplexing assay plate. Periodic vortexing avoids precipitation of the beads.

3.2.3. Securely insert the hand-held magnetic plate washer. Wait 2 min for the beads to accumulate on the bottom of each well and quickly invert both the hand-held magnetic plate washer and plate assembly, over a sink or waste container. Remember to use the hand-held magnetic plate washer to maintain the beads inside the wells.

3.2.4. Add 150 µL of wash buffer into each well and wait 30 s to allow the beads to accumulate on the bottom. Discard the contents as in step 3.2.3. Then, add 25 µL of universal assay buffer (provided in the kit) followed by 25 µL of prepared standards, samples, and controls.

3.2.5. Seal the plate and incubate for least 60 min at room temperature, light-protected, with constant shaking at 500 rpm. Alternatively, incubate overnight at 4 °C, light-protected, with constant shaking at 500 rpm if possible.

3.2.6. Wash 2x by adding 150 µL of wash buffer and wait 30 s. Discard the contents by inserting the hand-held magnetic plate washer. Wait 2 min and invert over a sink or waste container.

3.2.7. Incubate sequentially with 25 µL of detection antibody mixture followed by 25 µL of streptavidin-PE solution at room temperature for 30 min, sealed and light-protected, with constant shaking at 500 rpm. Wash 2x between incubations, as described in step 3.2.6.

3.2.8. Obtain the readings. Add 120 µL of reading buffer. Seal the plate and incubate 5 min at room temperature, light-protected, with constant shaking at 500 rpm. Run the reading on a multiplexing assay reader. Adjust the reading parameters according to each analyte.

REPRESENTATIVE RESULTS:

Coronary, venous sinus, and peripheral blood were collected from 52 patients that underwent coronary angiography (**Figure 1**) and showed a high prevalence of hypertension and dyslipidemia. At the clinical follow-up, 11 (21.1%) MACEs occurred 6 months after coronary angiography: death (n = 1), angina requiring hospital attendance (n = 6), myocardial infarction (n = 2), and/or evidence of heart failure (n = 4).

The baseline coronary concentration of most MPCs was significantly lower in patients who developed MACEs (**Figure 4**), with a larger decrease in MPC subpopulations $CD34^+CD133^+$ and $CD45^+CD34^+CD133^+CD184^+$. Likewise, patients who developed MACEs had an increased baseline in coronary amounts of sICAM-1 and lower MMP-9 (**Table 1**)**.**

Coronary MPCs (subpopulations $CD45^+CD34^+CD133^+$ and $CD45^+CD34^+CD133^+CD184^+$) and sICAM-1 (dichotomized by their median values) demonstrated prognostic ability for MACE-free survival (**Figure 5**).

We further characterized the dynamics of soluble biomarkers under different conditions, because there is very little information regarding coronary blood determination. The expression of tumor necrosis factor alpha (TNFα) showed variations according to the time of measurement (pre- or post-angioplasty) and the location of coronary sampling based on a comparison of different lumen areas at same coronary artery using intravascular ultrasound (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1: Coronary angiography and blood collection. The image shows heart catheterization using a radial approach, performed under a fluoroscopy guide in the hemodynamics room. Cardiology experts evaluate the coronary vessels during angiography and collect coronary blood from the closest location to the atheroma plaque and/or sinus blood through a heart catheter just before balloon angioplasty.

Figure 2: Blood sample preparation and MPCs determination by flow cytometry. (**A**) Density gradient after blood centrifugation (blue arrow = lymphocyte band). (**B**) Collection of the lymphocyte phase. (**C**) Washes with 1x PBS. (**D**) Centrifugation. (**E**) Pellet formation at the bottom of the test tube. (**F**) Neubauer cell suspension load. (**G**) Lymphocyte cell count using light microscopy. (**H**) Determination of cell subpopulations by flow cytometry.

Figure 3: Immunoassays to determine blood soluble mediators. Upper row: Enzyme-linked immunosorbent assay (ELISA). The image shows how information from the map samples (notebook) was transferred to the software to start the readings after sample preparation, antibody incubation, and washes. It also shows yellow color development, either in the standard wells (left columns in the plaque) or in the test samples (right columns in the plaque). Lower row: Immuno-magnetic multiplexing assay. After sample preparation, magnetic bead-antibody incubation, and washes, the sample information was transferred to the appropriate immuno-magnetic multiplexing assay system reader software, and a typical standard curve is shown in the screen.

Figure 4: Coronary circulating mononuclear progenitor cells (**MPCs**)**.** The figure shows baseline %MPCs subpopulations. (**A**) Representative readings from flow cytometry. (**B**) Quantification of %MPCs subpopulations with flow cytometry, plotted according to the presentation of MACEs (*) = significant difference, with $p < 0.05$. This figure has been modified from Suárez-Cuenca et al.[10].

Figure 5: Coronary circulating cellular (**MPCs**)**, soluble biomarkers and prognosis.** The figure shows baseline coronary blood amounts of (**A**) %MPCs subpopulations determined by flow cytometry and (**B**) plasma concentration of sICAM-1 determined by ELISA, both plotted according to the presentation of MACEs during the 6 month follow-up. The blue line indicates the number of individuals with risk values for each biomarker, such as lower %MPCs or higher sICAM-1. sICAM-1 = soluble intercellular adhesion molecule 1. This figure has been modified from Suárez-Cuenca, et al.[10].

Figure 6: Conditions determining variability of coronary circulating soluble biomarkers. The figure shows changes in the intracoronary concentration of tumor necrosis factor alpha (TNFα), according to the time of measurement (**A**: Pre-angioplasty or **B**: Post-angioplasty) as well as the location of coronary sampling (comparison between two coronary lumen diameters at a 3.5 mm cutoff, measured by intravascular ultrasound). (*) = $p < 0.05$ difference of biomarkers obtained pre- vs. post-angioplasty, and difference of sampling at locations of coronary lumen diameters ≤3.5 mm vs. >3.5 mm. This figure has been modified from Suárez-Cuenca et al.[11].

Table 1: Baseline blood soluble biomarkers. (*) indicates $p < 0.05$ difference biomarkers from coronary blood vs. peripheral circulation. (**) indicates $p < 0.05$, without MACEs vs. with MACEs; one-tailed independent T-test. Abbreviations: sICAM-1 = soluble intercellular adhesion molecule 1; IL-1β = interleukin 1 beta; MMP-9 = matrix metalloproteinase 9.

