Universidade de Aveiro Departamento de Biologia
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Avaliação de células progenitoras endoteliais circulantes por citometria de fluxo multicor em pacientes com doença renal crónica

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Janete Maria Quelhas dos Santos, Estagiária de Pós-Doutoramento do Instituto de Inovação e Investigação em Saúde (I3S)/Instituto Nacional de Engenharia Biomédica – Unidade de Investigação e Desenvolvimento de Nefrologia, e sob a co-orientação da Doutora Helena Cristina Correia de Oliveira, Estagiária de Pós-Doutoramento do Centro de Estudos do Ambiente e do Mar, Departamento de Biologia da Universidade de Aveiro.

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“Não é na ciência que está a felicidade, mas na aquisição da ciência”

Edgar Allan Poe

o júri

presidente

Prof. Doutora Maria de Lourdes Gomes Pereira
Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Doença Renal Crónica, disfunção endotelial, Células Endoteliais Circulantes, Células Progenitoras Endoteliais, capacidade regenerativa, técnica de citometria de fluxo multicor.

resumo

A disfunção endotelial e as alterações nos processos de regeneração endotelial podem desempenhar um papel determinante na patogénese da doença cardiovascular, que é uma das principais causas de mortalidade na doença renal crónica (DRC). As células endoteliais circulantes (CEC) podem ser um indicador de dano vascular, enquanto que as células progenitoras endoteliais circulantes (CPEC) pode ser um biomarcador de reparação vascular. No entanto, a avaliação simultânea dos níveis de CECs e de CPECs e sua relação não foram previamente avaliados numa população de doentes renais crónicos.

Amostras de sangue (18 mL) foram recolhidas a partir de indivíduos saudáveis (n = 10), e a partir de doentes renais crónicos em estadios precoces (n=10) e em estádios avançados (n=10), para se proceder ao isolamento de populações de CPECs imaturas e maduras, CECs e células hematopoiéticas. Estas populações de células foram identificadas por citometria de fluxo (sistema BD FACS Canto II) usando uma combinação de anticorpos primários conjugados com fluorocromos: CD31-PE, CD45-APC Cy7, CD34-FITC, CD117-PerCp Cy5.5, CD133-APC, CD309-PE Cy7 e CD146-Paciific blue. Para a exclusão das células mortas recorreu-se a um marcador de viabilidade (“fixable viability dye”). Este protocolo otimizado de citometria de fluxo de oito cores permitiu identificar simultaneamente e com precisão as subpopulações de CECs, CPECs e células hematopoiéticas. Além disso, também foi possível distinguir as duas subpopulações de CPECs, imaturas e maduras, por marcação múltipla CD45intCD31+ CD34+ CD117-CD133+ CD309-CD146- e CD45intCD31+ CD34+CD117- CD133-CD309+ CD146-, respetivamente. Adicionalmente, a identificação de CECs e células hematopoiéticas foi realizada por CD45-CD31+ CD34-/lowCD117- CD133-CD309- CD146+ e CD34+ CD117+, respetivamente.

Os níveis de CECs foram mais elevados em pacientes em estadios precoces de DRC (312,1±91,3) e em estadios avançados (191,4±49,9) comparativamente com o grupo controlo (103,23±24,13), n.s. Para além disso, os níveis de CPECs imaturas foram significativamente diminuídos em estadios avançados de DRC (17,1±3,2) em comparação com estadios precoces (32,3±4,9), p=0,04, e com o grupo controlo (36,3±6,2), p=0,03. Os níveis de CPECs maduras foram significativamente reduzidos em estadios avançados de DRC (6,6±1,9), p=0,01 e em estadios precoces (8,4±2,6), p=0,01, em comparação com o grupo controlo (91,5±29,1). Estes resultados foram acompanhados por uma diminuição acentuada nos índices de capacidade de recrutamento, diferenciação e regeneração na população de doentes renais crónicos. Globalmente, estes resultados sugerem um desequilíbrio no processo de reparação endotelial na DRC, e sugerem ainda, que os índices de recrutamento, diferenciação e regeneração podem ajudar na seleção de pacientes que possam beneficiar de estratégias de intervenção para melhorar a saúde cardiovascular induzindo proteção vascular.

keywords

Chronic Kidney Disease, endothelial dysfunction, Circulating Endothelial Cells, Endothelial Progenitor Cells, regenerative capacity, multicolor flow cytometry technique.

abstract

Endothelial dysfunction and impaired endothelial regenerative capacity play a key role in the pathogenesis of cardiovascular disease, which is one of the major causes of mortality in chronic kidney disease (CKD) patients. Circulating endothelial cells (CEC) may be an indicator of vascular damage, while circulating endothelial progenitor cells (EPC) may be a biomarker for vascular repair. However, the simultaneously evaluation of CEC and EPC circulating levels and its relation were not previously examined in CKD population.

A blood sample (18ml) of healthy subjects (n=10), early CKD (n=10) and advanced CKD patients (n=10) was used for the isolation of early and late EPCs, CECs, and hematopoietic cells, identified by flow cytometry (BD FACSCanto™ II system) using a combination of fluorochrome-conjugated primary antibodies: CD31-PE, CD45-APC Cy7, CD34-FITC, CD117-PerCp Cy5.5, CD133-APC, CD146-Pacific Blue, and CD309-PECy7. Exclusion of dead cells was done according to a fixable viability dye staining. This eight-color staining flow cytometry optimized protocol allowed us to accurate simultaneously identify EPCs, CECs and hematopoietic cells. In addition, it was also possible to distinguish the two subpopulations of EPCs, early and late EPCs subpopulation, by CD45intCD31+CD34+CD117-CD133+CD309-CD146- and CD45intCD31+CD34+CD117-CD133-CD309+CD146- multiple labeling, respectively. Moreover, the identification of CECs and hematopoietic cells was performed by CD45-CD31+CD34-/lowCD117-CD133-CD309-CD146+ and CD34+CD117+, respectively.

The levels of CECs were non-significantly increased in early CKD (312.06 ± 91.34) and advanced CKD patients (191.43±49.86) in comparison with control group (103.23±24.13). By contrast, the levels of circulating early EPCs were significantly reduced in advanced CKD population (17.03±3.23) in comparison with early CKD (32.31±4.97), p=0.04 and control group (36.25 ± 6.16), p=0.03. In addition the levels of late EPCs were significantly reduced in both advanced (6.60±1.89), p=0.01, and early CKD groups (8.42±2.58), p=0.01 compared with control group (91.54±29.06). These results were accompanied by a dramatically reduction in the recruitment, differentiation and regenerative capacity indexes in CKD population.

Taken together, these results suggest an imbalance in the process of endothelial repairment in CKD population, and further propose that the indexes of recruitment, differentiation and regenerative capacity of EPCs, may help to select the patients to benefit from guiding intervention strategies to improve cardiovascular health by inducing vascular protection.

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
BH4	Tetrahydrobiopterin
CAC	Circulating Angiogenic Cells
CAD	Coronary Artery Disease
CEC	Circulating Endothelial Cells
CEPC	Circulating Endothelial Progenitor Cells
CFU	Colony Forming Unit
CFU-Hill	Colony Forming Unit – Hill cells
CVD	Cardiovascular Disease
CKD	Chronic Kidney Desease
ECFC	Endothelial Colony Forming Cells
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cells
EPO	Erytropoietin
ESRD	End-stage Renal Disease
ET-1	Endothelin-1
FMO	Fluorescence Minus One
FSC	Forward Scatter
G-CSF	Granulocyte-colony Stimulating Factor
GFR	Glomerular Filtration Rate
GM-CSF	Granulocyte Monocyte-colony Stimulating Factor
HIF-1	Hypoxia Inducible Factor
ICAM-1	Intercellular Adhesion Molecule 1
KDR	Kinase insert Domain Receptor

Kit L	Soluble Kit Ligand
MDRD	Modification of diet in renal disease
mKit L	Membrane-bound Kit Ligand
MMP-9	Matrix Metalloproteinase-9
NO	Nitric Oxide
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PGI2	Prostacyclin
ROS	Reactive Oxygen Species
SDF-1	Stromal-cell Derived Factor
SSC	Side Scatter
TXA2	Tromboxane A2
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Type 2 receptor of Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1 Chronic Kidney Disease

Chronic Kidney Disease (CKD) is defined as the presence of kidney damage that persists for more than 3 months, manifested by abnormal albumin excretion or decreased kidney function, quantified by measured or estimated glomerular filtration rate (GFR) (Thomas *et al.*, 2008). It is common, frequently unrecognized and often exists together with other conditions (such as cardiovascular disease and diabetes). Moderate to severe CKD is also associated with an increased risk of other significant adverse outcomes such as acute kidney injury, falls, frailty and mortality. This disease is usually asymptomatic, but it is detectable, and tests for CKD are simple and freely available. There is evidence that treatment can prevent or delay the progression of CKD, by early detection and treatment, reduce or prevent the development of complications, and reduce the risk of cardiovascular disease (Levey *et al.*, 2003). To facilitate assessment of CKD severity, the National Kidney Foundation developed criteria as part of its Kidney Disease Outcomes Quality Initiative (NKF K/DOQI) to stratify CKD patients (Table 1).

Table 1 – Classification of CKD in 5 stages based on the combination of GFR and markers of kidney damage, by National Kidney Foundation (Thomas *et al.*, 2008).

	GFR (mL/min/1,73m²)	**Terms**
Stage 1	≥90	Normal
Stage 2	60 - 89	Mildly decreased
Stage 3a	45-59	Moderately to Severely decreased
Stage 3b	30-44	
Stage 4	15-29	Severely decreased
Stage 5	<15	Kidney Failure

1.2 Cardiovascular risk in CKD

Cardiovascular disease (CVD) is one of the major causes of morbidity and mortality in CKD population, 10 to 20 times higher than in general population. Association between CKD and cardiovascular complications is linked to a number of factors including traditional risk factors, such as age, gender, obesity, hypertension, hyperlipidemia, and nontraditional risk factors typical of CKD like uremic toxins, proteinuria, inflammation, alterations of mineral metabolism, and increased oxidative stress (Zhang *et al.*, 2014). Cardiovascular morbidity and mortality are inversely and independently associated with kidney function, particularly at estimated GFR<15 ml/min per 1.73 m^2 (Herzog *et al.*, 2011).Cardiovascular involvement in CKD can be evaluated by both serological and instrumental tests. (Di Lullo *et al.*, 2015).

1.2.1 Endothelial dysfunction in association with CKD

Endothelial dysfunction is a condition in which the endothelium (inner lining) of blood vessels does not function normally and is observed in CKD patients, sometimes even as early as in stage 1 (Zhang *et al.*, 2014; Zhang *et al.*, 2014).Increasing documents indicate that prolonged exposure to risk factors, such as inflammation and oxidative stress chronically present in CKD patients, may alter the normal homeostatic properties of the endothelium and active endothelial cells (De Groot, K. *et al*, 2004). It participates in the development of atherosclerosis and could partially explain the high incidence of vascular complications in this population (Jourde-Chiche *et al.*, 2009). This manifestation of endothelial dysfunction is not only associated with CVD but may also precede its development (Endemann & Schiffrin, 2004). Endothelial dysfunction and impaired endothelial regenerative capacity play a key role in the pathogenesis of CVD (Hadi *et al.*, 2005).

1.3 Endothelium

The endothelium is the monolayer of endothelial cells (Fig. 1) mechanically and metabolically strategically located, lining the lumen of the vascular beds and separating the vascular wall from the circulation and the blood components (Lerman & Zeiher, 2005). The healthy endothelium is a major player in the control of blood fluidity, platelet aggregation and vascular tone, a major actor in the regulation of immunology, inflammation and angiogenesis, and an important metabolizing and an

endocrine organ. This organ weighs approximately 1 kg and consists of 1 to-6x 10^{13} cells. Endothelial cells controls vascular tone, and thereby blood flow, by synthesizing and releasing relaxing and contracting factors such as nitric oxide (NO), metabolites of arachidonic acid via the cyclooxygenases, lipoxygenases and cytochrome P450 pathways, various peptides (endothelin, urotensin, nautriuretic peptide type C, adrenomedullin, etc.), angiotensins, prostaglandins, reactive oxygen species (ROS), among others. Normally these factors act in a coordinated manner so that the vasodilator and vasoconstrictor influences are locally balanced and regulate the resistance of the vascular tone to maintain steady tissue perfusion (Kharbanda & Deanfield, 2001). Additionally, these mediators have effects on other endothelial functions such as regulation of cell-cell adhesion, thrombosis and fibrinolysis. Some of these relaxing and contracting factors will be presented in more detail below.

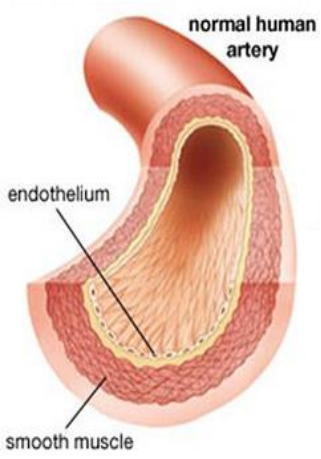

Figure 1 – Representation of the healthy endothelium in a human artery.
(Adapted from http://www.examiner.com/article/prevent-a-heart-attack-tomorrow-mind-your-endothelium-today)

1.3.1 Nitric Oxide

Nitric Oxide is an endothelium-derived relaxing factor, generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin (BH4), and released in response to stimuli that act on the endothelial cell surface (Davignon & Ganz, 2004). Once activated this gas diffuses to the vascular smooth muscle cells and activates guanylate cyclase, which reduces intracellular calcium within the smooth muscle cell, causing smooth muscle relaxation or vasodilatation (Kharbanda & Deanfield, 2001). Shear stress is a key activator of eNOS in normal physiology, besides other signaling molecules that can promote eNOS activation, such as bradykinin, adenosine, vascular endothelial growth factor (in response to hypoxia), and serotonin (released during platelet aggregation) (Endemann & Schiffrin, 2004).

1.3.2 Prostaglandin

Prostaglandins are lipid autacoids derived from arachidonic acid. The main prostaglandin molecules produced by endothelial cells are prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) (Ricciotti & FitzGerald, 2011). PGI_2 is a vasodilator that binds to specific receptors on the target cell and activates adenylate cyclase to increase cyclic AMP levels, which causes relaxation of smooth muscle. On the other hand TXA_2 has vasoconstrictor properties and aggregates platelets. Under normal physiological conditions the effects of prostacyclin predominate but when this physiology is disturbed by disease then vasoconstrictor prostanoids become more important (Kharbanda & Deanfield, 2001).

1.3.3 Endothelin

Endothelins are a group of three peptide hormones that have paracrine activity and are potent vasoconstrictors. Human endothelial cells secrete endothelin-1 (ET-1), which induce several biological effects, such as profound vasoconstriction, pro-inflammatory actions, mitogenic and proliferative effects, stimulation of free radical formation and platelet activation (Davignon & Ganz, 2004). In addition, ET-1 has been implicated as an important factor in the development of vascular dysfunction and cardiovascular disease. Under physiological conditions, ET-1 is produced in small amounts mainly in endothelial cells, primarily acting as an autocrine/paracrine mediator, whereas under pathophysiological conditions, the production is stimulated in a large number of different cell types, including endothelial cells, vascular smooth muscle cells, cardiac myocytes, and inflammatory cells such as macrophages and leukocytes (Bohm & Pernow, 2007).

1.3.4 Angiotensin

The endothelium modulates vasomotion, not only by releasing vasodilator substances, but also by an increase in constrictor tone via generation of endothelin and vasoconstrictor prostanoids, as well as via conversion of angiotensin I to angiotensin II at the vessel wall in endothelial surface (Deanfield *et al.*, 2007). In turn, Angiotensin II is a peptide that is generated by tissue angiotensin converting enzyme (ACE) and has vasoconstrictor, prothrombotic, oxidant and atherogenic properties (Endemann & Schiffrin, 2004).

