To:

European Society of Cardiology

The European Heart House

2035 Route des Colles – Les Templiers

06903 Sophia Antipolis

France

Bucharest, 1st June 2016

ESC First Contact Initiative Grant Report

Dear Council Members,

I would like to thank the European Society of Cardiology for awarding me with the First Contact Initiative Grant in March 2016. This grant gave me the opportunity to visit the Cardiovascular Research Center, CSIC-ICCC, IIB-Sant Pau, Barcelona, Spain and to work in the research team of Prof. Dr. Lina Badimon. Her group has a strong experience in studying cardiovascular disease, provides a scientific environment of the highest standard and conducts innovative research in this field.

In the present final report I have the pleasure to inform you on the outcome of the initiative, which has been very successful. The main goal of this grant was to learn new techniques on flow cytometry for the identification of novel potential predictive biomarkers of cardiac ischemia severity. Interestingly, I also had the possibility to enrich my own scientific knowledge and establish new contacts for future collaborations.

Acute coronary syndromes (ACS) are the main cause of mortality and morbidity worldwide myocardial infarction being one of the main causes of death. ACS are associated to a series of pathological events such as release of inflammatory cytokines, oxidative reactions, hypoxia and cardiomyocyte necrosis. The exact mechanism of atherosclerotic plaque disruption and thrombus formation are still unknown. Current state-of-the-art research is mainly focused on searching more specific and sensitive new markers with high predictive value such as circulating microparticles.

Circulating microparticles (cMPs) are small phospholipid microvesicles (ranging from 0.1 to 1 μm in diameter). They are released to the blood from activated or apoptotic cells of the vascular compartment and contain phosphatidylserine and distinct surface proteins depending on their parental cells, which enable their characterization. However, their specific functions are not fully known. Recent studies showed that they are involved in cell-to-cell communication with special importance in atherothrombosis. Indeed, high levels of

procoagulant MP subpopulations have been found in the circulation of patients with ACS [1]. Platelet and endothelial cell-derived MPs correlate with the size of myocardium damage in ST-elevation myocardial infarction (STEMI) patients [2], indicating their involvement in disease severity. Elevation of procoagulant MPs has also been detected in the occluded coronary artery of patients with STEMI [3,4] showing that they might contribute to the formation of intracoronary thrombi and on microembolization [5].

By applying flow cytometry studies, I have learned to determine levels of different subpopulations of microparticles in blood-derived samples. Specifically, cMPs from plasma have been well-characterized in terms of cell origin and activation status of the parental cell. For this aim, blood samples were collected in EDTA-anticoagulated tubes. Within 2 h of collection, blood cells were removed by a double low-speed centrifugation step (20 minutes at 1260 xg at 20°C) to obtain the platelet-free plasma (PFP), which was snap-frozen in liquid nitrogen and stored at −80 °C until flow cytometric studies were performed, as previously described[6,7].

After thawing plasma samples in melting ice, the cMP-fraction was washed and isolated from PFP by a two- step high-speed centrifugation (30 minutes at 20.000 xg at 20°C). Briefly, after first centrifugation, the supernatant was carefully removed and the cMP pellet was washed by adding phosphate buffered saline (PBS)- citrate buffer (citrate-PBS; 1.4mM phosphate, 154mMNaCl, 10.9mM trisodium citrate, pH 7.4) and centrifuging again. Finally, the supernatant was removed and MPs were resuspended with PBS-citrate buffer.

After isolation, cMPs were triple-label stained by flow cytometric analysis [6,7]. Washed cMPs were incubated 20 min at RT in the dark with combinations of annexin V with two specific cell-surface monoclonal antibodies (mAbs) labeled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), or with the isotype-matched control antibodies. After incubation, samples were analyzed on a FACSCantoII™ flow cytometer (BD). Acquisition was performed at 1 min per sample at low flow rate mode. Flow rate was measured before each experiment (mean of 17 ± 0.5μL/min). Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with the settings in the logarithmic scale. cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific mAb. Gate limits were established as described [6,7]. The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer and a threshold was set at SSC parameter. Data were analyzed with FACSDiva™ software (version 6.1.3). The concentration was based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events. During this experimental procedure is of highly importance to filter all the solutions before used and to centrifuge the antibodies prior to labelling, in order to avoid any potential background noise signal on the flow cytometer.

Different microparticle subpopulations were analyzed by using distinct combinations of cell-surface markers: monocyte-derived AV+-mMPs (CD14+ and CD14+/CD11b+), lymphocyte-derived AV+-ℓMPs (CD3+/CD45+), endothelial-derived AV+-eMPs (CD146+ and

CD31+/CD41a-+), activated EC-derived AV+-eMPs (CD62E+, CD62E+/CD146+), , platelet-derived AV+-pMPs (CD41a+ and CD41a+/CD31+), activated platelet-derived AV+-pMPs (PAC1+ and PAC1+/CD62P+); erythrocyte-derived AV+-ErMPs (CD235a+), progenitor neuronal cell-derived AV+MPs (CD56+/CD34+) and those MPs of distinct origin rich in tissue factor (CD142+/CD62E+, (CD142+/CD41a+, and (CD142+/CD14+).

MPs can be considered as signaling components favouring cell interactions and cell-to-cell communication [8]. Current data indicates that combining distinct cMP subsets is significantly superior in predicting cardiovascular events than one type of cMPs alone and that the combination adds value to their prognostic value [1]. New combinations between vascular resident, blood and neuronal cell markers in this ongoing studies will provide further insights on the atherothrombotic pathophisiology and novel prognostic strategies for CVD.

Because circulating microparticles tend to become a new surogate marker in cardiac pathology and furthermore a novel therapeutic target, learning new techniques for isolation and identification MPs using flow cytometry approaches is of great relevance for future reserach in our laboratory. We want to move forward with the study of cardiovascular disease and atherosclerotic pathology in order to understand the mechanisms involved in its progresion and to advance on biomarker discovery. I look forward to establish future collaborations between both research centers.

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Bibiography

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