DISCUSSION:

Blood collection from the affected coronary artery may be difficult. Sometimes, the coronary artery is barely accessible. In this case, sampling from the venous sinus may be an alternative. We performed validation tests comparing circulating biomarkers in coronary artery vs. venous sinus, with no significant differences. However, the performance of circulating biomarkers was validated only for coronary sampling. Therefore, the performance of biomarkers obtained from the venous sinus remains to be explored.

It is best to process the samples for MPCs within the first 3 h after blood collection. Therefore, good communication should be established between the cardiology team and the lab researchers. During MPCs isolation, care should be taken when depositing blood samples during density gradient preparation when washing the MPCs pellet. Finally, for convenience, we always transfer cells into a cytometry tube, add the primary antibodies, fix and store the cells overnight at 4 °C, and perform the flow cytometry reading the day after. Regarding the biomarker role of circulating MPCs, important efforts have been taken to standardize the most clinically useful immunophenotypes between progenitor cells[12], but one limitation of the study may be the fact that specific subpopulations of circulating progenitor cells have not been fully characterized for all clinical scenarios within CAD or other vascular diseases. Therefore, different circulating progenitor cell subpopulations should be explored in each study.

During the determination of soluble markers some general recommendations for ELISA and multiplexing assays include the use of a multichannel pipette, depositing solutions at the bottom of each well without touching the side walls, and avoiding the drying out of the wells during the assay. Always check the sample distribution in the plate, particularly for the multiplexing assay, to avoid precipitation of the magnetic beads by constant vortexing. Also, make sure to insert the bottom plate into the hand-held magnetic plate washer to maintain the magnetic beads inside the wells, otherwise the samples will be lost during the washes.

We found that coronary circulating MPCs, mainly those from hematopoietic origin, as well as sICAM-1 and MMP-9, were outstanding biomarkers for prediction and prognosis of MACEs. This is consistent with the notion that inflammatory response and/or vascular damage mediators stimulate homing signals for MPC mobilization and recruitment, promoting local tissue repair[4]. Accordingly, we found variations in these biomarkers in several settings. Changes in relation to angioplasty and/or location of coronary sampling may be explained by the effect of the impact over the atheroma plaque during angioplasty, the size of the plaque, and the release of soluble mediators sequestered within the plaque into the coronary flow[11]. Increased IL-1β has been consistently involved in the development of the plaque and clinical complications[13].

To our knowledge, this is the first study prospectively evaluating the role of coronary circulating MPCs and soluble mediators of vascular injury and repair as prognostic biomarkers in a population with CAD submitted to coronary angioplasty, including characterization of changes related to angioplasty, location of coronary sampling, and comparison of coronary vs. peripheral sampling. We think that the method can be easily established in any hospital carrying out coronary angiography. However, one limitation is that we applied this methodology mainly in patients with chronic stable angina released from an emergency room department.

Current traditional methods used for MACEs prediction or prognosis in CAD have moderate predictive ability. There has been an increasing amount of interest in finding novel biomarkers based on the pathophysiology mechanisms responsible for repair and regeneration occurring after CAD and angioplasty. Such biomarkers have shown similar or better predictive performance compared with traditional methods[3–5,14,15]. Thus, we think that the role of coronary circulating MPCs and soluble mediators in predicting the risk for MACEs will be explored further in future prospective studies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1.eps