1.3.5 Endothelial dysfunction and Circulating Endothelial Cells (CECs)

Endothelial dysfunction is also referred as endothelial activation, by some authors, which represents a switch from a quiescent phenotype toward one that involves the host defense response (Deanfield *et al.*, 2007). In general, cardiovascular risk factors promote endothelial dysfunction, which is characterized by reduction of bioavailability and impairment of vasodilator effect of endothelium-derived relaxing factors, such as NO, prostacyclin or endothelium-derived hyperpolarizing factor (Kharbanda & Deanfield, 2001). In addition, can occur an increased production and biological activity of the potent vasoconstrictor and pro-inflammatory peptide endothelin (ET-1). The decrease of NO is an important factor in this process that results from reduced activity of eNOS, (as a result of endogenous or exogenous inhibitors) and to decreased bioavailability of NO (Endemann & Schiffrin, 2004).This results in an increase of ROS formation (in the presence of superoxide dismutase), lead to generation of hydrogen peroxide, which, can diffuse rapidly throughout the cell and react with cysteine groups in proteins to alter their function. Moreover, hydrogen peroxide leads to degradation of the eNOS cofactor BH4, leading to “uncoupling” of eNOS, and results in superoxide formation. When ROS are generated at low concentrations can function as signaling molecules participating in the regulation of fundamental cell activities such as cell growth and cell adaptation responses, whereas at higher concentrations, results in very different consequences, such as phosphorylation of transcription factors, induction of nuclear chromatin remodeling and transcription genes, and protease activation (Davignon & Ganz, 2004). In certain circumstances, when exposure to cardiovascular risk factors is prolonged and repeated, chronic production of ROS may exceed the capacity of cellular enzymatic and nonenzymatic anti-oxidants, as a consequence, the endothelium not only becomes dysfunctional, but endothelial cells can also lose integrity, progress to senescence, and detach into the circulation (Deanfield *et al.*, 2007).

Therefore, the number of circulating endothelial cells (CECs) may reflect the state of endothelium dysfunction. Circulating endothelial cells have been recognized as a potential marker of endothelial damage in a variety of vascular disorders. A number of antigens have been used to identify cells of endothelial origin such as Muc-18 (CD146), Thrombomodulin (CD141), VE-cadherin (CD 144), vascular cell adhesion molecule 1 (CD106), Endoglin (CD105), E-selectin (CD62e), intercellular adhesion molecule 1 (CD54) and platelet endothelial cell adhesion molecule-1 (CD31) (Zhang *et al.*, 2014). According to some authors, CECs have high expression for CD34 marker (Kraan *et al.*, 2012). In addition mature endothelial cells may express endothelial-specific markers, including type 2 receptor

of vascular endothelial growth factor (VEGFR-2), also designated CD309, however they lose this marker when beginning the senescence process (Ramcharan *et al.*, 2013). In other hand, these cells have negative expression to leukocyte common antigen (CD45) and Prominin 1 (CD133) (Flores-Nascimento *et al.*, 2015).

However, endothelial integrity depends not only on the extent of injury, but also on the endogenous capacity for repair. Over time two mechanisms of repair have been identified. One through the adjacent mature endothelial cells that can replicate locally, and replace the lost and damaged cells, another through the repairment by circulating endothelial progenitor cells (CEPCs) (Fig. 2) recruited from the bone marrow. These cells, once in circulation can differentiate into mature cells with endothelial characteristics (Zampetaki *et al.*, 2008).

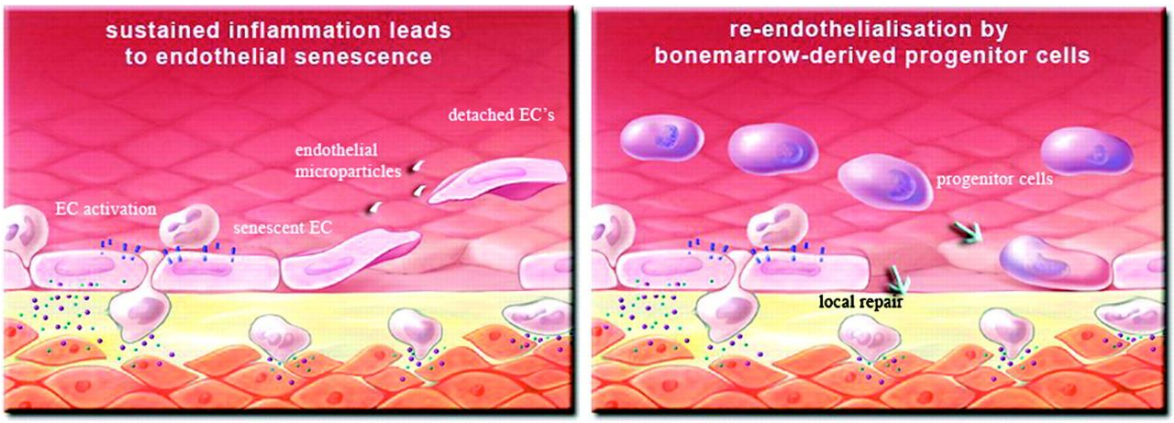

Figure 2 – Representation of endothelial dysfunction by prolonged ROS signaling, that induces senescence of endothelial cells, and the repairment process by adjacent mature endothelial cells and circulating endothelial progenitor cells. (Adapted from: Deanfield *et al.* 2007).

1.4 Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are small, immature precursor and bone marrow derived cells that can be found in the peripheral and umbilical cord blood (Burger & Touyz, 2012). They are extremely rare events in normal peripheral blood, representing somewhere between 0.01% and 0.0001% of peripheral mononuclear cells (Khan *et al.*, 2005). These cells were first isolated from adult peripheral blood (PB) in 1997 by Asahara and their collaborators using magnetic micro beads, on the basis of proteins in cell surface (also known as surface markers); in this case, they based on expression of hematopoietic progenitor cell antigen marker (CD34) (Asahara, 1997). With this discovery, the dogma that differentiation of mesodermal cells to angioblasts and subsequent

endothelial differentiation exclusively occur in embryonic development was overturned, since EPCs from adults can differentiate *ex vivo* to an endothelial phenotype (Urbich & Dimmeler, 2004). In addition to the aforementioned markers, EPCs show expression of various endothelial markers in their surface, such VE-cadherin (CD144), platelet endothelial cell adhesion molecule-1 (CD31), endothelial NO synthase, E-selectin (CD62E) and von Willebrand factor (CD41), show expression of stem cell markers such Prominin 1 (CD133), and also express surface markers of Hematopoietic System such c-kit (CD117) and Leukocyte Common Antigen (CD45) (Burger & Touyz, 2012) (Yoder, 2012). However, no marker was identified as specific for EPCs.

Moreover, the mentioned surface markers are dependent on the state and localization of the EPCs, because, the surface markers presented in early EPCs are different from the surface markers expressed by mature EPCs. The surface markers present in early EPCs are principally CD133, CD34 and VEGFR-2, termed also kinase insert domain receptor (KDR) or CD309. In the peripheral circulation of adults, more mature EPCs are found that obviously have lost CD133 but are positive for CD34 and VEGFR-2 (Hristov *et al.*, 2012). It seems, therefore, that the loss of CD133 reflects the transformation of circulating EPCs into more mature endothelial-like cells. However, it is not clear at which time point the EPCs begin to lose CD133, either during their transmigration from the bone marrow into the systemic circulation or later during their mobilization. This indicates that are found two types of EPCs in the peripheral blood and that the cells change their progenitor properties in the circulation (Urbich & Dimmeler, 2004).

1.4.1 Functions of Endothelial Progenitor Cells

Endothelial progenitor cells are involved in physiological neovascularization (new blood vessel formation), wound healing, tissue regeneration in ischemia, tissue remodeling and growth of tumors (George *et al.*, 2011). To aid in neovasculogenesis, EPCs are mobilized from the bone marrow in response to endogenous or exogenous signals and home to peripheral tissue sites to participate in endothelial repair. Thus a reduction in EPCs may contribute to the development of endothelial dysfunction. (Burger & Touyz, 2012). Endothelial progenitor cells are postulated to arise from an earlier progenitor, termed hemangioblast, which also generates hematopoietic stem cells (Urbich & Dimmeler, 2004). Although EPCs have the same precursor of stem cells, there are differences between these two cell populations.

The stem cells are a class of undifferentiated cells that are the remarkable potential to dividing and renewing themselves for long periods, and can give rise to specialized cell types. These cells are classified as embryonic stem cells (pluripotents) if they are formed during embryological development, or as adult stem cells (multipotents), if they are formed in adult tissue. The type of cells that are found in bone marrow is the adult stem cells, and can be hematopoietic stem cells (which can produce blood cells) and stromal (which can produce fat, cartilage and bone) (Morrison & Scadden, 2014). During injuries or neoplastic proliferations, adult stem cells are recruited from the bone marrow and migrate to target places to complete self-renewal and differentiation to achieve tissue reconstruction (Zhang *et al.*, 2014). Whereas, EPCs derived from multipotent stem cells, are also from bone marrow and are able to promote the survival and proliferation of endothelial cells, contribute to vessel formation and/or stabilization of new blood vessels.

1.4.1.1 Vasculogenesis and Angiogenesis

Neovascularization is an essential mechanism determining the formation, but also the maintenance, of the cardiovascular system. It is thought to depend mainly on two processes, angiogenesis and vasculogenesis (Fig.3) (Eibel *et al.*, 2011) (Russell, 2013). Angiogenesis is the process by which new vessels are formed from pre-existing vessels by the activation, proliferation and migration of endothelial cells (ECs). Vasculogenesis is defined as the process by which new vessels are generated when there are no pre-existing vessels, by the migration and differentiation of vascular endothelial growth factor receptor 2 positive (VEGFR-2^{+}) mesodermal precursors, into ECs that adhere to form a primary vascular plexus during embryonic development (Caiado & Dias, 2012). Accumulating evidence suggests that EPCs have an important role in homeostasis of the vascular network, when vasculogenesis occurs (Yoder, 2012). However, EPCs might not only be involved in the formation of new vessels in ischemic tissues, but might also contribute to the repair of pre-existing vessels. Thus, EPCs might be interesting candidates for novel therapeutic approaches, such the repair of injured vessel wall, the neovascularization or regeneration of ischemic tissue, and the coating of vascular grafts (Distler *et al.*, 2009). However, the therapeutic applications of post natal EPCs have a critical limitation that is their low number in circulation.

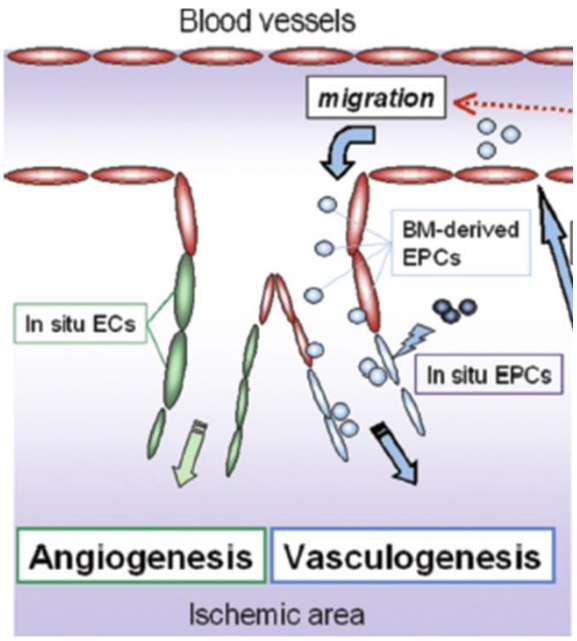

Figure 3 – Representation of angiogenesis and vasculogenesis processes.
(Adapted from: Eibel *et al*. 2011).

1.4.2 Mechanisms by which EPCs improve Neovascularization

In the past, the regeneration of injured endothelium has been attributed to the migration and proliferation of neighboring endothelial cells. More recent studies, indicate that additional repair mechanisms may exist to replace denuded or injured arteries and EPCs are involved in this process (Urbich & Dimmeler, 2004). The process from which EPCs mobilize from the bone marrow and circulate in peripheral circulation to ischemic tissues and tumors, comprises several steps that are recruitment, mobilization, differentiation, homing and regenerative potential of EPCs (Hristov *et al.*, 2012).

1.4.2.1 Recruitment

Recruitment and incorporation of EPCs requires a coordinated sequence of multistep adhesive and paracrine signals termed chemoattraction, which have utmost importance to allow for recruitment of reasonable numbers of progenitor cells to the ischemic or injured tissues (Goon *et al.*, 2006). Those paracrine signals (growth factors or cytokines) generated by ischemic tissue and tumor

cells include vascular endothelial growth factor (VEGF) and stromal-cell derived factor (SDF-1) production. However, additional factors inducing mobilization of progenitor cells from the bone marrow, as granulocyte-colony stimulating factor (G-CSF), the granulocyte monocyte-colony stimulating factor (GM-CSF), erythropoietin (EPO) and chemokines such SDF-1 also increased the levels of EPCs (Aicher *et al.*, 2006).

1.4.2.2 Mobilization and Differentiation

Physiologically, ischemia, caused by hypoxia, is believed to be the predominant signal to induce mobilization of EPCs from the bone marrow, because hypoxia, in tumors and ischemic tissues, mediate activation of hypoxia inducible factor gene (HIF-1) (Ceradini & Gurtner, 2005).

This gene is a heterodimeric transcription factor consisting of a β-subunit and an oxygen-regulated-α-subunit. The HIF-1α and HIF-1β proteins both contain basic helix-loop-helix motifs that bind DNA and cause subunit dimerization. This gene, whose activation is prompted by hypoxia conditions, can interact with enzymes and other transcription factors in other to control vascularization and tissue growth. Therefore, HIF-1 activation promotes an increase synthesis of a potent angiogenic factor, termed VEGF, which is a major regulator of angiogenesis, which promotes endothelial cell migration toward a hypoxic area. This happens, since during hypoxia, HIF-1 binds the regulatory region of the VEGF gene, inducing its transcription and initiating its expression (Ziello *et al.*, 2007).

In turn, the expression of VEGF will promote activation of matrix metalloproteinase-9 (MMP-9) in bone marrow, which will cleave the membrane-bound kit ligand (mKitL) and will induce the release of soluble Kit ligand (KitL, also known as stem cell factor, SCF). Subsequently, cKit-positive stem and progenitor cells, including also a common hematopoietic and angioblast precursor cells (Hemangioblast, HABL), moves to the vascular zone of the bone marrow microenvironment. This translocation activates the cells from a quiescent to a proliferative state. The signals, which initiate the diversion of the hemangioblast to either hematopoietic precursor cells or EPCs, are largely unknown, but may include angiogenic growth factors from the periphery, as VEGF and SDF-1 (Hristov *et al.*, 2012) (Fig.4).

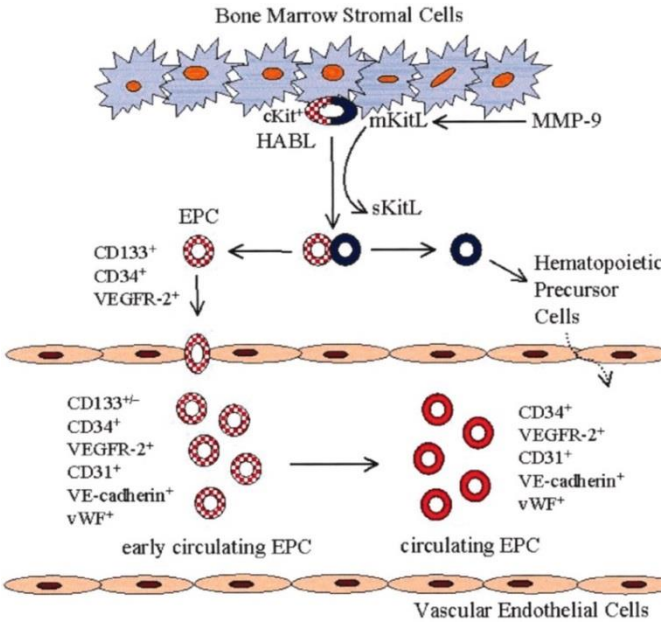

Figure 4 – Mechanisms by which EPCs are recruited and mobilized from bone marrow to peripheral circulation. (Adapted from: Hristov *et al*. 2012).

1.4.2.3 Homing and regenerative potential of EPCs

It is known that after their differentiation, EPCs leave the bone marrow and move through systemic circulation to the ischemic tissues and contact with injured endothelial cells, this process is known as homing. It is thought that EPCs mobilization from the bone marrow is mediated by integrins. This class of proteins is responsible for cellular tissue architecture and also functions as signal transducers regulating survival, proliferation, differentiation and migratory signaling pathways (Caiado & Dias, 2012). The main integrins that regulate the mobilization of EPCs from the bone marrow microenvironment are the α4 integrins, namely α4β1 and α4β7. The α4β1 integrin mediates cell adhesion to vascular cell adhesion molecule-1 (VCAM-1) the α4β7 integrin is important in lymphocyte homing and it also binds to VCAM-1 (Chavakis *et al.*, 2005). This data suggests that EPC mobilization is an active process involving direct interaction between molecular targets expressed on homing tissues and adhesion molecules, namely integrins, expressed by EPCs. The next step of homing of progenitor cells to ischemic tissue involves adhesion of these cells to endothelial cells activated by cytokines, and the transmigration of the progenitor cells through the endothelial cell monolayer. It is known that adhesion of various cells, including hematopoietic stem cells and leukocytes to endothelial

cells, is also mediated by integrins (Zampetaki *et al.*, 2008). The integrins that are capable to mediate cell-cell interactions are the β2-integrins and the α4β1-integrin. The latter are expressed by several cell types including endothelial cells and hematopoietic cells, whereas β2-integrins are found preferentially on hematopoietic cells. β2-integrins not only mediate the adhesive interactions of EPCs to mature endothelial cells and to extracellular matrix proteins but are also critical for chemokine-induced transendothelial migration of EPCs. During firm adhesion of leukocytes to the endothelium, members of the β2-integrin family, interact with endothelial counter ligands such as ICAM-1, VCAM-1, and surface-associated fibrinogen (Chavakis *et al.*, 2005) (Fig.5).