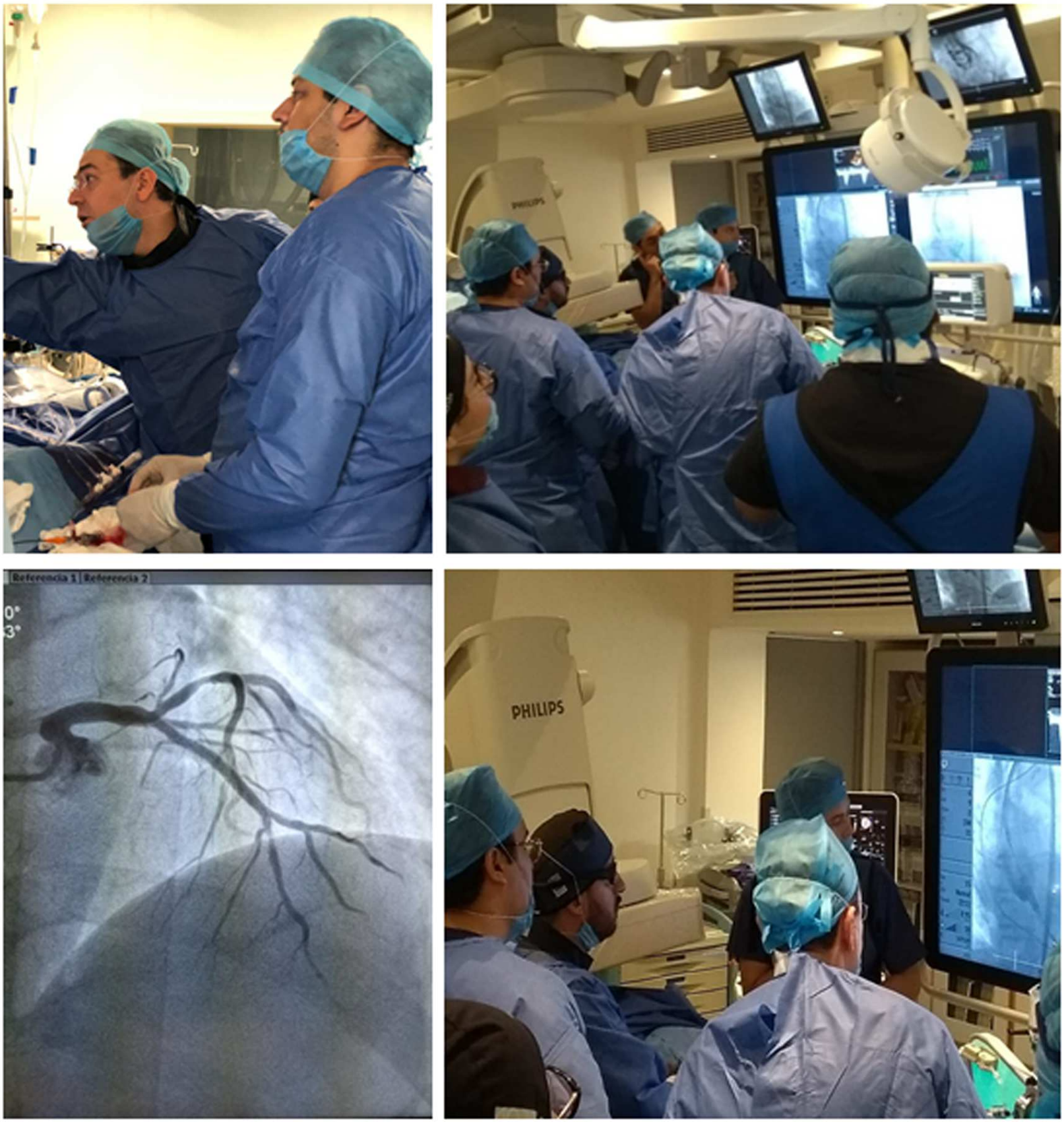


Figure 2

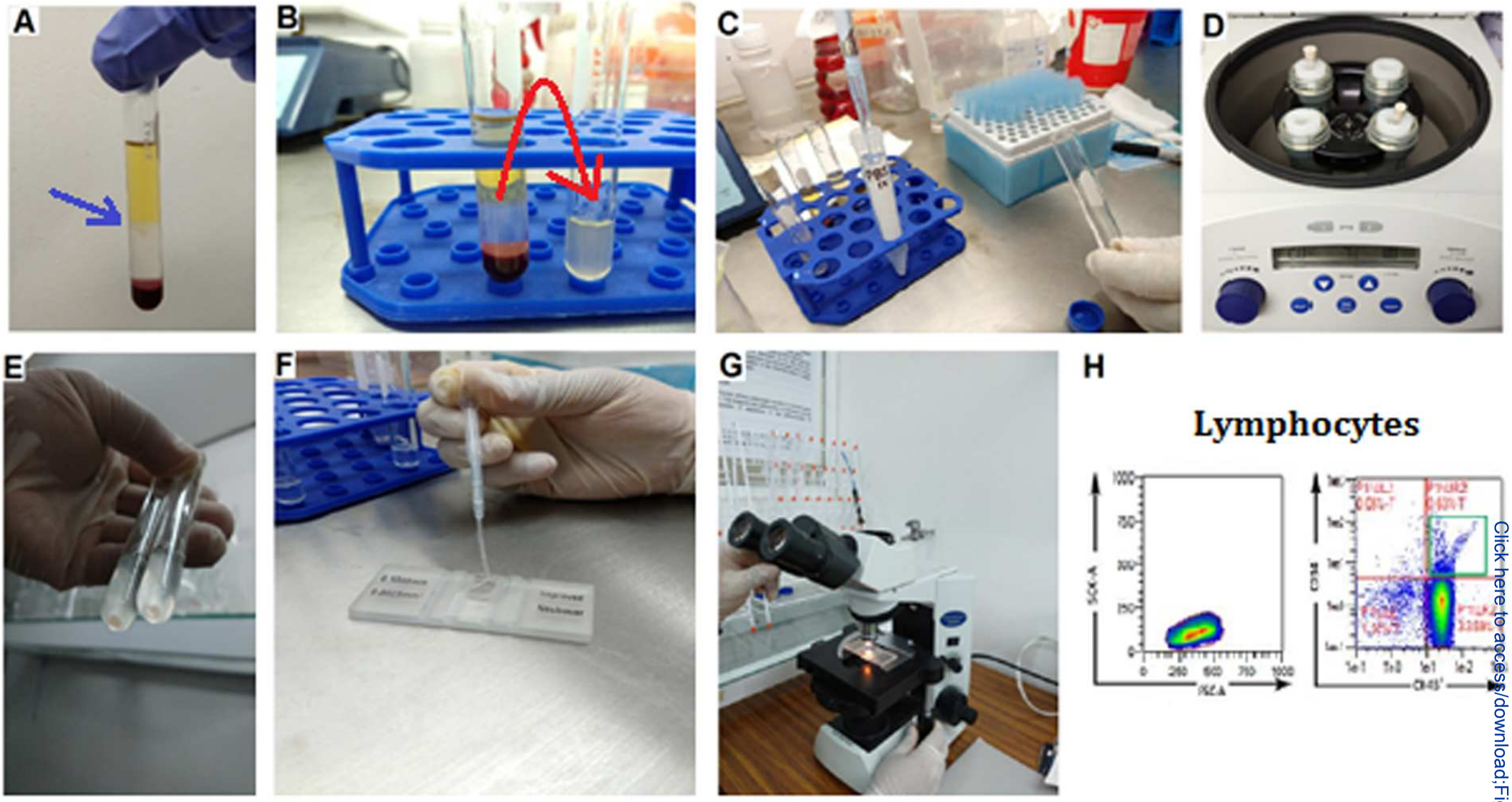
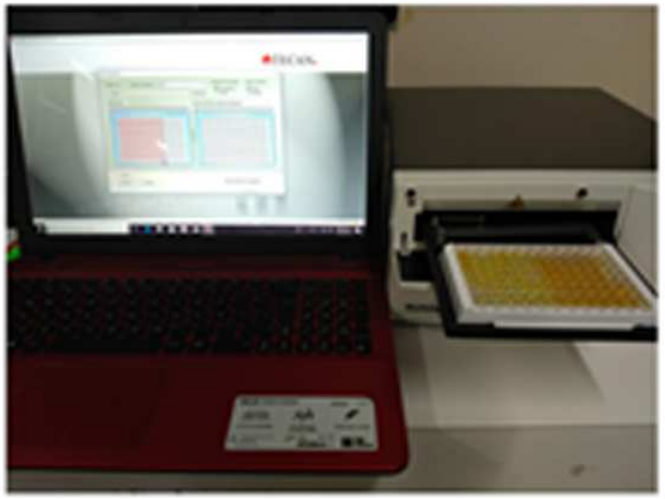
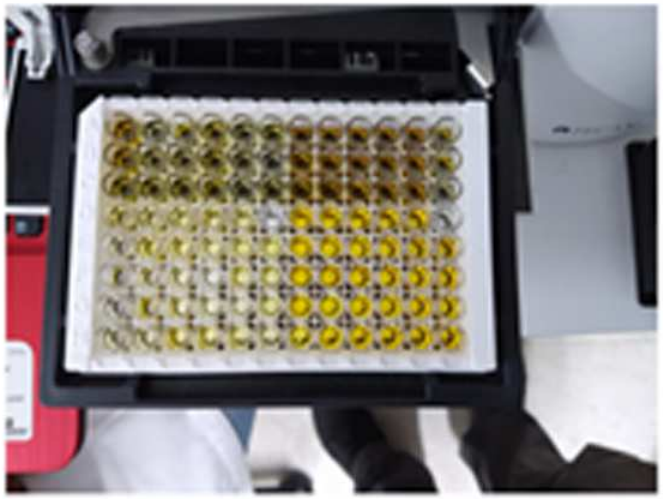


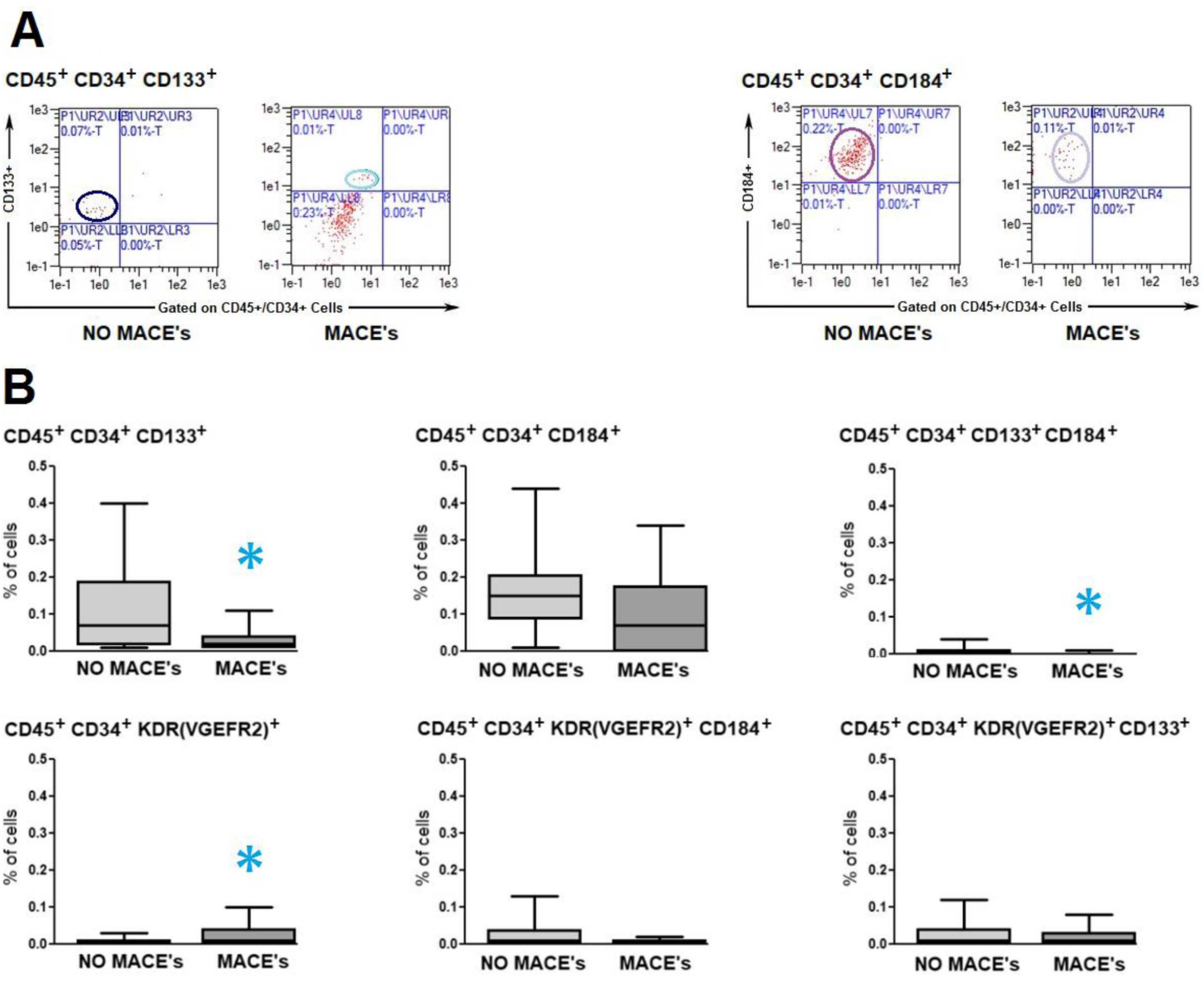
Figure 3

Enzyme-linked Immunosorbent *Assay (ELISA)*





Immuno-Magnetic Multiplexing Assay

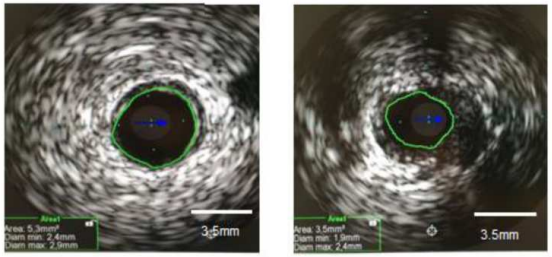


A. Pre-Angioplasty

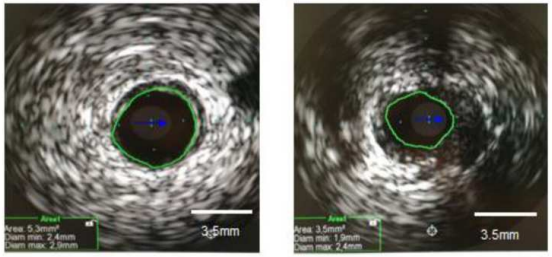
B. Post-Angioplasty

Intravascular Ultrasound

>3.5mm ≤3.5mm



>3.5mm ≤3.5mm



Circulating Biomarker (TNFα)