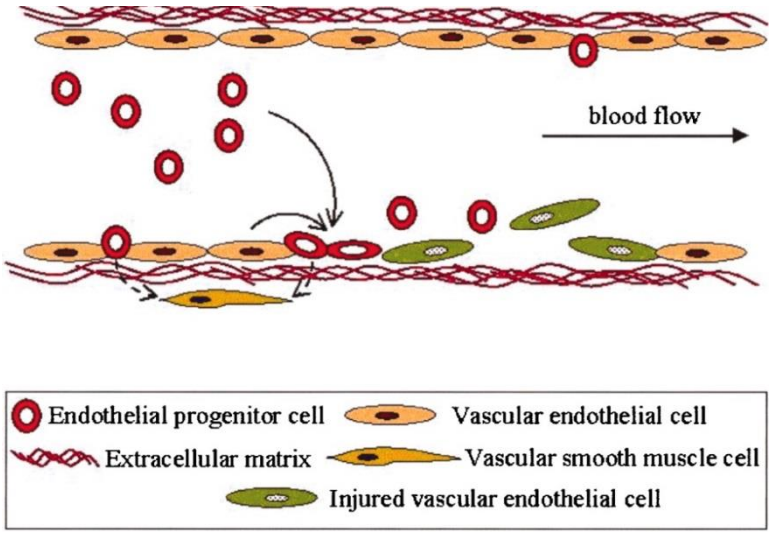

Figure 5 – Representation of EPCs homing and their adhesion on injured endothelium. (Adapted from: Hristov *et al.* 2012).

After adhesion and insertion into the monolayer of surrounding mature vascular ECs, this process may be completed, and the injured monolayer is repaired. Thus, EPCs derived from the hematopoietic tissue of postnatal bone marrow may possess highly regenerative potential and some characteristics of embryonic stem cells (Hristov *et al.*, 2012).

1.4.3 Methods for EPC's Assessment

In 1997, Asahara and their collaborators isolated $CD34^+$ mononuclear blood cells (EPCs) from human peripheral blood by means of magnetic beads coated with antibody to CD34 (Asahara 1997) .Since this discovery of EPCs, significant steps forward have been taken to reach a better definition

and a detailed functional characterization of these cells. However, the outcome and success of several studies have been limited by the lack of unambiguous and consistent definitions of EPCs (Fadini *et al.*, 2008). Actually, there are a variety of procedures that one can use to assist in the isolation and quantification of EPCs, but these can be simplified into two approaches: *in vitro* adhesion and growth and selection by cell surface phenotype using fluorescent labeled antibodies or flow cytometry (Hirschi *et al.*, 2008). Of note, all current methods for identifying or quantifying the endothelial lineage potential of circulating cells have limitations in that none has been shown to reliably predict the behavior of the circulating cells in a relevant *in vivo* context (Fadini *et al.*, 2008).

1.4.3.1 *In Vitro* Culture of EPC's

Most culture assays were used to obtain circulating EPCs from peripheral blood for identification of EPCs as biomarkers for cardiovascular disease, for analysis of intracellular signaling pathways, or for enriching cells for therapeutic angiogenesis (Fadini *et al.*, 2008). After isolation of Peripheral Blood Mononuclear Cells, the cells are cultured in medium with specific growth factors (eg, VEGF, bovine brain extract, and epidermal growth factor), which facilitates the growth of endothelial-like cells. The incubation *in vitro* with a mixture of growth factors, the adhesion of specific substrates (eg. Fibronectin), and the contact with the extracellular matrix or the surrounding mature ECs *in vivo* will probably influence the proliferation or differentiation of bone marrow-derived EPCs (Hristov *et al.*, 2003). The vast majority of studies used one of the following three culture media: Medium 199 (Gibco, Carlsbad, California, USA) has been used for the Culture of coloning forming unit (CFU) assay with fetal bovine serum only, endothelial growth medium (EGM; Clonetics, San Diego, California, USA) supplemented with bovine brain extract and human epidermal growth factor, and EGM-2 (Clonetics, San Diego, California, USA) that contains defined concentrations of VEGF-2, human fibroblast growth factor 2, human epidermal growth factor, insulin-like growth factor 1, ascorbic acid, heparin and hydrocortisone. In addition to different culture media, different extracellular matrix proteins have been used for the coating of cell culture dishes, such cell culture dishes coated with collagen, fibronectin or gelatin, which might also influence the outcome (Distler *et al.*, 2009). Considering this, through time three major methods have been used for EPCs *in vitro* culture (Fig.6).

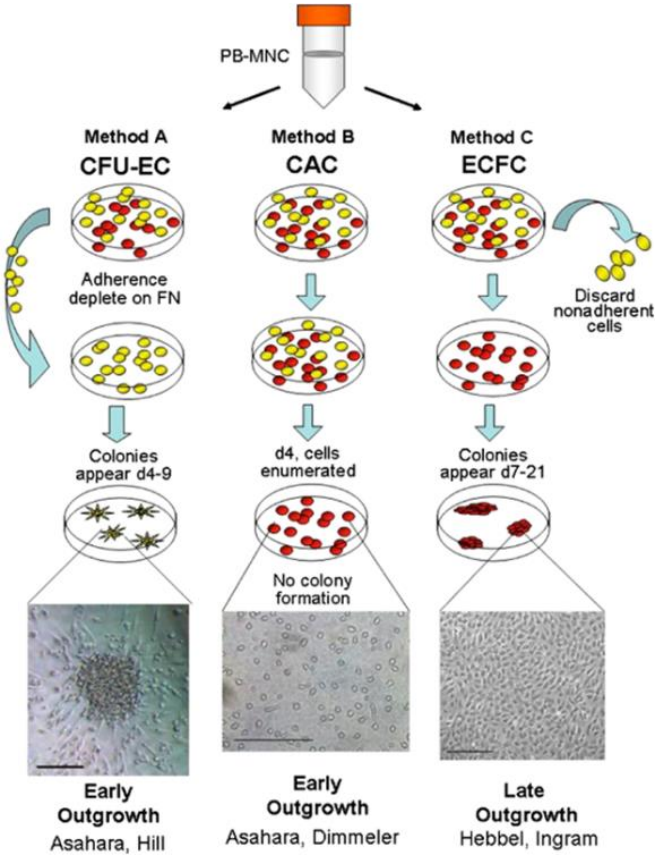

Figure 6 – Schematic representation of common methods of EPCs *in vitro* culture.

(Adapted from: Hirschi, Ingram et al. 2008).

Because the proliferative capacity might be one criterion to define a progenitor cell, several groups established colony assays (Fadini *et al.*, 2008). The most prominent assay, termed Culture of colony forming unit–Hill cells (CFU-Hill), which cells are plated and after 4-9 days, the nonadherent peripheral blood mononuclear cells give rise to the colony. In this method all cell populations of the PBMC fraction are cultured together, which implies the risk of contamination with mature circulating endothelial and monocytic cells. To minimize contamination, some authors included a pre-plating step, which the nonadherent cells are removed, based in principle that mature endothelial cells should adhere to the culture surface (Hirschi *et al.*, 2008). Another method the Circulating Angiogenic Cells (CAC) that consists in culture of adherent mononuclear cells along 4-7 days. In this case, colony formation not occurs. Finally, the another method commonly used is termed Endothelial colony forming cells (ECFC), which mononuclear cells are plated, and the nonadherent cells are discarded. The remaining cells, which are, the adherent cells are cultured along 7-21 days in endothelial conditions, and after this time colonies with cobblestone morphology appear. With this method high proliferation

capacity was verified. The CFU-Hill and CAC methods present an early outgrowth, whereas ECFC method presents a late outgrowth (Hirschi *et al.*, 2008). In addition to the efforts made to improve the techniques of EPCs *in vitro* culture, there will always be a high risk of contamination, and also it has been demonstrated that the frequency of EPCs quantified by culture methods does not correlate with the number of EPCs quantified by flow cytometry (Hristov *et al.*, 2003).

1.4.3.2 EPCs identification by flow cytometry

Flow cytometry is a technique defined as the simultaneous measurement of multiple physical characteristics of a single cell as the cell flows in suspension through a measuring device (Givan, 2001). This technique allows measurements on cells (prokaryotic and eukaryotic) or particles (cytokines, chromosomes and beads) that are in single cell liquid suspension.

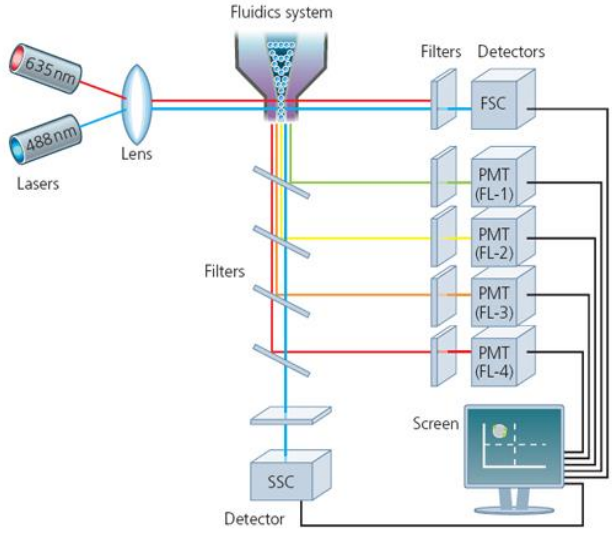

Figure 7 - Representation flow cytometry principles and components.

(Adapted from: http://www.abdserotec.com/flow-cytometry-signal-processing.html)

A flow cytometer is a system consisting of five elements (Fig.7): a light source (mercury lamp or laser), a flow chamber, units of optical filters for selecting a range of specific wavelength, the spectral range from a more wide, photodiodes or photomultiplier for sensitive detection and signal processing with interest and a unit that processes data collected .

In the most common scenario, one or more lasers cross each particle or cell, and the light scatter properties are recorded, namely the side scatter (indicator of the particle's complexity) and forward scatter (indicator of the particle's size) (Givan, 2001). When the laser strikes the cell, the light is diffracted around the edges of the cell, producing a diffraction pattern along the path of the laser beam. This scattered light (forward scatter and side scatter) is approximately equivalent to the cell circumference and is the same wavelength as the exciting laser light.

The sample cells in suspension can be labeled with specific antibodies linked to fluorochromes, which allows the identification and quantification of cells with specific features based on the fluorescence (Mund *et al.*, 2012). The fluorochromes emit light when excited by light of a shorter wavelength, and can be conjugated directly to the primary or secondary antibody, or to streptavidin. The important properties of a fluorochrome are its absorption spectrum, its extinction coefficient at a wavelength convenient for excitation, its emission spectrum and its quantum efficiency. The fluorochromes conjugated with antibodies are very helpful in flow cytometry technique, some of the more widely used fluorescent labels are listed in Table 2.

This technique has several advantages, since it allows multiparameter analysis in a large number of cells and in short time, allowing the identification of a homogeneous population within a heterogeneous population. In addition, also allows the detection of extremely rare populations of events (frequencies less than 10^{-6}), such as stem cells, dendritic cells, endothelial cells, among others (Bakke, 2001). Flow cytometry is currently the best method to obtain pure quantitative data on putative EPCs. Being sensitive, specific and reproducible, should be considered the gold-standard when count of peripheral blood EPC is conceived as a disease biomarker (Fadini *et al.*, 2008). In addition, it is a rapid and convenient way to measure rare events, thus, this method is clearly well suited for detection and quantitation of EPCs.

Table 2 - Representation of more widely used fluorochromes in Flow Cytometry, as well their fluorescence color, and the wavelength of maximal absorbance and of maximal emission.

Adapted from: http://www.bdbiosciences.com/reagents/custom_conjugation/index.jsp

Fluorochrome	Fluorescence color	Maximal absorbance (nm)	Maximal emission (nm)
AlexaFluor™405	Blue	401	421
PacificBlue™	Blue	410	455
AmCyan	Blue	415	500
AlexaFluor™488	Green	495	519
FITC	Green	490	525
PE	Yellow	496	578
APC	Red	650	661
PerCp	Red	490	675
PE-Cy™5	Red	496	667
PerCP-Cy™5.5	Far Red	496	695
PECy™7	Infrared	496	785
APC-Cy7	Infrared	650	785

Nevertheless, the development of cytometric assays was constrained by the lack of reasonably specific monoclonal antibodies for this task (Khan *et al.*, 2005). However, several methodologies have been suggested for EPCs identification but there is not a consensual definition by cell surface antigen expression. In addition, according to Mund J, some of the clinical trials published claiming to quantify EPCs actually quantified hematopoietic stem cells, because they did not use enough surface markers (Mund *et al.*, 2012).

Delorme, B. *et al*, proceed to distinction of CECs and EPCs, in samples of cord and peripheral blood, based essentially on the surface marker CD146, which is an adhesion molecule present in endothelial cells. According these authors, using 4-color flow cytometry analysis, they discriminate EPCs ($CD146^+$ $CD34^+$ $CD45^+CD133^+$ or $CD117^+$) and CECs ($CD146^+$ $CD34^+$ $CD45^-$ $CD133^-$ or $CD117^-$), in samples of peripheral blood collected from patients after myocardial infarction. As results of four color cytometry analysis, the authors obtained, according to CD45 expression, two distinct subpopulations of CD34+ cells, respectively $CD34^+CD45^+$ cells, representing more than 90% of the

circulating $CD34^+$ cells, and $CD34^+CD45^-$ cells, representing less than 3% of the $CD34^+$ cells. These two subpopulations were analyzed individually for the co-expression of CD146, CD117 and CD133, among the $CD34^+CD45^+$, a low percentage of cells co-expressed the referred surface markers, and this subpopulation was defined by authors as EPCs. In turn, the $CD34^+CD45^-$ subpopulation, co-expressed CD146 in a higher percentage, but not co-expressed CD117 and CD133. This subpopulation defined mature CECs. According these authors, a 4-color cytometry analysis of selected CD34+ cells from cord and peripheral blood clearly discriminated between two subsets of circulating CD146+ cells (Delorme *et al.*, 2005).

On the other hand, Khan, S. and collaborators in their review assume that CD31 and CD146 are present on CECs but not on EPCs or hematopoietic stem cells, and in addition assume that CD133 will help to identify EPCs because it is not present on CECs or any mature endothelial cells. However, CD133 provided a means for detecting primitive stem cells in the circulation without the use of CD34. In addition, according these authors, the $CD34^+CD309^+$ combination, is a potential combination of surface markers for EPCs identification. Concerning to CD45 expression of these cells, has been reported for various groups to be positive or negative, due to its Dim expression (slightly increased when compared to the negative control) in these cells (Khan *et al.*, 2005). Some studies suggest that particularly the fraction of CD45- cells may harbor the "true" circulating EPCs. For prove this, some authors such as Schmidt-Lucke, C. *et al*, through samples of patients with coronary artery disease (CAD), considered, in flow cytometry analysis, the $CD45^-$ and $CD45^{dim}$ expression, and in their quantification using CD45, CD34 and CD309 surface markers and gating strategy, $CD34^+$ cells were subdivided in $CD45^-$, $CD45^{dim}$ and $CD45^{bright}$. The data obtained with this protocol showed numbers of $CD45^{dim}CD34^+CD309^+$ cells significantly higher in healthy controls compared to patients with CAD and according the authors, this study confirm that indeed only the fraction of $CD45^{dim}$ cells harbours the "true" circulating EPCs (Schmidt-Lucke *et al.*, 2010). Another parallel analysis of CD45 expression has been also proposed to distinguish EPCs, and most (90%) $CD34^+$ progenitor cells express CD45 at low intensity ($CD45^{dim}$), whereas less than 10% are $CD45^-$(Fadini *et al.*, 2012). Despite this controversy, EPCs were assessed by Hristov, M. *et al*, in the peripheral venous blood of patients with stable coronary artery disease by 3-color flow cytometry, by $CD34^+CD309^+CD45^{-/low}$ combination. As result, in flow cytometry analysis, circulating EPCs were obtained in very low percentages, and to improve this, the authors recommended additional strategies in order to increase the sensitivity and accuracy of the method. These included the use of specific high-quality monoclonal antibodies, selection of high-

intensity fluorochromes to reveal low-density markers, automatic compensation, exclusion of dead cells, and multiparameter gating (Hristov *et al.*, 2012).

In other study, EPCs and CECs were quantified in patients with venous thromboembolism and myeloproliferative neoplasms, by Torres, C. *et al*. CECs and EPCs were quantified in peripheral blood samples by $CD45^{-}CD146^{+}CD133^{-}$ and $CD45^{+low}CD146^{+}CD133^{+}$ immunophenotyping, respectively. The chosen strategy used CD146 to recognize CEC, and also to identify EPC and CD133 to distinguish between CEC and EPC, since that CD133 is absent in CEC. The results of this study indicate that both patients groups had a significant increase in the CEC numbers, as compared to controls, and a decrease of EPC numbers (in both patient groups) relatively to controls, although differences were not statically significant (Torres *et al.*, 2013). In turn, Rustemeyer, P.*et al*, through umbilical cord blood, bone marrow and whole blood samples, selected the $CD309^{+}CD34^{+}$ cells, because the EPCs should be contained in this fraction. In addition, to exclude a major portion of shredded cells from the vessel wall, they measured the number of $CD133^{+}CD34^{+}$ cells. This fraction showed more $CD309^{+}CD34^{+}$ cells than $CD133^{+}CD34^{+}$ (Rustemeyer *et al.*, 2006). In other hand, CD133 is expressed on more immature cells than CD34 and, for that reason, $CD133^{+}CD309^{+}$ cells are rarer than $CD34^{+}CD309^{+}$ cells in the circulation, in steady-state conditions (Fadini *et al.*, 2008).

Some authors, such as Distler, J. *et al* defended that EPCs identification requires a multicolor approach, that is, the use of several surface markers labeled with fluorochromes. They also recommend the use of CD34, CD133 and CD309 to increase the specificity of the analysis (Distler, Allanore et al. 2009). In addition, Mund, J. *et al*, assume that in several studies of EPCs identification the contamination with false-positive events and nonspecific fluorescent event readings may occur. Specifically monocytes, red blood cells, and dead cells autofluorescence and nonspecifically bind antibodies. In his study, trough peripheral blood samples and cord blood samples, a population of cells containing endothelial colony-forming cell (ECFCs) and mature circulating endothelial cells was determined by varying expressions of CD34, CD31, and CD146, but not CD133 and CD45. The results show that if red blood cells, monocytes, and dead/apoptotic (LIVE/DEAD) cells are not excluded, it may lead to occurrence of false-positive events (Mund *et al.*, 2012). In review Fadini G., *et al*, based on the definition of EPCs, recommend that the minimal antigenic profile should include at least 1 marker of stemness/immaturity (usually CD34 and/or CD133), plus at least 1 marker of endothelial commitment (usually CD309) (Fadini *et al.*, 2012).

Beyond all controversy, some authors agree in several issues of quality in flow cytometry, such as, the use of blocking serum to inhibit nonspecific binding, the use of real-time viability stain, the establishment of a dump channel to exclude cells not of interest to analysis, the collection of a large number of events to identify adequate numbers of the rare event population, and clean the cytometer before data acquisition, to remove amounts of cellular debris that have the potential to contaminate the sample of interest. In CEC and EPC assays, at least 500,000 to 1 million list mode events should be collected (Khan *et al.*, 2005). In addition, is recommended the setting and monitoring of fluorescence detectors sensitivity and the use of a multicolor approach, because no markers are entirely specific for these cells (Distler *et al.*, 2009).

1.5 **Evaluation of EPCs by flow cytometry in CKD patients**

Despite the rare nature of EPCs in peripheral blood, several studies identify CKD patients taking into account the advantages of EPCs evaluation. Several authors have suggested that EPCs are reduced in CKD population. In addition to this decrease, EPCs functions such as migratory activity, adhesion to matrix proteins or adhesion to mature endothelial cells are also impaired (Jourde-Chiche *et al.*, 2009).

In 2003 Einzawa, T, *et al*, aimed to determine the number and functional activity of EPCs in hemodialysis patients and control subjects. In this study the numbers of $CD34^+$ MNC and $CD133^+$ MNC in the peripheral blood were quantified using flow cytometry. In results, the numbers of $CD34^+$ MNCs and $CD133^+$ MNCs were significantly reduced in hemodialysis patients compared with control subjects (Eizawa *et al.*, 2003).

On other hand the study of De Groot, K *et al*, 2004, for explore whether uremia influences the number of EPCs, only used CD34 surface marker for assess EPCs in 46 patients with advanced renal failure. The results of this study document that the number of EPCs is significantly reduced in patients with advanced renal failure as compared with healthy subjects. In conclusion, the authors assumed that differentiation of EPCs is inhibited in uremia (de Groot *et al.*, 2004). Moreover, Westerweel, P *et al*, 2007, studied 45 end-stage renal disease patients (ESRD) on hemodialysis, and EPCs are identified by $CD34^+CD309^+$ using flow cytometry. In this study, levels of circulating EPC were also reduced in the peripheral blood of ESRD patients on hemodialysis treatment compared with healthy controls (Westerweel *et al.*, 2007).

In turn, Krening, G. *et al*, 2009, assessed the numbers and the angiogenic function of EPC from 50 patients with varying degrees of CKD, considering that in patients with CKD, the number and function of EPC may be affected by kidney dysfunction. Mononuclear cells were isolated, and circulating EPC were quantified by flow cytometry based on expression of CD14 and CD34. The results suggested that the numbers of circulating $CD34^+$ decreased with increasing kidney disease, since was observed marked decrease in the number of circulating EPC as early as stage 1 of CKD. In addition the results suggested that adherence and endothelial outgrowth of EPC from patients with CKD is progressively reduced during kidney disease. In contrast to the reduction in CD34 EPC numbers, there was no effect of CKD on the number of circulating CD14 EPC. Therefore, the authors hypothesize that the chemoattractants for CD14 EPC and CD34 EPC are differentially expressed in patients with CKD (Krenning *et al.*, 2009). However, according to bibliography, CD14 is a monocyte marker, not being considered a surface marker of EPCs.

Jourde-Chiche, D *et al*, 2009, studied 38 hemodialysis patients, and after peripheral blood mononuclear cells isolation, EPCs were identified by flow cytometry, using $CD34^+CD133^+CD309^+$ labelling. In results, the absolute number of EPCs was reduced in comparison with healthy subjects, and the authors concluded that uremic toxins and vascular injury may be among the factors affecting EPC numbers in uremia. However, their results suggest that even in a context of reduced EPC production induced by uremia, vascular damage may still stimulate EPC release (Jourde-Chiche *et al.*, 2009). Furthermore, Jie, K *et al*, 2010, studied 49 patients with different stages of CKD and EPC were identified as $CD34^+CD309^+$-cells. In results, they observed that $CD34^+CD309^+$ levels were lower in CKD. In addition, the authors compared EPC levels of CKD patients with a history of CVD and CKD patients without a history of CVD. In results CKD patients with a history of CVD showed lower EPCs in comparison to other patients. This study shows that pre-dialysis CKD patients on regular medical therapy have lower levels of circulating EPC and reduced EPC outgrowth compared to healthy controls (Jie *et al.*, 2010).

Taken together in all of these studies was observed a reduced number of EPCs, these alterations in vascular progenitor cell levels may advance progression of CKD as it has been reported that EPC contribute to glomerular endothelial repair (Jie *et al.*, 2010). Therefore, early intervention may reduce cardiovascular morbidity in CKD patients through increased physiological vascular regeneration (Krenning *et al.*, 2009).

2. AIM

Taking into consideration that there is no specific surface marker and there is no an optimized protocol for EPCs identification, the main goal of this study was the establishment of a standardized protocol for simultaneously identification of EPCs and CECs and their distinction, using a multicolor flow cytometry technique. Furthermore in this study we aimed to evaluate the circulating levels of both EPCs and CECs, in a CKD population.

3. MATHERIAL AND METHODS

3.1 Study Subjects

In this study were selected a representative number of CKD patients (n=25) with GFR included in stages 1 to 5. Patients were distributed in the 5 stages of CKD according to the glomerular filtration rate calculated by MDRD formula (ml/min/1.73m2): Stage 1, > 90; Stage 2, between 60–90; Stage 3 (comprises 3a and 3b), between 30-59; Stage 4, between 15–29; and Stage 5, < 15. We defined two subgroups of CKD patients, the "Early CKD", with stages comprises between stage 1 and stage 3a, and the "Advanced CKD", with the stages comprises between stage 3b and stage 5. In addition to the selected CKD patients, it was selected healthy subjects (n=15), consisting in a control group.

Patients with CKD included in several stages and followed-up in the outpatient clinic of Nephrology department of "Centro Hospitalar de São João" were invited to participate in the present study. The informed consent process and the study protocols were submitted and approved by the Ethics Committee of "Centro Hospitalar de São João, EPE" (see Annex 2).

3.2 Isolation of Peripheral Blood Mononuclear Cells

Samples of peripheral blood were collected through the antecubital veins to EDTA tubes (0.5M EDTA, 3.8% w / v sodium citrate). In the initial step it was collected more volume of blood samples (36 mL) to flow cytometry optimizations. However this volume of blood was collected from healthy subjects for ethical reasons. Taking into account that these cells are extremely rare events, we tried to use the greatest amount of blood possible. After these optimizations it was collected a fixed volume of peripheral blood samples (18 mL).

For isolation of mononuclear cells from peripheral blood sample, it was used the same amount of Histopaque-1077 (Sigma-Aldrich, St. Louis, Missouri, United States) and of whole blood sample in each falcon (18 mL of blood and 18 mL of Histopaque-1077). In this step it was necessary that the blood would be carefully and slowly added in the falcon with Histopaque-1077, which must be on a 45º inclination. Then, a gradient-density centrifugation was performed during 30 minutes, at 400g and

room temperature. Initially, the stipulate centrifugal force would be 800g but a higher layer was at 400g. After gradient density centrifugation, several layers were observed, as can be seen in Fig.8.

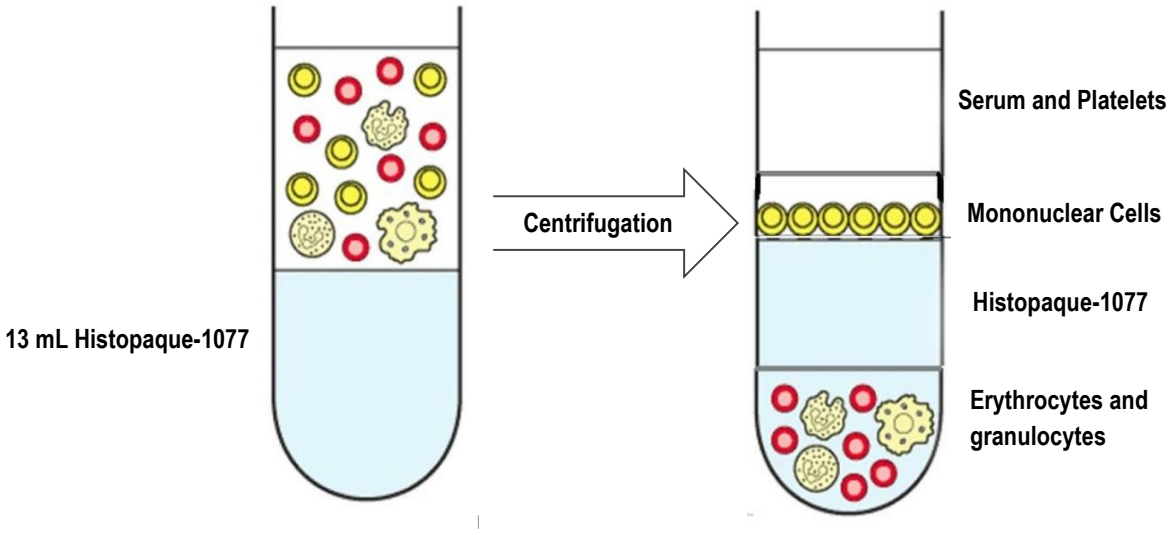

Figure 8 -– Schematic representation of the blood sample before and after gradient-density centrifugation.

The mononuclear cells layer was removed carefully by a micropipette to another falcon of 50 mL, and was resuspended in phosphate buffered saline (PBS) to make up 50 mL and centrifuged for 10 minutes, at 300g, room temperature. This step was repeated twice, if necessary a second washing.

3.3 Preparation of MNC for flow cytometry staining

Afterwards the supernatant was removed and 200 µL of FACS buffer (50 mL PBS, 250 mg BSA, 5µL azide) was added and then the mononuclear cells were incubated with FACS buffer in ice during 30 minutes to avoid cell aggregation. During this time stained cells with Tripan Blue (Sigma-Aldrich, St. Louis, Missouri, United States) 1:20, were counted in a Neubauer chamber. One and seven millions of cells were counted for the controls and for each mix, respectively, and were transferred to a 96 well round bottom ELISA plates (Orange Scientific, Braine-l'Alleud, Belgium). The plate was centrifuged during 4 minutes, at 400g, 4ºC.

3.4 Incubation of MNCs with antibodies

After centrifugation, the supernatant was discarded, and the MNC were subsequently labeled using the fluorochrome-conjugated primary antibodies CD31-PE (eBioscience, San Diego, USA), CD34-FITC (eBioscience, San Diego, USA), CD45-APC Cy7 (eBioscience, San Diego, USA), CD117-PerCP Cy5.5 (Biolegend, San Diego, California, USA), CD133-APC (Miltenyi Biotec, Bergisch Gladbach, Germany) , CD146-eFluor450 (eBioscience, San Diego, USA), CD309-PE Cy7 (Biolegend, San Diego, California, USA) (Table 3). In addition, cells were labelled with Fixable viability dye – eFluor506 (eBioscience, San Diego, USA), for dead cells exclusion. In Table 3 are represented the several antibodies used, associated to fluorochromes and their respective characteristics.

Table 3 - Representation of fluorochromes conjugated with primary antibodies that were used and their maximal absorbance and emission wavelength.

Antibody	Fluorochrome	Fluorescence color	Maximal absorbance (nm)	Maximal emission (nm)
CD146	PacificBlue™	Blue	410	455
Fixable Viability Dye	AmCyan eFluor506	Blue	415	500
CD34	FITC	Green	490	525
CD31	PE	Yellow	496	578
CD133	APC	Red	650	661
CD117	PerCP-Cy™5.5	Far Red	496	695
CD309	PECy™7	Infrared	496	785
CD45	APC-Cy7	Infrared	650	785

Antibody (Ab) titration was performed in order to determine the optimal amount of Ab for cell staining. For all the Abs tested, the optimal concentration was 1µl of antibody per 1 million of cells. For staining 7 million of cells, it was scaled up and used 7µl of each antibody for the antibody cocktail preparation. For the negative control (unstained) and for FMO control (Fluorescence Minus One- to determining positive and negative limits) it was used 1 µL of each Ab to label 1 million of cells. For the fixable viability dye, it was used 3.5µl to label 7 million cells. As we wanted to study a rare population,

in order to spare cells, the automatic compensation settings was done by using UltraCompeBeads (eBioscience, San Diego, USA) that bind to the antibodies, with the exception for the viability dye, in which were used labeled cells. Briefly, for the UltraCompBeads, we used 1 drop of beads (which correspond to 50µL) to label with one specfic antibody; which means a total of 7 drops of beads, each one used to be labeled with each of the 7 antibodies used in the staining mix. The table 4 shows the constitution of unstained, monolabel controls, FMO controls and Mix, relatively to antibodies and FACS buffer. The MNC and the beads with antibodies were incubated during 30 min on ice, protected from light. Afterwards they were centrifuged for 4 minutes, at 400g, at temperature of 4ºC. The supernatant was discarded and washed twice with 100 µL of FACS buffer, before fixation. The fixation was performed with 100 µL paraformaldehyde 4% (Alfa Aesar, Ward Hill, Massachusetts), during 20 minutes. To remove the excess of paraformaldehyde, 100 µL of PBS were added and cells were washed twice by centrifugation for 4 minutes, at 400g, 4ºC. Cells were then stored at 2-8ºC, protected from the light, overnight. In the next day, before flow cytometry analysis, it was added 100µL of PBS to the samples and then, the cells were filtered with a nylon mesh 1mx1m 100µm (Thermo Fisher Scientific, Waltham, Massachusetts, USA), to FACS tubes (VWR International, Radnor, PA, USA) (Fig.10).