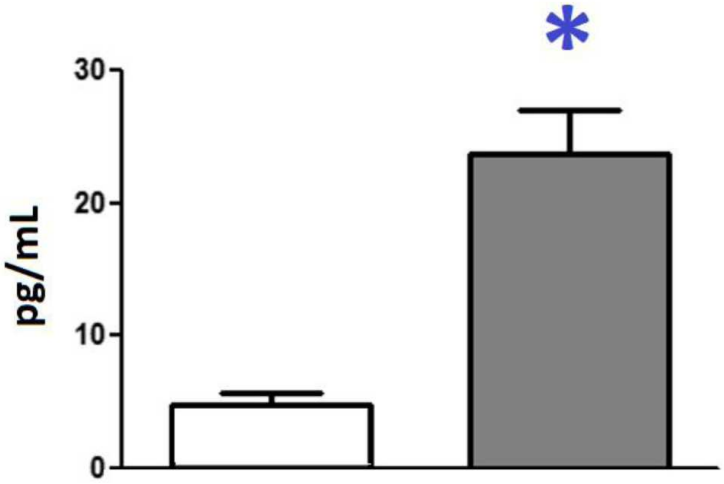
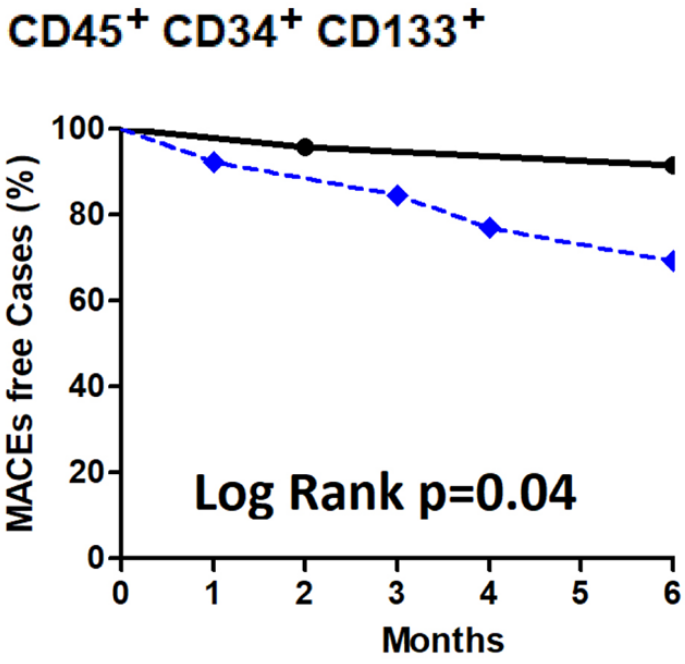
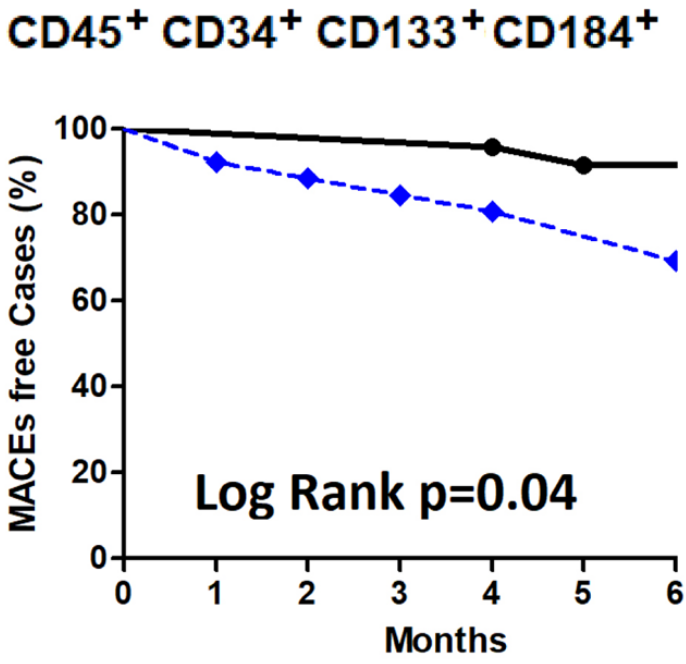


Figure 5

A. Cellular Circulating Biomarkers (MPCs)





B. Soluble Circulating Biomarkers

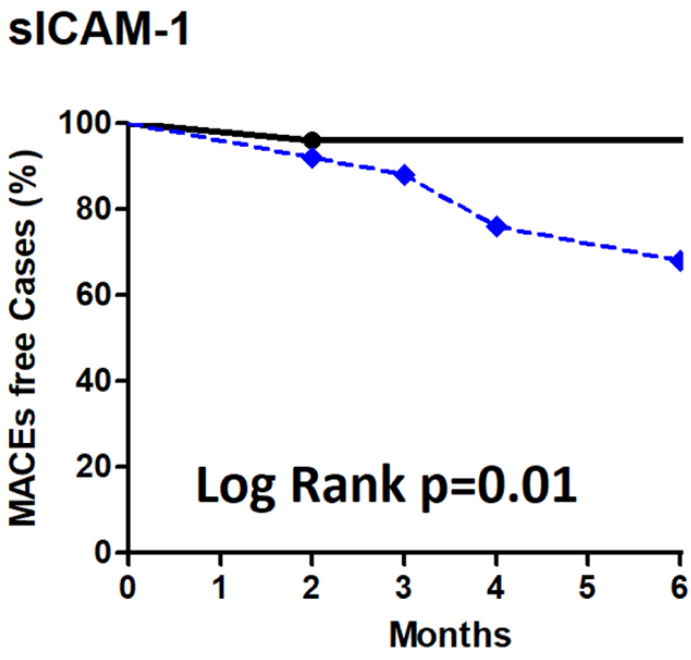


Table 1. Baseline Blooc

	no MACEs (r
	Coronary
sICAM-1 (pg/mL)	17.7 ± 4.8
IL-1β(ng/mL)	1.2 ± 0.75
MMP-9 (pg/mL)	113 ± 59

(*) indicates p<0.05 difference biomarkers from coronary w/o MACEs *vs*. MACEs; one-tail, independent T-test A Molecule 1; IL-1β, Interleukin 1 beta; MMP-9, Matrix Meta

l Soluble Biomarkers

ı = 32)	**MACEs (n = 20)**	
Peripheral	**Coronary**	**Peripheral**
17.9 ± 3.0	**25.8 ± 9.9****	**25.6 ± 9.7****
1.3 ± 0.94	1.2 ± 1.15	1.9 ± 2.02
208 ± 165	**66 ± 18*/****	132 ± 73

blood *vs.* peripheral circulation. (**) indicates p<0.05, bbreviatures: sICAM-1, soluble InterCellular Adhesion lloproteinase 9.

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

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RESPONSE TO COMMENTS FROM REVIEWERS (FORMAT COMMENTS) 3	
REVIEWER COMMENT	**RESPONSE AND ACTION(S) TAKEN**
Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	**Response:** We appreciate the reviewer's comment. **Actions:** We have performed a thoroughly proofread of the manuscript, searching for spelling or grammar issues.
Comment 2: The Abstract is over the 50 word limit.	**Response:** We appreciate the reviewer's comment. **Actions:** Abstract extension was limited to 49 words.
Comment 3: Please do not use more than 1 note for each step.	**Response:** We appreciate the reviewer's comment. **Actions:** Only one note was used for each step, if a note was required.
Comment 4: To film a step, all details need to be included. For example, in order to film "wash the pellet several times" in step 2.5, "Aspirate off previous solution and gently resuspend cell pellet in fresh PBS. For subsequent washes, centrifuge at 1800 x g, 4°C for 2 min and repeat the process during 6 times" also need to be highlighted. Please highlight fewer steps and highlight enough details for each highlighted step.	**Response:** We appreciate the reviewer's comment. **Actions:** Highlighted steps were edited to contain enough details, and fewer steps were considered.
Comment 5: Step 3.2.1: Please write this step in the imperative tense.	**Response:** We appreciate the reviewer's comment. **Actions:** the step 3.2.1 was re-written in imperative tense.
Comment 6: After you make all changes, please ensure that the highlighted protocol steps are fewer than 2.75 pages including headings and spacing.	**Response:** We appreciate the reviewer's comment. **Actions:** Highlighted protocol steps (including headings and spacing) were checked to meet a limit of 2.75 pages.
Comment 7: Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite the note after step 1.2, step 2.6, step 3.1.7, step 3.1.12, step 3.2.4.2, step 3.2.4.3.	**Response:** We appreciate the reviewer's comment. **Actions:** Indicated steps and notes were re-written. Likewise, techniques were described again, using more common language, and more similar to other published works.

RESPONSE TO COMMENTS FROM REVIEWERS (FORMAT COMMENTS) 2	
REVIEWER COMMENT	**RESPONSE AND ACTION(S) TAKEN**
Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	**Response:** We appreciate the reviewer's comment. **Actions:** We have performed a thoroughly proofread of the manuscript, searching for spelling or grammar issues.
Comment 2: Please highlight complete sentences (not parts of sentences) for filming.	**Response:** We appreciate the reviewer's comment. **Actions:** Complete sentences were highlighted (text for filming)
Comment 3: Please avoid long steps (more than 4 lines).	**Response:** We appreciate the reviewer's comment. **Actions:** Steps were reduced to ≤4 lines.
Comment 4: Please use a single space between numerical values and their units.	**Response:** We appreciate the reviewer's comment. **Actions:** Numerical values and their units were edited to contain single space between them.
Comment 5: Please use h, min, s for time units.	**Response:** We appreciate the reviewer's comment. **Actions:** recommended abbreviations for time units were replaced.
Comment 6: Please define all abbreviations before use.	**Response:** We appreciate the reviewer's comment. **Actions:** Abbreviations were defined before use.
Comment 7: Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).	**Response:** We appreciate the reviewer's comment. **Actions:** Personal pronouns were deleted from protocol text.
Comment 8: Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.	**Response:** We appreciate the reviewer's comment. **Actions:** Techniques were described again, using more common language, and more similar to other published works.

RESPONSE TO COMMENTS FROM REVIEWERS	
REVIEWER COMMENT	**RESPONSE AND ACTION(S) TAKEN**
General Comment: Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.	**Response:** We appreciate the comment. **Actions:** Figures at 300 dpi were saved as .eps. Table was saved ad .xlsx file.
Reviewer 1, Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version	**Response:** We appreciate the reviewer's comment. **Actions:** We have performed a thoroughly proofread of the manuscript, searching for spelling or grammar issues.
Reviewer 1, Comment 2: Figure 3: Please remove commercial language: MAGPIX	**Response:** We appreciate the reviewer's comment. **Actions:** Commercial name "MAGPIX" was deleted along all the text and modified for: Immuno-Magnetic Multiplexing Assay
Reviewer 1, Comment 3: Figure 6: Please include a space between numbers and units. 3.5 mm instead of 3.5mm	**Response:** We appreciate the reviewer's comment. **Actions:** Figure 6 was modified. We inserted a space between numbers and units as follows: "3.5 mm".
Reviewer 1, Comment 4: Materials Table: Please sort alphabetically by material name.	**Response:** We appreciate the reviewer's comment. **Actions:** Materials Table were alphabetically sorted by material name; as well as in Materials Table inserted at the end of the text.
Reviewer 1, Comment 5: Please shorten the title to be more concise.	**Response:** We appreciate the reviewer's comment. **Actions:** The title was shorten, as recommended by reviewer. *Old title:* "Coronary Circulating Mononuclear Progenitor Cells and Soluble Biomarkers to Estimate Cardiovascular Prognosis after Coronary Angioplasty". *New Title:* "Coronary Progenitor Cells and Soluble Biomarkers in Cardiovascular Prognosis after Coronary Angioplasty".
Reviewer 1, Comment 6: JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Lymphoprep, MAGPIX, Luminex, etc.	**Response:** We appreciate the reviewer's comment. **Actions:** All trademarks symbols, company names and any term containing a commercial implication was removed.

Reviewer 1, Comment 7: Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.	**Response:** We appreciate the reviewer's comment. **Actions:** The following statement: *"This protocol meets institutional guidelines from human research Ethics Committee"* has been included just below the subtitle "PROTOCOL:"
Reviewer 1, Comment 8: Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.	**Response:** We appreciate the reviewer's comment. **Actions:** Essential steps of the protocol were highlighted.
Reviewer 1, Comment 9: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."	**Response:** We appreciate the reviewer's comment. **Actions:** Statements of copyright permission for reuse figure(s) from a previous publications are provided.
Reviewer 1, Comment 10: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations. While some of these topics are discussed, please add some more depth. 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	**Response:** We appreciate the reviewer's comment. **Actions:** Discussion was modified as shown below, and two references were added: • Schmidt-Lucke, C., Fichtlscherer, S., Aicher, A., Tschöpe, C., Schultheiss, H.P., Zeiher, A.M., et al. Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. *PLoS One.* **5** (1), e13790 (2010). • Morales-Portano JD, Peraza-Zaldivar JÁ, Suárez-Cuenca JA, Aceves-Millán R, Amezcua-Gómez L, Ixcamparij-Rosales CH, et al. Echocardiographic measurements of epicardial adipose tissue and comparative ability to predict adverse cardiovascular outcomes in patients with coronary artery disease. *International Journal of Cardiovascular Imaging.* **34** (9), 1429-1437 (2018). "Blood collection from the affected coronary artery may show technical difficulties. Sometimes, coronary artery is little accessible and sampling from venous sinus may be an alternative. We performed validation tests comparing circulating biomarkers in coronary artery *vs* venous sinus, with no significant differences. However, clinical performance of circulating biomarkers was validated only for coronary sampling; therefore, performance of biomarkers obtained from venous sinus remains to be explored. After blood collection, sample processing for MPCs determination within the first 3-hours is recommended; therefore a good communication should be established between cardiology team and lab researchers. During MPCs isolation, careful should be paid in deposing blood samples through the walls during density gradient preparation, as well as delicate washes of MPCs pellet. Finally, for time-administration convenience, we always transfer cells into cytometry tube, add primary antibodies, fix and store cells overnight at 4°C; then perform Flow Cytometry Reading the day after. Regarding biomarker role of circulating MPCs, important efforts have been done to standardize most clinically useful immunophenotypes between progenitor cells[12], but one limitation may be the lack of specific subpopulations of circulating progenitor cells fully characterized for all clinical scenarios within CAD or other vascular diseases. Therefore, different circulating progenitor cells subpopulations should be explored in each study.