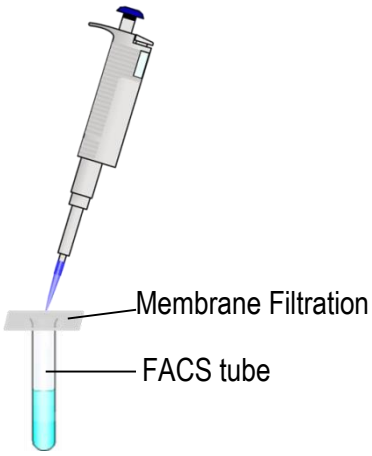

Figure 9 - Schematic representation of methodology used to cells filtration before flow cytometry analysis.

Table 4 - Representation of the several conditions used for Flow Cytometric analysis.

	CD45	CD34	CD31	CD117	CD133	CD146	CD309	Viability dye	FACS volume
Unstained Control									
Cells without Ab	-	-	-	-	-	-	-	-	50µl
Compensation Controls									
Beads+CD45	1 drop	-	-	-	-	-	-	-	-
Beads+CD34	-	1 drop	-	-	-	-	-	-	-
Beads +CD31	-	-	1 drop	-	-	-	-	-	-
Beads+CD117	-	-	-	1 drop	-	-	-	-	-
Beads+CD133	-	-	-	-	1 drop	-	-	-	-
Beads+CD146	-	-	-	-	-	1 drop	-	-	-
Beads+CD309	-	-	-	-	-	-	1 drop	-	-
Cells + Violet Dye	-	-	-	-	-	-	-	0.5µL	49.5 µL
Fluorescence Minus One (FMO) Controls									
FMO- CD45	-	1 µL	1µL	1µL	1µL	1µL	1µL	0.5µL	43.5µL
FMO- CD34	1µL	-	1µL	1µL	1µL	1µL	1µL	0.5µL	43.5µL
FMO- CD31	1µL	1µL	-	1µL	1µL	1µL	1µL	0.5µL	43.5µL
FMO- CD117	1µL	1µL	1µL	-	1µL	1µL	1µL	0.5µL	43.5µL
FMO- CD133	1µL	1µL	1µL	1µL	-	1µL	1µL	0.5µL	43.5 µl
FMO- CD146	1µL	1µL	1µL	1µL	1µL	-	1µL	0.5µL	43.5µL
FMO- CD309	1µL	1µL	1µL	1µL	1µL	1µL	-	0.5µL	43.5µL
MIX (All Abs)									
Cells + MIX	7 µL	7 µL	7 µL	7 µL	7 µL	7 µL	7 µL	3.5 µL	-

3.5 Flow cytometry data acquisition and analysis

Flow cytometry was performed on a BD FACSCanto™ II system BD Biosciences flow cytometer (Becton-Dickenson, New Jersey, U.S.A). The optics of the BD FACSCanto™ II system consist of an excitation source with three lasers: blue (488-nm, air-cooled, 20-mW solid state), red (633-nm, 17-mW HeNe), and violet (405-nm, 30-mW solid state). The blue laser comprises the following detectors, forward scatter (FSC), side scatter (SSC), FITC, PE, PerCp and PECy7, in turn the red laser comprise APC and APC Cy7 parameters, while violet laser comprise Pacific blue and AmCyan parameters. For EPCs identification it was used all data acquisition parameters of Flow cytometer. In first place, the unstained control was acquired for voltages adjustments, and then UltraCompeBeads were acquired for automatic compensation.

Data analysis was done with FlowJo10 (Treestar, San Carlos, CA) with multiparameter logical gating of defined regions. Positive staining and gating strategy was determined by comparison to the FMO controls.

3.6 EPC characterization

Using the FlowJo10 software, debris such as platelets and other nonspecifically stained events were removed based on SSC-A and FSC-A, then dead cells were excluded based on fixable viability dye positive expression, and based on FCS-H and FSC-A, the single cells were selected, meaning that the doublets and triplets were excluded. For identification of EPCs and ECs, it was tried several strategies of gating (see Annex I), until it was identified the optimal strategy.

3.6.1 Strategy adapted from Torres C. *et al*, 2013

As a starting point for our strategy optimization, an analysis based on bibliography (Torres, C. et al, 2013) was performed, using only some antibodies (CD45, CD133, CD309, CD146). For EPCs identification the strategy used was the following: $CD45^{low}$ > $CD133^{+}CD309^{+}$ > $CD146^{-}$ cells selection (Fig.11.I), and for CECs identification: $CD45^{-}$ > $CD309^{-}CD133^{-}$ > $CD146^{+}$cells (Fig.11.II).

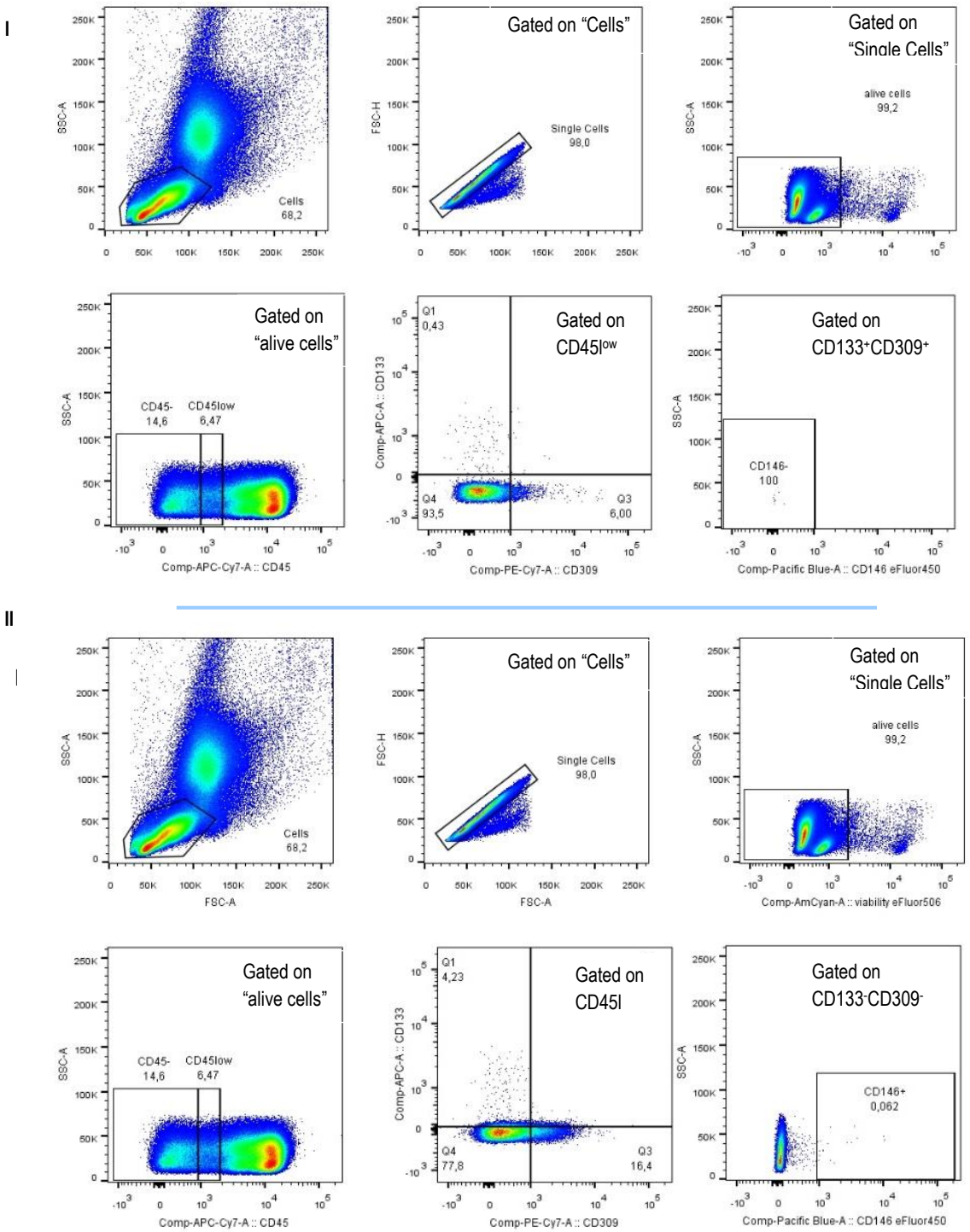

Figure 10 - Representation of gating strategy according Torres *et al*, 2013. **I** - EPCs were identified by CD45low cells selection, CD133+CD309+ selection, and CD146+ cells selection. **II** – ECs were identified by CD45- cells selection, CD133-CD309- and CD146+ cells selection.

3.6.2 Optimized strategy

According to the literature, for identification of EPCs there are several strategies that use different markers. In addition, there is some controversy between articles relatively to expression of some markers by EPCs. However, it does not exist a specific antibody able to identify these rare cells. For that we defined a panel that encompasses many of these antibodies that are referred in bibliography. Using these antibodies (CD31, CD45, CD34, CD117, CD133, CD309 and CD146) it was defined a single strategy that allows to identify two subpopulations of EPCs (early EPCs and late EPCs). Early EPCs, immediately after their recruitment from the bone marrow, express specific cell marker and with their migration along blood circulation they lose this marker and acquire new surface marker, being called late EPCs. For this, it was important the establishment of a single strategy able to identify these two subpopulations of EPCs.

In addition, the optimized strategy allows to identify a population of circulating ECs and a population of hematopoietic progenitor cells that have a common precursor with EPCs. Over the time, our strategy has changed in the order of gates, until we achieve the final strategy.

For early EPCs identification the strategy used was the following: $CD45^{-/int}CD31^{-/+}>CD117^{-}CD34^{+}>CD34^{+}CD133^{+}>CD309^{-}CD146^{-}$ (Fig.12.II) and for late EPCs identification: $CD45^{-/int}CD31^{-/+}>CD117^{-}CD34^{+}>CD34^{+}CD133^{-}>CD309^{+}CD146^{-}$ (Fig.12.III). For CEC identification the gating strategy was the following: $CD45^{-/int}CD31^{-/+}>CD117^{-}CD34^{-/low}>CD34^{-}CD133^{-}>CD309^{-}CD146^{+}$ (Fig.12.IV). Taking into account that the putative population of hematopoietic progenitor cells express CD34 and CD117 markers in their surface, this subpopulation was identified from the following gating strategy: $CD45^{-/int}CD31^{-/+} >CD34^{+}CD117^{+}$ (Fig.12.I).

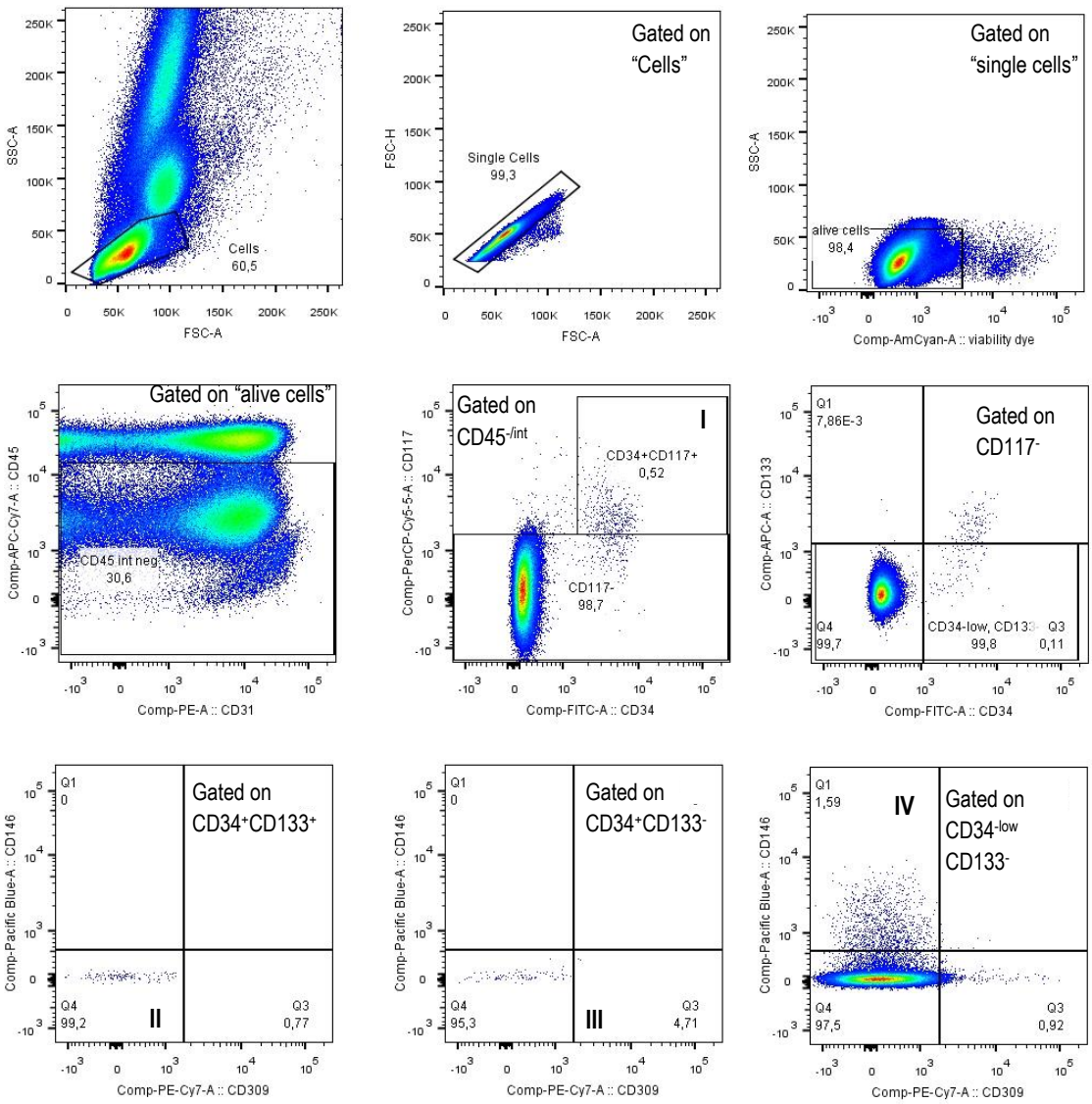

Figure 11 - Representation of Gating Strategy for Hematopoietic progenitor cells (I), Early EPCs (II), Late EPCs (III) and CEC (IV) identification.

3.7 **Statistical experimental design**

Data are expressed as the mean of the individual values ± standard error of the mean (SEM) of values. To analyze mean differences between patient groups and healthy controls, data were analyzed using the software's Microsoft Excel 2010 (Washington, USA) and GraphPad Prism 6 (California, USA), through the use of T-test or 1 way Anova. A P value <0.05 was considered statistically significant.

To assess the recruitment, differentiation and the reparative capacity of these subpopulations of cells were established three indexes that was obtained using the following formulas:

$$Index\ of\ recruitment\ capacity = \frac{\text{number of "early" EPCs}}{\text{number of CECs}}$$

$$Index\ of\ differentiation\ capacity = \frac{\text{number of "late" EPCs}}{\text{number of "early" EPCs}}$$

$$Index\ of\ regenerative\ capacity = \frac{\text{number of "late" EPCs}}{\text{number of CECs}}$$

4. RESULTS

4.1 Antibody concentration optimization

While establishing the optimal antibodies concentration, we initially observed that with 2 µL of antibody to several million of cells (1 until 5 million), the antibodies loss their specificity. Therefore, it was necessary an Ab titration. The optimal volume was 1µL of antibody per million of cells, in order to avoid non-specific biding. Initially the concentration of fixable viability dye that we used was 1µL, but in the first flow cytometry analysis it was observed that numerous cells were marked as dead. Therefore, the optimal volume of fixable viability dye was 0.5µL per million of cells.

4.2 EPCs and CECs phenotype

The figure12.A refers to the FMO control for CD45 (Leukocyte common antigen marker) staining that determines the positive/negative boundaries for this antibody. According to this FMO control, we can observe, in figure12.B that most of CD34+ cells (putative EPCs) have intermediate expression to CD45 marker and positive expression to CD31.

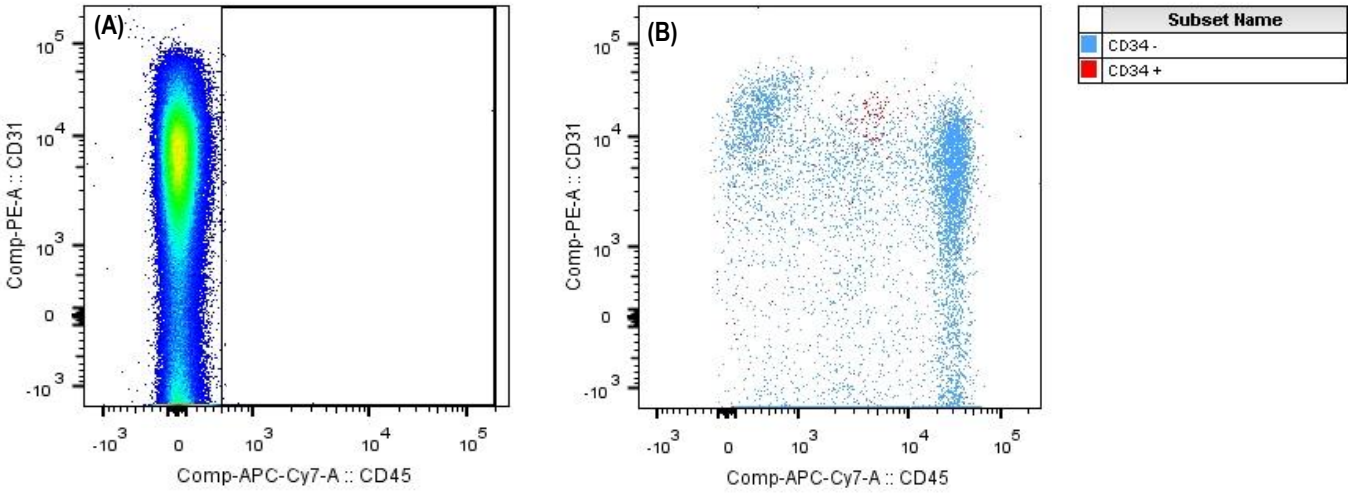

Figure 12 - (A) Dot Plot representing the FMO control for CD45 marker. (B) Dot Plot representing different CD45 expression in CD34+ (red color) and CD34- (blue color) cell populations. Gated from "alive cells".

The figure 13.A shows the FMO control for CD34 marker (hematopoietic stem cell marker). According to this FMO control, we can observe in figure 13.B that CD309-CD146+ cells (putative CECs subpopulation) have both negative and low levels of CD34 expression.

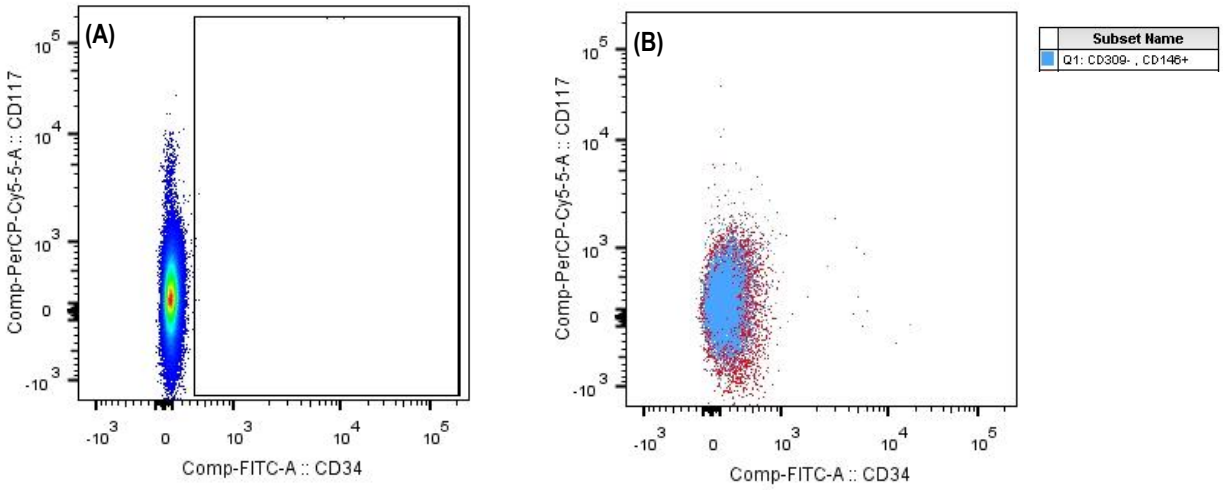

Figure 13- (A) Dot Plot representing a FMO control for CD34 marker **(B)** Dot Plot representing CD34 expression in CEC population. CD309-CD146+ (blue color) have negative and low CD34 expression. Gated from: "CD45$^{-/int}$".

In figure14 we can observe that all most CD34$^{-/low}$ cells (putative CECs) presented negative expression of CD45 and positive expression of CD31. Once that this pattern was reproducible in all analysis we selected cells with intermediate and negative expression of CD45 to identify EPCs and CECs.

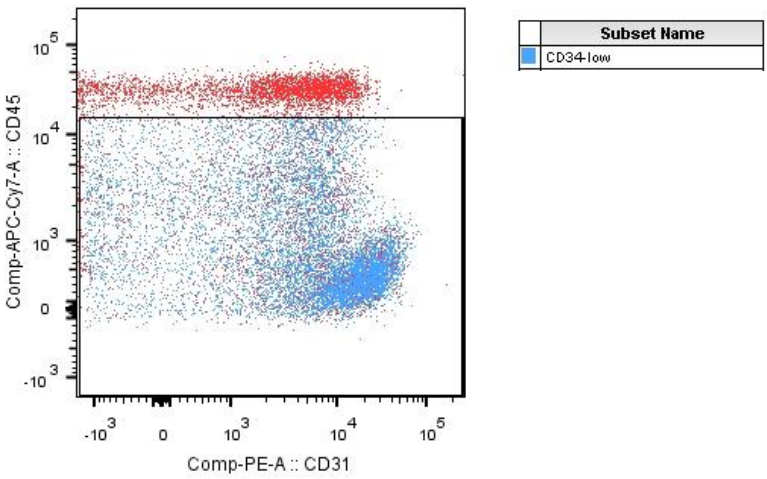

Figure 14 - Dot Plot representing CD31 and CD45 expression in CEC population (blue color).

Regarding EPCs characterization, with this protocol it was possible to distinguish two different subpopulations of EPCs. The figure 15 shows that $CD34^{+}CD133^{+}$ cells (putative early EPCs subpopulation) do not express neither CD146 nor CD309 markers in their surface. Once that this results was reproducible in all analysis we selected cells with $CD31^{-/+}CD45^{-/int}$>CD34+CD117-$^{-}$>$CD34^{+}$CD133+>$CD309^{-}CD146^{-}$ to identify early EPCs. Furthermore, we selected cells with $CD31^{-/+}CD45^{-/int}$>$CD34^{+}CD117^{-}$>$CD34^{+}CD133^{-}$>$CD309^{+}CD146^{-}$ to identify late EPCs.

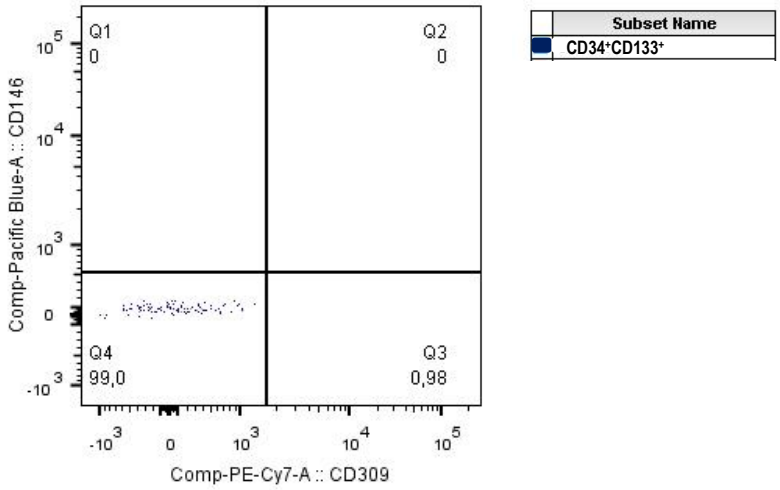

Figure 15 - Dot Plot representing early EPCs expression for CD309 and CD146 markers. Gated from CD34+CD133+ cells.

4.3 EPC characterization

4.3.1 Strategy adapted from Torres C. *et al*, 2013

In the course of technique optimization, the cytometer voltages have suffered some adjustments. The results of this strategy comprised a control group (n=5) and a CKD group (n=5) (stages 2 to 5).

The results show that EPCs levels were decreased in CKD patients group (0.3±0.1 cell/mL) in comparison with control group (2.3±0.8 cells/mL), without statistical significance (Fig.16.A), whereas, the levels of ECs were increased in CKD patients (185.5±76.2 cell/mL) in comparison with the control group (1.8±0.8 cells/mL), also without statistical significance (Fig.16.B). However, the index of regenerative capacity was significantly lower in CKD patients group (0.007 ± 0.03) than in control group (3.9±1.5), p=0.008 (Fig.17).

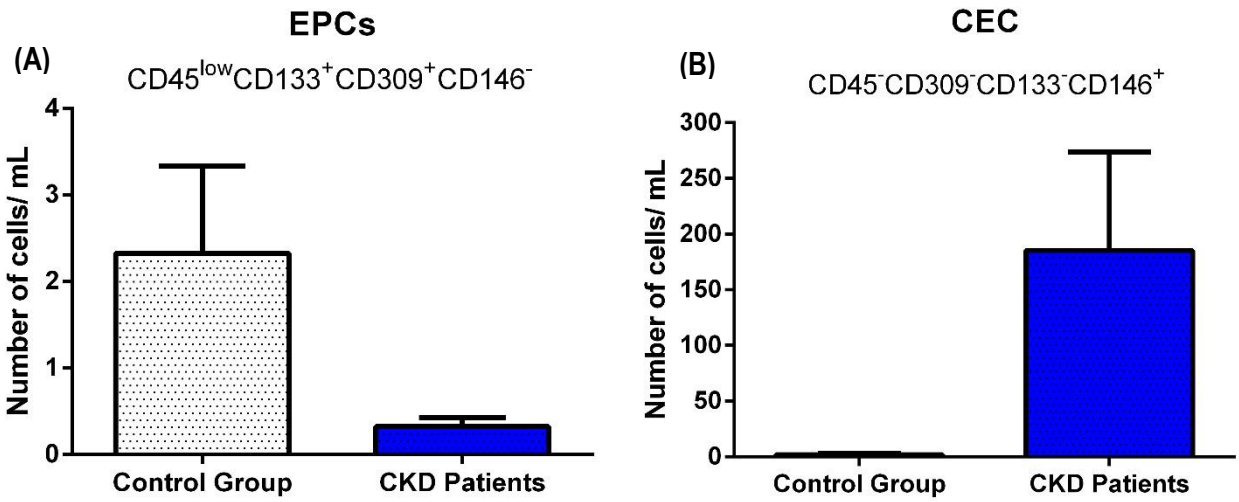

Figure 16 - Levels of EPCs (A) and CECs (B) among CKD patients (n=5) and control subjects (n=5).

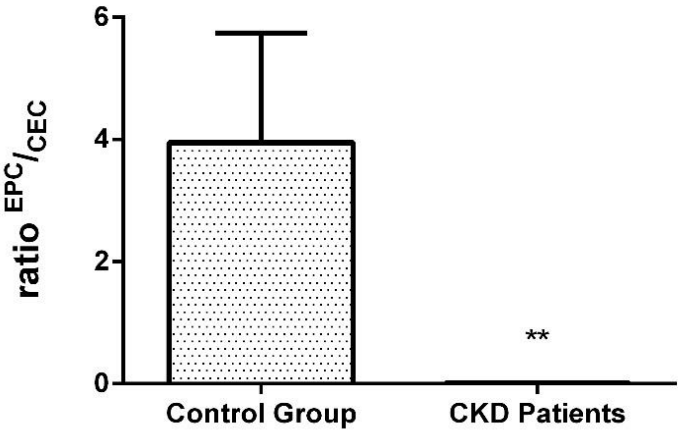

Figure 17 – Levels of ratio EPC/CEC among CKD patients (n=5) and control subjects (n=5).

**P<0.01 compared with control group.

4.3.2 Optimized strategy

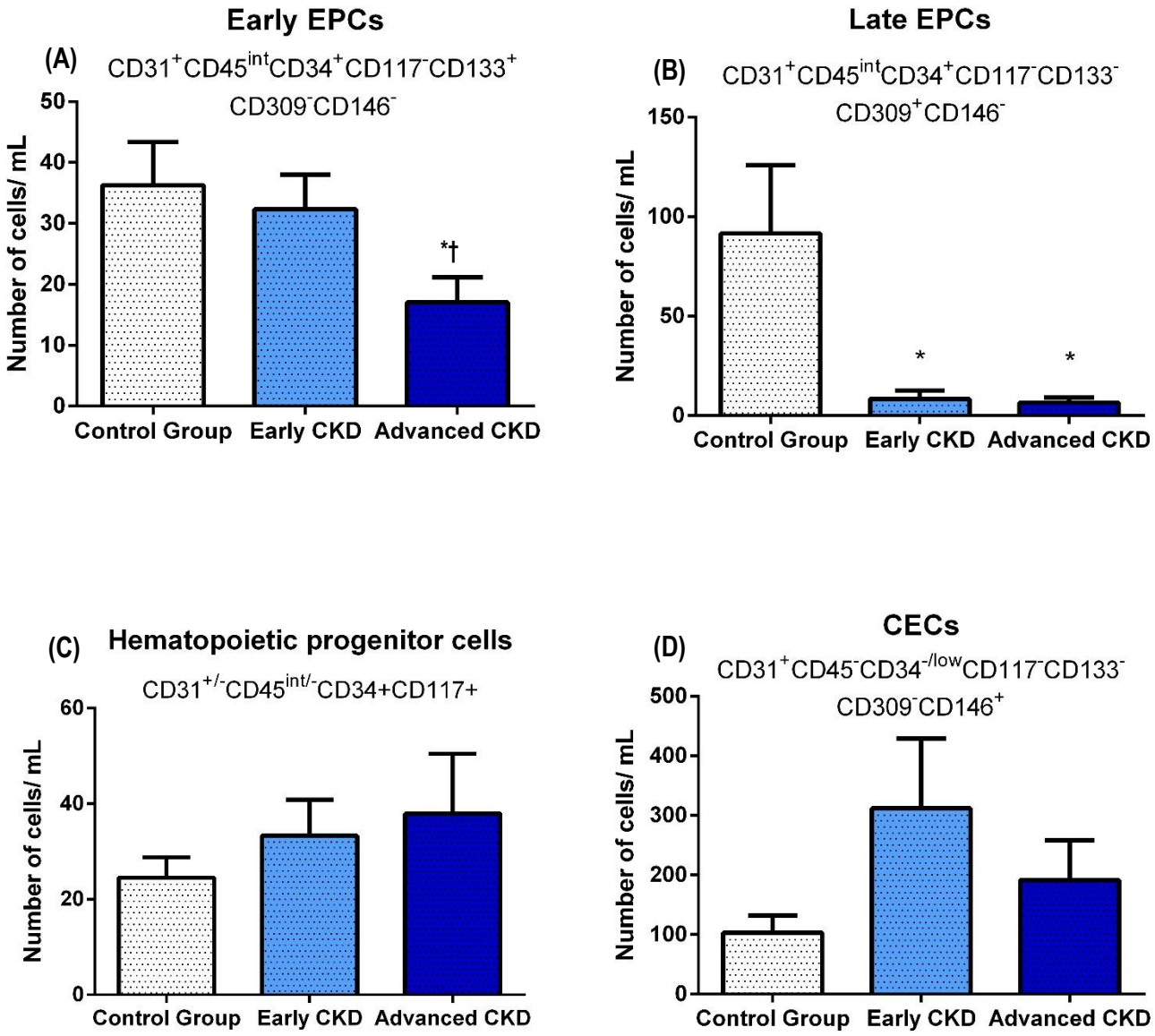

Figure 18 - Levels of early EPCs (A), late EPCs (B), hematopoietic progenitor cells (C) and CECs (D) among early CKD (n=10) and advanced CKD (n=10) patients and control subjects (n=10).

*P<0.05 compared with control group. †P<0.05 compared with Early CKD group.