	During determination of soluble markers some general recommendations for ELISA and Multiplexing assays include the use multichannel pipette, deposit solution at the bottom of each well without touching the side wall, and avoid the wells get completely dry during the assay. Always register sample distribution in the plaque, and particularly for Multiplexing assay avoid precipitation of magnetic beads by constant vortexing and insert the Bottom Plate into the Hand-Held Magnetic Plate Washer to maintain magnetic beads inside the wells; otherwise samples will be lost during washes. We found that coronary circulating MPCs, mainly those from hematopoietic origin, as well as sICAM-1 and MMP-9, were outstanding biomarkers that impacted prediction and prognosis of MACEs. This is consistent with the notion that inflammatory response and/or vascular damage mediators stimulate homing signals for MPC mobilization and recruitment, promoting local tissue repair[4]. Accordingly, we found variations of these biomarkers when measured under several possible settings. Changes in relation to angioplasty and/or location of coronary sampling may be explained by the effect of the impact over the atheroma plaque during angioplasty, the size of the plaque, and the release into coronary flow of soluble mediators sequestered within the plaque[11]. Consistently, increased IL-1β has been involved in the development of the plaque and clinical complications[13]. To our knowledge, this is the first study prospectively evaluating the role of coronary circulating MPCs and soluble mediators of vascular injury and repair as prognostic biomarkers in population with CAD submitted to coronary angioplasty, including characterization of changes related to angioplasty, location of coronary sampling and comparison of coronary *vs* peripheral sampling. We think that the method is feasible to establish in any hospital carrying out coronary angiography. However, one limitation is that we applied this methodology mainly in patients with chronic stable angina, out from an emergency room department. Current traditional methods used for MACEs prediction or prognosis in CAD own moderate predictive ability. There has been an increasing interest to study novel biomarkers based on pathophysiology mechanisms responsible for repair and regeneration occurring after CAD and angioplasty. Such biomarkers have shown similar or better clinical performance as compared with traditional methods[3-5,14,15]; then, we think that the role of coronary circulating MPCs and soluble mediators in staging risk for MACEs will be addressed in future prospective studies".
Reviewer 1, Comment 11: Please do not abbreviate journal titles in the references.	**Response:** We appreciate the reviewer's comment. **Actions:** Journal titles were re-written without abbreviations.
Reviewer 2, Comment 1: Manuscript Summary: The manuscript describes a method for the evaluation of intracoronary circulation biomarkers for the early identification and prognosis of MACEs within 6 months from coronary angioplasty. In particular, the amount of subpopulations of Mononuclear Progenitor Cells as well as the concentration of soluble molecules reflecting inflammation, cell adhesion and repair, are evaluated after 10 ml coronary blood withdrawal. Major Concerns: - The main concern is that the authors' purpose is to explore the role of intracoronary circulating biomarkers rather than showing a method useful for patients' stratification. As JOVE is a methods journal, the suggestion is that authors show the efficacy of the protocol as a prerequisite for the method application, reorganizing the structure of the paper.	**Response:** We appreciate the reviewer's comment. **Actions:** The purpose of the study was re-written as shown below. Introduction: "…Therefore, the purpose of the present study was to describe a method to determine the amount of coronary circulating MPCs and soluble molecules, both reflecting vascular injury and repair, and to show whether these biomarkers associated with MACEs and clinical prognosis of patients with CAD submitted to coronary angioplasty…". Some results were eliminated, since these data didn´t support usefulness of the method to stratify cardiovascular risk: "…as well as variation in oxidative stress mediators, exclusively observed in peripheral circulation and not reflected in coronary circulation." Likewise, discussion was significantly modified to focus on methodological issues, more than the results obtained.

Reviewer 2, Comment 2: Another concern is that it is not clear which soluble molecules are evaluated with ELISA and MAGPIX.	**Response:** We appreciate the reviewer's comment. **Actions:** In the section of methods, subtitles were modified as follows: "Enzyme-linked Immunosorbent Assay (ELISA) – Determination of SICAM-1 and MMP-9.". "Immuno-Magnetic Multiplexing Assay (Figure 3, lower row) – Determination of TNFα".
Reviewer 2, Comment 3: Minor Concerns: - Description of gating strategy for different subpopulations of MNCs is too little exhaustive.	**Response:** We appreciate the reviewer's comment. **Actions:** Gating strategy for subpopulation of MNCs was better described, as follows: "We use the following stragegy for cell subpopulation analysis; first, select lymphocytes spread, as determined by their size (FSC) and granularity (SSC) in the FSC/SSC plot, trying to exclude residual granulocytes, cellular debris and other particles, which are usually located in the lower, left distributed in the plot. Such distribution is considered as 100%. Then, to select further cell subpopulations, use a gate for high number of cells with common immunophenotype $CD45^+$ and $CD34^+$; then, for double positive immunophenotypes use a gate that identify previous $CD45^+$, $CD34^+$ immunophenotype with the addition of either $KDR(VEGFR\text{-}2)^+$, $CD133^+$ or $CD184^+$. Corresponding isotype controls for each antibody were used for the initial setup…"
Reviewer 2, Comment 4: - Some references need to be added regarding soluble biomarkers and MACEs (see, for instance, Samman Tahhan A, Hammadah M, Raad M, et al. Progenitor Cells and Clinical Outcomes in Patients With Acute Coronary Syndromes. Circ Res (United States), May 25 2018, 122(11) p1565-1575.)	**Response:** We appreciate the reviewer's comment. **Actions:** Reference was added: Samman Tahhan, A., Hammadah, M., Raad, M., Almuwaqqat, Z., Alkhoder, A., Sandesara, P.B., et al. Progenitor Cells and Clinical Outcomes in Patients With Acute Coronary Syndromes. *Circulation Research.* **122** (11), 1565-1575 (2018). And text in "discussion" was added and modified: "Coronary circulating MPCs, mainly those from hematopoietic origin, as well as sICAM-1 and MMP-9 were outstanding biomarkers that impacted prediction and prognosis of MACEs. This is consistent with the notion that inflammatory response and/or vascular damage mediators stimulate homing signals for MPC mobilization and recruitment, promoting local tissue repair[4]. Accordingly, we found variations of these biomarkers when measured under several possible settings…"