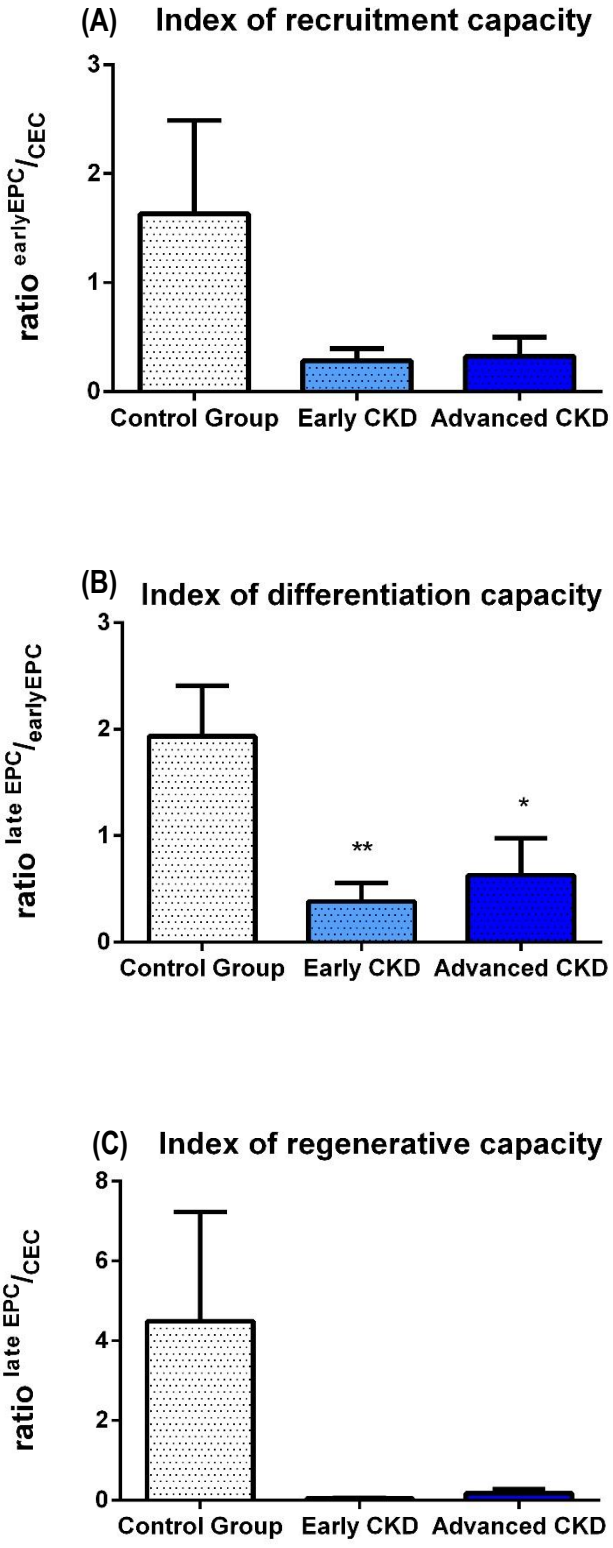

Figure 19 – Levels of Index of recruitment capacity (A), Index of differentiation capacity (B) and Index of regenerative capacity (C), among early CKD (n=10) and Advanced CKD (n=10) patients and control group (n=10). *P<0.05, **P<0.005 compared with control group.

The results shows that the levels of early EPCs (Fig.18.A) were significantly lower in advanced CKD group (17.0±3.2 cells/mL) compared with early CKD group (32.3±4.9 cells/mL), p=0.04, and control group (36.3±6.2 cells/mL), p=0.03, and the levels of late EPCs (Fig.18.B) were significantly lower in both advanced CKD (6.6±1.9 cells/mL), p=0.01, and early CKD groups (8.4±2.6 cells/mL), p=0.01, than in control group (91.5±29.1 cells/mL). By contrast, the levels of CECs (Fig.18.D) were higher in early CKD group (312.1 ± 91.3 cells/mL) than in advanced CKD (191.4 ± 49.9 cells/mL) and control group (103.2 ± 24.1 cells/mL), without reaching statistical significance. The levels of hematopoietic progenitor cells (Fig.18.C) was higher in advanced CKD group (37.9 ± 8.7 cells/mL) and in early CKD group (33.3 ± 6.4 cells/mL) than in control group (24.5 ± 3.3 cells/mL), however without reaching statistical significance.

The ratio between early EPCs and CECs (Fig.19.A) is considerable reduced in both early CKD (0.3 ± 0.1) and advanced CKD groups (0.3 ± 0.1) in comparison with control group (1.6 ± 0.7), however the values did not achieve statistical significance. In addition the ratio between late EPCs and early EPCs (Fig.19.B) is significantly reduced in both early CKD (0.4 ± 0.1), p=0.0045, and advanced CKD groups (0.6 ± 0.3), p=0.04, in comparison with control group (1.9 ± 0.4). Moreover, the ratio between late EPCs and CECs (Fig.19.C) is also considerable reduced in both early CKD (0.04 ± 0.02) and advanced CKD groups (0.2 ± 0.1) in comparison with control group (4.5 ± 2.1), but the values did not achieve statistical significance.

Table 5 represents the percentages (%) of hematopoietic progenitor cells, early EPCs, late EPCs and ECs in relation with the total number of MNCs in all studied groups. The % of hematopoietic, early EPCs and late EPCs subpopulations ranged between 0.01 and 0.07%, while the % of CECs subpopulation ranged between 0.11 and 0.26%.

The percentage of late EPCs subpopulation was significantly lower in both early and advanced CKD groups compared with control group. By contrast, the percentage of CECs subpopulation was significantly higher in advanced CKD group compared with in control group.

Table 5 - Representation of percentages values obtained to hematopoietic progenitor cells, early EPCs, late EPCs and CEC, in a total of mononuclear cells. *P<0.05; **P<0.01 compared with group control, n=10.

	Control Group	Early CKD	Advanced CKD
Hematopoietic progenitor cells	0.04 %	0.04%	0.04%
Early EPCs	0.03%	0.03%	0.02%
Late EPCs	0.07%	0.01%**	0.01%**
CECs	0.11%	0.24%	0.26%*

4.4 Evaluation of EPCs by flow cytometry in CKD population

In the *Introduction* section several works are cited referring the use of different strategies and combinations of markers for EPCs identification by flow cytometry. In order to compare our results with these strategies, we tested different combinations of markers, in early (n=10) and advanced CKD (n=10) patients and in control subjects (n=10). In table 6 are present the results that were obtained for each strategy, according to the literature.

Taking account only CD34 expression used by De Groot, K et al, 2004 and Asahara et al, 1997, our results show a non-significant decrease in the levels of these cells only in advanced CKD group compared with the control group. Using only CD133 marker used by Eizawa, Murakami et al. 2003, the levels of cells were slight increased in early CKD in comparison with control group, without reach statistical significance. Relatively to CD34+CD309+ strategy used by Peter E. Westerweel et al. 2006, the levels of these cells were non-significantly lower in early CKD group than in control group. Finally, using the CD34+CD309+CD133+ strategy, used by Jourde-Chiche, Dou et al. 2009, the levels of these cells obtained were also non-significantly lower in early CKD than in control group.

Table 6 - Representation of different strategies for EPCs identification and the number of cells/mL obtained in each strategy in early CKD, advanced CKD and control group.

Strategy for EPCs identification	Authors	Control Group (cells/mL)	Early CKD (cells/mL)	Advanced CKD (cells/mL)
$CD34^+$	de Groot *et al*., 2004 Asahara, 1997	200.5±31.1	195.5±37.8	165.6±33.8
$CD133^+$	Eizawa *et al*., 2003	33.4±3.7	54.5±9.5	39.3±8.7
$CD34^+CD309^+$	Westerweel *et al.*, 2007	27.0±5.7	21.7±4.2	25.9±6.7
$CD34^+CD309^+CD133^+$	Jourde-Chiche *et al.*, 2009	3.5±0.8	1.7±0.2	3.0±0.9

5. DISCUSSION

Circulating endothelial cells are released to the blood circulation after vascular injury, while EPCs are recruited from bone marrow and mobilized to damage sites to perform vascular repair. Which means that CECs and EPCs have been considered as biomarkers of endothelial injury and endothelial repair, respectively (Zhang *et al.*, 2014). However, the identification and quantification of CECs and EPCs in the blood is technically very difficult and not yet well standardized (Khan *et al.*, 2005). Therefore, the present thesis was designed to develop an optimized protocol of flow cytometry for accurately and simultaneously identification of EPCs and CECs in the same blood sample.

To date, the most commonly used methods for EPCs identification were adhesion and growth *in vitro*; and selection by cell surface phenotype using fluorescent labeled antibodies by flow cytometry (Hirschi *et al.*, 2008). However the culture assay requires a large blood sample and a long assay time. Whereas, flow cytometry technique offers the promise of a highly sensitive, accurate and reproducible approach. In addition this technique allows exactly identification and enumeration of rare and complex cell subpopulations, including overlapping phenotypes (Fadini *et al.*, 2008). One of the major problems resides in the correct marker combination for EPCs identification. Based on literature, there are several strategies for EPCs identification, but the most common combination of markers is based on surface expression of CD34 and CD309 (Westerweel *et al.*, 2007) (Jourde-Chiche *et al.*, 2009), as hematopoietic marker and adhesion marker, respectively. However, there is some controversy and doubts regarding the expression of some surface markers of EPCs. Taking account the several surface markers that are referred in the current literature, it was defined in our work a panel that encompasses many of these antibodies. Therefore, in our study, EPCs and CECs were simultaneously identified based on the differential expression of CD45, CD31, CD34, CD117, CD133, CD146 and CD309.

Regarding to the optimization of the flow cytometric protocol and because of the low percentage of analyzed circulating cells, additional strategies were applied in order to increase the sensivity and accuracy of our assay. This included the selection of high-intensity fluorochromes (PE, APC, PE-Cy7) to reveal low-density markers (CD31, CD133, CD309), and the selection of low-intensity fluorochromes (FITC, PerCp-Cy5.5, APC-Cy7, Pacific blue) to reveal high-density markers (CD34, CD117, CD45, CD146). In addition, was performed automatic compensation, exclusion of dead cells and doublets, and multiparameter gating. For setting gates and determining positive and negative

limits, were used FMO controls. With these adjustments it was possible to demonstrate, in the present study, that all the fraction of $CD34^+$ cells that may harbor the true circulating EPCs, have consistently intermediate expression of CD45 and positive expression to CD31, contradictorily to the literature (Delorme *et al.*, 2005) (Torres *et al.*, 2013) (Fadini *et al.*, 2008). Additionally, it was possible to demonstrate that all the fraction of $CD34^+CD133^+$ cells (putative early EPCs subpopulation) do not yet express adhesion markers such as $VEGFR_2$ (CD309), also in contradiction to what was described in the literature (Hristov *et al.*, 2003). Therefore, we defined a gating strategy able to distinguish early EPCs subpopulation phenotypically identified as $CD31^+CD45^{int}CD117^-CD34^+CD133^+CD309^-CD146^-$ cells, and late EPCs subpopulation phenotypically identified as $CD31^+CD45^{int}CD117^-CD34^+CD133^-CD309^+CD146^-$ cells. Moreover, with a different gating strategy it was possible also to identify CECs ($CD31^{-/+}CD45^{-/int}CD117^-CD34^{-/low}CD133^-CD309^-CD146^+$) as well as hematopoietic progenitor cells that have the same precursor of EPCs ($CD34^+CD117^+$), using the same panel of antibodies in the same peripheral blood sample (see Fig.20). To the best of our knowledge, this is the first report in which early and late EPCs, CECs and hematopoietic progenitor cells were simultaneously quantified.

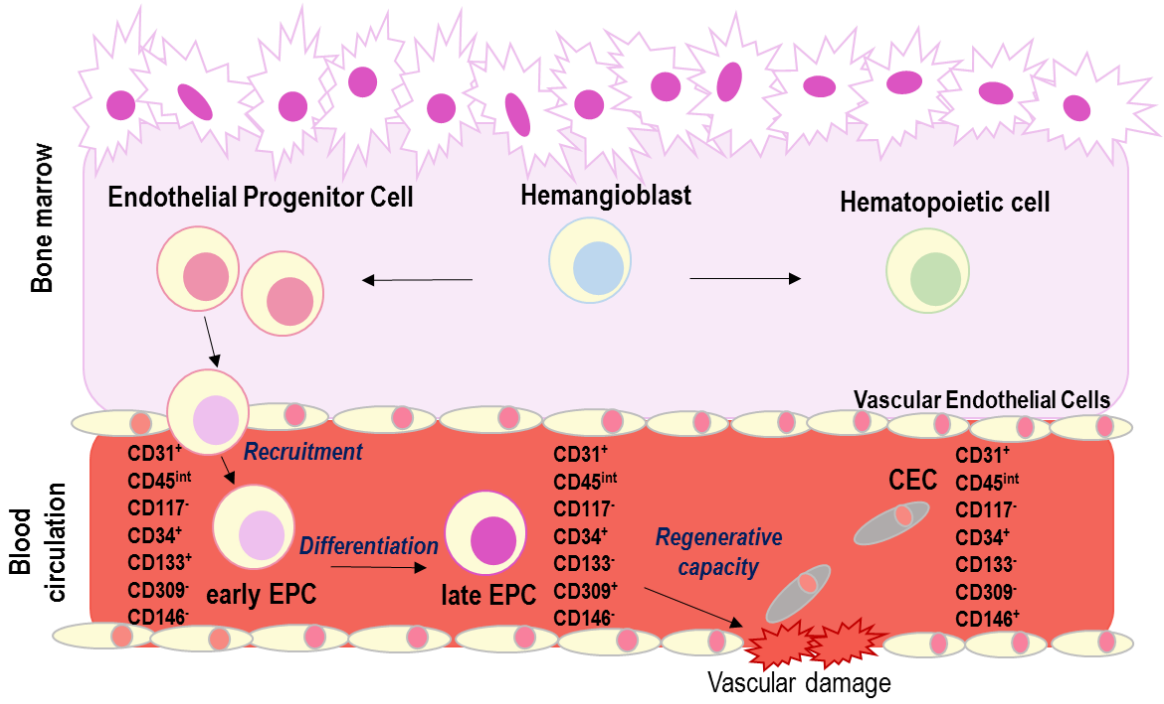

Figure 20 – Schematic representation of EPCs mobilization from bone marrow in cases of vascular damage, as well the phenotype of the different subpopulations identified (early EPCs, late EPCs, CEC).

The present thesis also aimed to evaluate the influence of kidney function in the circulating levels of both EPCs and CECs, in a CKD population. Despite EPCs and CECs have been already identified in other diseases, such as in patients with myocardial infarction (Delorme *et al.*, 2005),

venous thromboembolism and myeloproliferative neoplasms (Torres *et al.*, 2013) and colorectal cancer (Ramcharan *et al.*, 2013), the present work is the first to identify and distinguish EPCs and CECs in CKD patients.

In this study, is presented for the first time an assay that allows the distinction and quantification of different subpopulations of circulating progenitor cells. Globally, the results show a drastically reduction in EPCs levels accompanied by a slight increase in CECs levels in CKD patients compared with control subjects, according to the literature (Jourde-Chiche *et al.*, 2009). In healthy individuals there is a balance between EPCs and CECs levels reflecting a preservation of endothelium integrity and homeostasis. This balance is disrupted in CKD patients. The increased levels of CECs found in CKD patients are indicative of an endothelial dysfunction status characteristically observed in this population. On the other hand, the levels of early EPCs subpopulation were significantly reduced in advanced CKD group compared with controls, whereas the levels of late EPCs subpopulation were significantly reduced in both advanced and early CKD patients in comparison with control group. These results were accompanied by a dramatically reduction in the recruitment, differentiation and regenerative capacity indexes in CKD population, indicating a disruption in endothelial repairment process in these patients.

Our findings suggest that in CKD patients the differentiation step is more impaired than the recruitment step (see Fig.21), once that the levels of early EPCs subpopulation were higher than late EPCs. Excessive oxidative stress, which is known to be related to increased cardiovascular risk in CKD associated with injured vascular endothelium, may inhibit the differentiation of early EPC into late EPC, contributing to the compromised reparative mechanisms in this population (Toshio Imanishi, 2003). EPCs represent a promising therapeutic approach that may contribute to treatment of cardiovascular disease in CKD patients. Early intervention and an improvement of the EPCs differentiation step may reduce cardiovascular morbidity in CKD patients through increased physiological vascular.

Early CKD patients presented higher levels of CECs in circulation than advanced CKD group, which was not accompanied by a significant reduction in early EPCs levels, suggesting a compensatory recruitment of this subpopulation of cells. Several studies have reported, in these patients an increasing of VEGF production associated with endothelial dysfunction status that mediates not only the proliferation of endothelial cells but may also stimulate the recruitment of EPCs from bone marrow (Zampetaki *et al.*, 2008).