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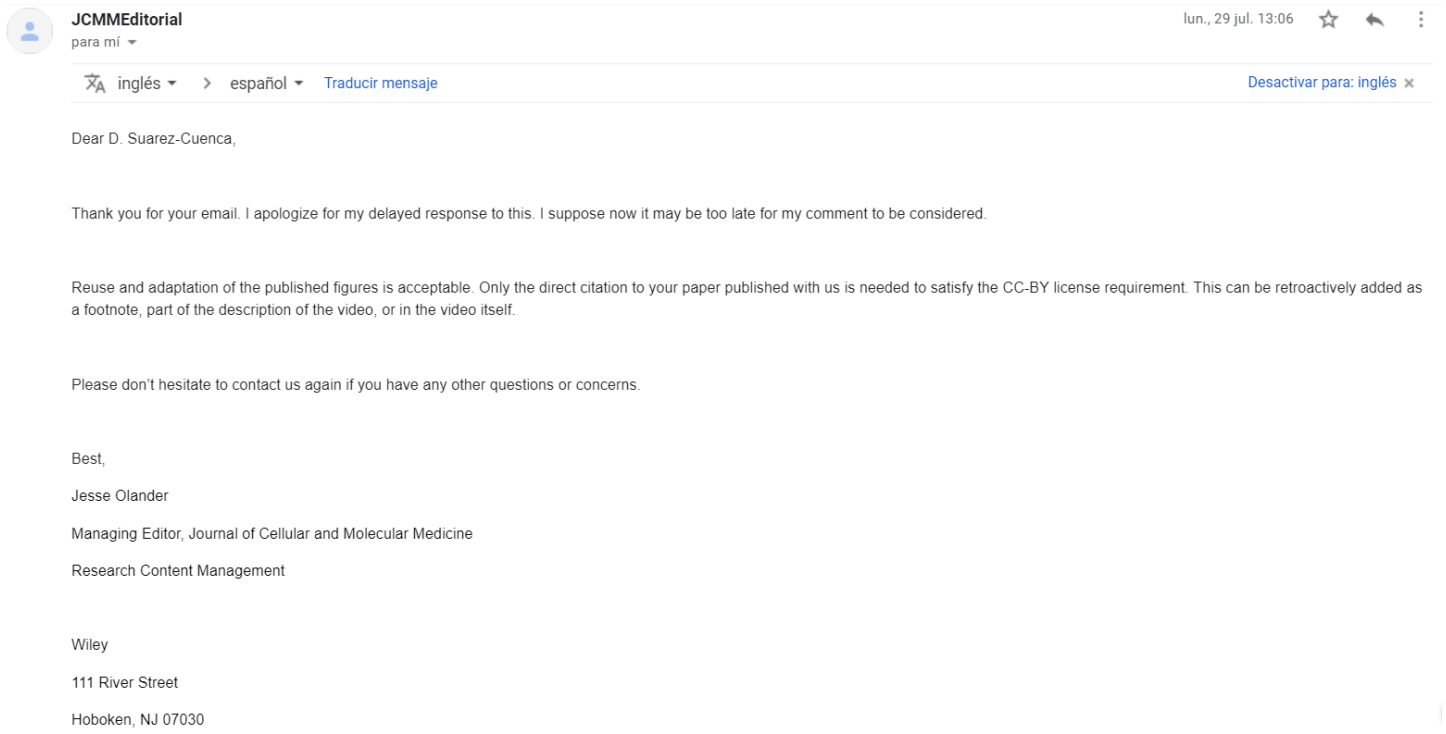

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Jesse Olander

Managing Editor, Journal of Cellular and Molecular Medicine

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Regarding figure adapted, to be used in other manuscript. Article entitled: “Relation of Coronary Artery Lumen with Baseline, Post-angioplasty Coronary Circulating Pro-Inflammatory Cytokines in Patients with Coronary Artery Disease”

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Dear Dr. Juan A. Suárez-Cuenca,

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Editorial Coordinator

Juan A. Suarez-Cuenca <suarej05@gmail.com> vie., 23 ago. 18:11 (hace 5 días)

para mí, angiology, angiology, Paul

Dear Dr. Brad A. Bryan
Editor-in-Chief Angiology Open Access
Center of Excellence in Cancer Research,
Paul Foster School of Medicine
Texas Tech University, USA

I hope this e-mail finds you well.
Regarding our article already published in Angiology, Open Access, entitled: "**Relation of Coronary Artery Lumen with Baseline, Post-angioplasty Coronary Circulating Pro-Inflammatory Cytokines in Patients with Coronary Artery Disease**"

I let you know that our study reached a lot of visibility thanks to the publication in your renowned journal, which is of high acknowledge. I was invited to publish on this subject in a Video Journal, mainly devoted to describe useful methods used in biomedical research. Although we focused our Video Journal manuscript in the "step-by-step" description of our methods, there is a section of the manuscript where some results obtained with the detailed methods are requested.
Therefore, I have prepared figures, which were adapted from the manuscript already published in your Journal. But before continue with the process of submittion to the video Journal, I want to make sure that there is no ethical or copyright issues that might be involved from this action; considering that citation acknowledge ("adapted from...Angiology Open Access") was stated in the figure prepared for the manuscript to submit to the video Journal.

I would appreciate your advice, and do not hesitate to let me know if any additional information or action is required.

Warm regards,

Dr. Juan A. Suárez-Cuenca

Figure Legends

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Title of Article: Coronary Circulating Mononuclear Progenitor Cells and Soluble Biomarkers to Estimate Cardiovascular Prognosis after Coronary Angioplasty

Author(s): Juan Antonio Suárez-Cuenca, Rogelio Robledo-Nolasco, Marco Antonio Alcántara-Meléndez, Luis Javier Díaz-Hernández, Eduardo Vera, et al.

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