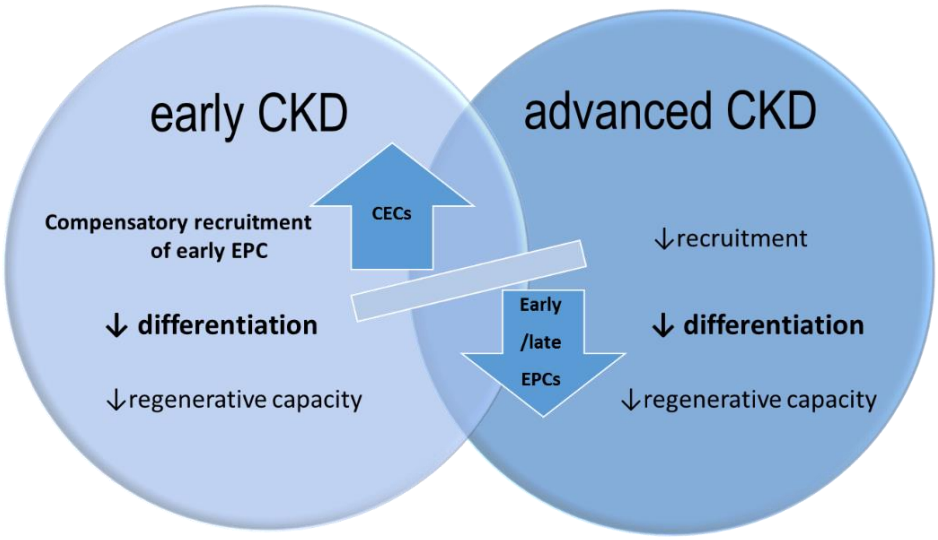

Figure 21 – Representation of the differences between early and advanced CKD in EPCs mobilization steps (recruitment, differentiation and regenerative capacity).

In the present work are presented some strategies described in the literature in order to compare with the results obtained by our eight-color staining flow cytometry optimized protocol. The strategies were based only in the expression of one, two or three surface markers that may be not enough to accurate identify EPCs, and could also causes false positive values, making it difficult to compare data. Despite the different combinations of markers, all works of EPCs identification in CKD population have reported a reduction of EPCs numbers in patients with advanced CKD as compared with healthy subjects. To explain the results, some authors assumed that differentiation of EPCs is inhibited, whereas other authors suggested that adherence of EPCs is impaired in CKD (De Groot, K *et al*, 2004) (Krenning *et al.*, 2009).

In summary, multicolor flow cytometry can abruptly increase the discriminatory power of cell analysis, which is very important to identify rare events as EPCs. In that context, a fast, simultaneous analysis of EPCs and CECs may substantially contribute to a coherent, prognostic, and diagnostic definition to the monitoring of vascular homeostasis. Taking account the main goal, our optimized flow cytometric protocol can be effectively applied as the basis for a standardized, sensitive and current technique in clinical studies, namely in CKD population. Moreover, our data demonstrate that EPCs are numerically and functionally impaired in CKD patients. Therefore, the results presented here reinforce the use of EPCs and CECs as vascular biomarkers. Our results further reinforce the potential

value of the established indexes as a useful indicator of recruitment, differentiation and endothelial regenerative capacity. These indexes could help to select the patients to benefit from guiding intervention strategies to improve cardiovascular health by inducing vascular protection.

6. CONCLUSIONS

What is new?

- The development of an eight-color staining flow cytometry optimized protocol to accurately and simultaneously identify EPCs, CECs and hematopoietic progenitor cells;
- The distinction of two subpopulations of EPCs (early and late EPCs) in the same sample;
- The establishment of three indexes indicatives of recruitment, differentiation, and regenerative capacity of these cells.

And now?

- Increase the number of patients in different stages of CKD as well as control subjects.
- Develop a personalizing analysis appropriate to each patient, once that it was verify physiological differences between patients;
- Using *in vitro* colony-forming assays to confirm the phenotype of the subpopulations of circulating cells that were identified by flow cytometry technique.

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8. ANNEXES

Annex 1: Different strategies used until strategy optimization

- **Strategy 1**

In the strategy 1 were used all antibodies. After excluding debris, dead cells, doublets and triplets, we gated on $CD117^{+}CD34^{+}$, and within this gate we selected the $CD31^{-}CD45^{-}$ cells, and within this population we checked for $CD133^{+}CD309^{+}$ cells and CD146-cells for EPCs identification (Fig. 1.I). For CECs identification, the gating hierarchy was: $CD117^{-}CD34^{-}$ > $CD31^{+}CD45^{-}$ > $CD133^{-}CD309^{+}$ > $CD146^{+}$ cells selection (Fig. 1.II).

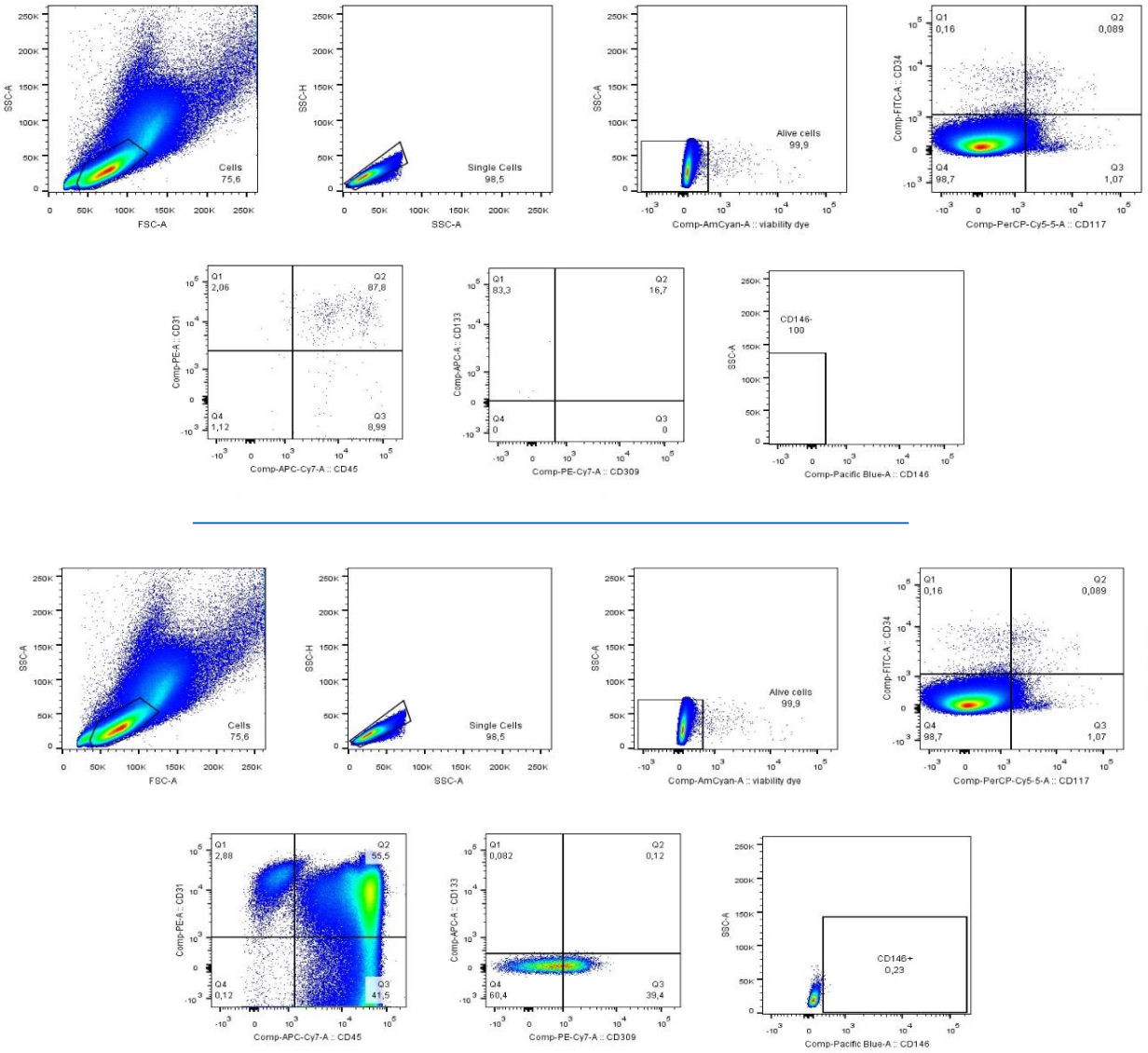

Figure 1 – Representation of first strategy of analysis. **I** – Enumeration of EPCs was performed by $CD117^{+}CD34^{+}$ selection, $CD31^{-}CD45^{-}$ selection, $CD133^{+}CD309^{+}$ selection, and finally selection of $CD146^{-}$cells. **II** – Enumeration of ECs was performed by CD117-CD34- selection, CD31+CD45- selection, CD133-CD309+ selection, and CD146+ cells selection.

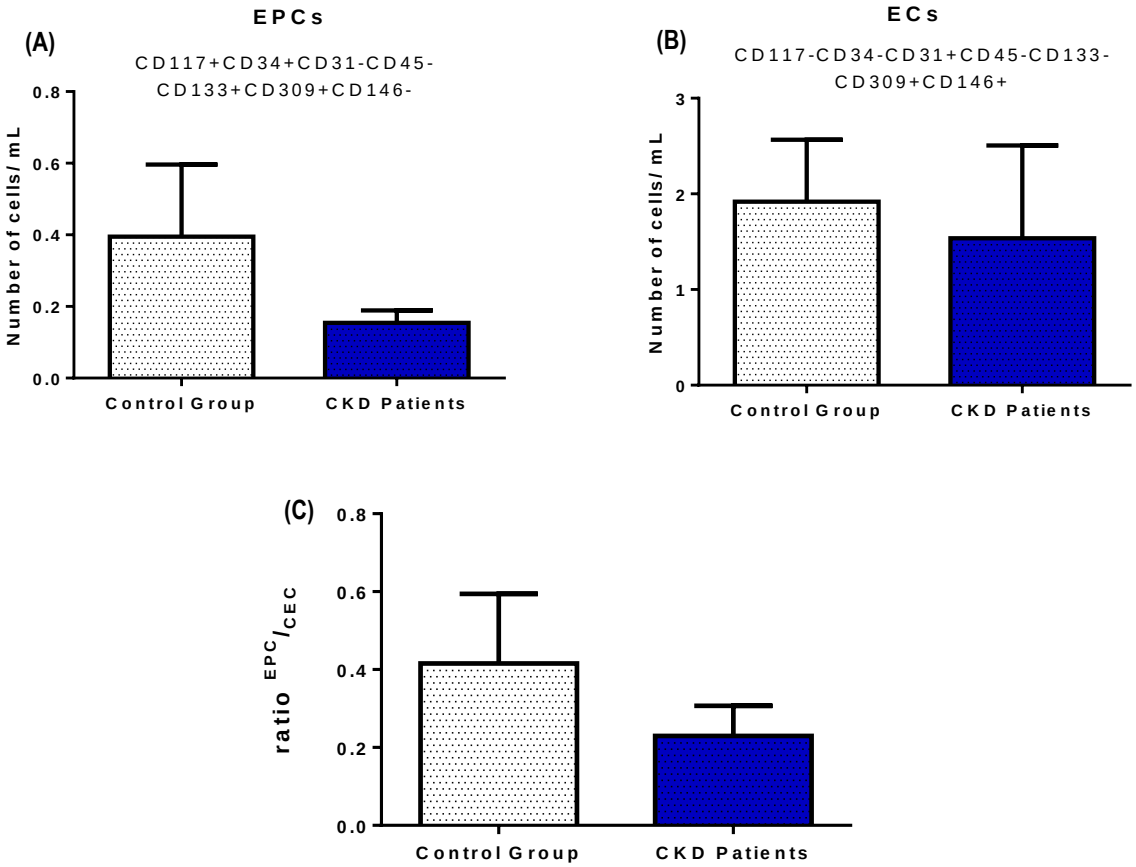

Figure 2 – Levels of EPCs (A), ECs (B) and of ratio EPC/CEC (C) among CKD patients (n=5) and control subjects (n=5).

The results obtained with this strategy shows that EPCs are lower in CKD patients (0.15±0.02 cell/mL) than in control group (0.39±0.14 cell/mL), (Fig.2.A). The absolute number of ECs was lower in CKD patients group (1.53±0.68 cell/mL) than in control group (1.92±0.55 cell/mL), p=0.75 (Fig.2.B). The index of regenerative capacity (Fig.2.C) was lower in CKD patients (0.23±0.06 cell/mL) than in control group (0,42 ± 0,13), p=0.37.

- **Strategy 2**

For EPCs identification were selected the $CD45^{-}$ and intermediate (int) cells and $CD31^{-/+}$ cells, then were selected $CD34^{+}CD133^{+}$, $CD309^{+}CD146^{-}$, and finally were selected $CD117^{+}$ cells (Fig.3.I). For ECs identification were selected the same cells $CD45^{-/int}$ $CD31^{-/+}$, $CD34^{-}CD133^{-}$, $CD309^{+}CD146^{+}$ and finally were selected $CD117^{-}$ cells (Fig.3.II).

I

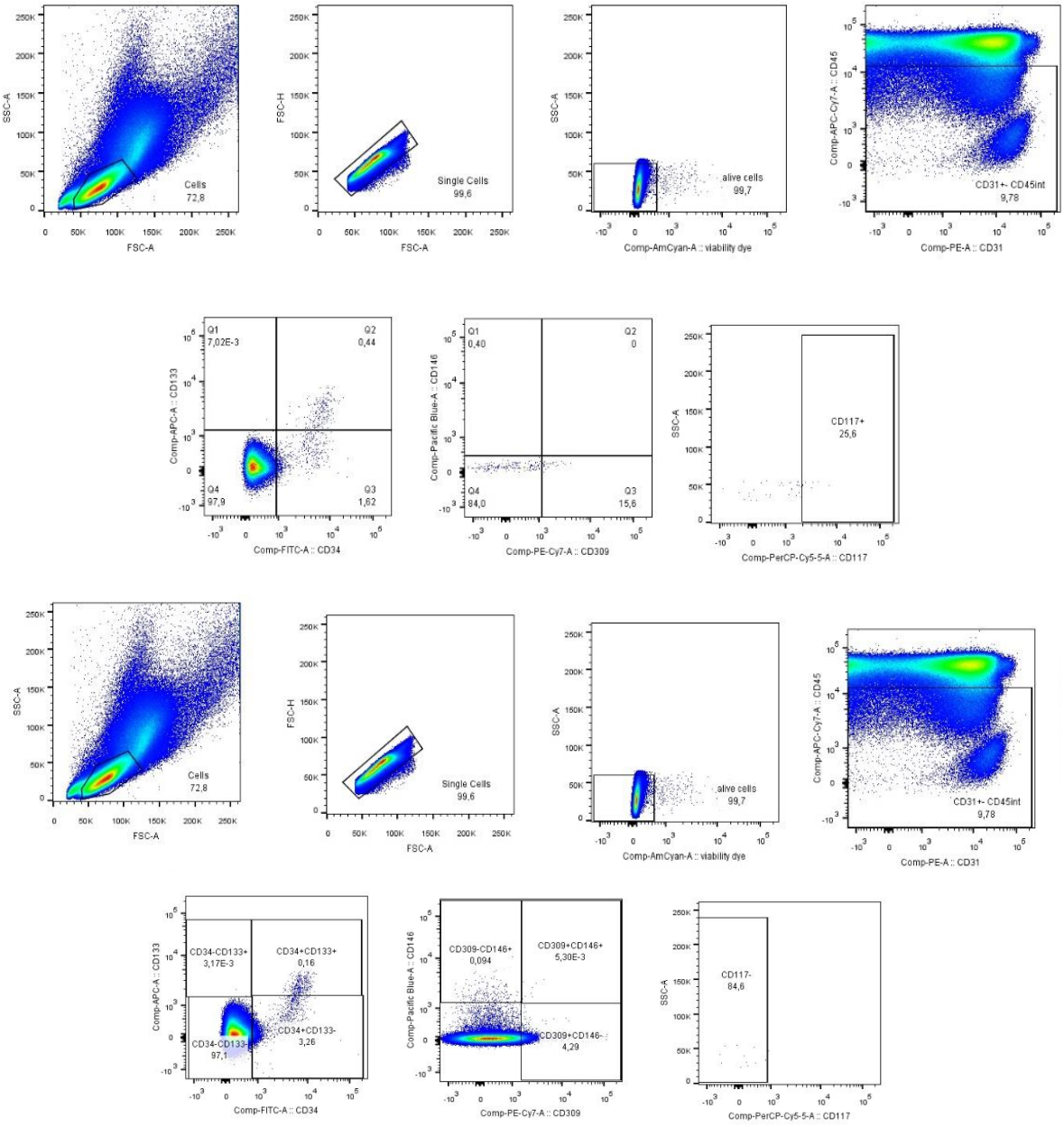

Figure 3 – Representation of Strategy 2 of analysis. **I** - EPCs were identified by $CD45^{-/int}$ $CD31^{-/+}$ cells, $CD34^{+}CD133^{+}$, $CD309^{+}CD146^{-}$, and CD117+ cells. **II** – ECs were identified by $CD45^{-/int}$ $CD31^{-/+}$ cells, $CD34^{-}CD133^{-}$, $CD309^{+}CD146^{+}$ and $CD117^{-}$ cells.

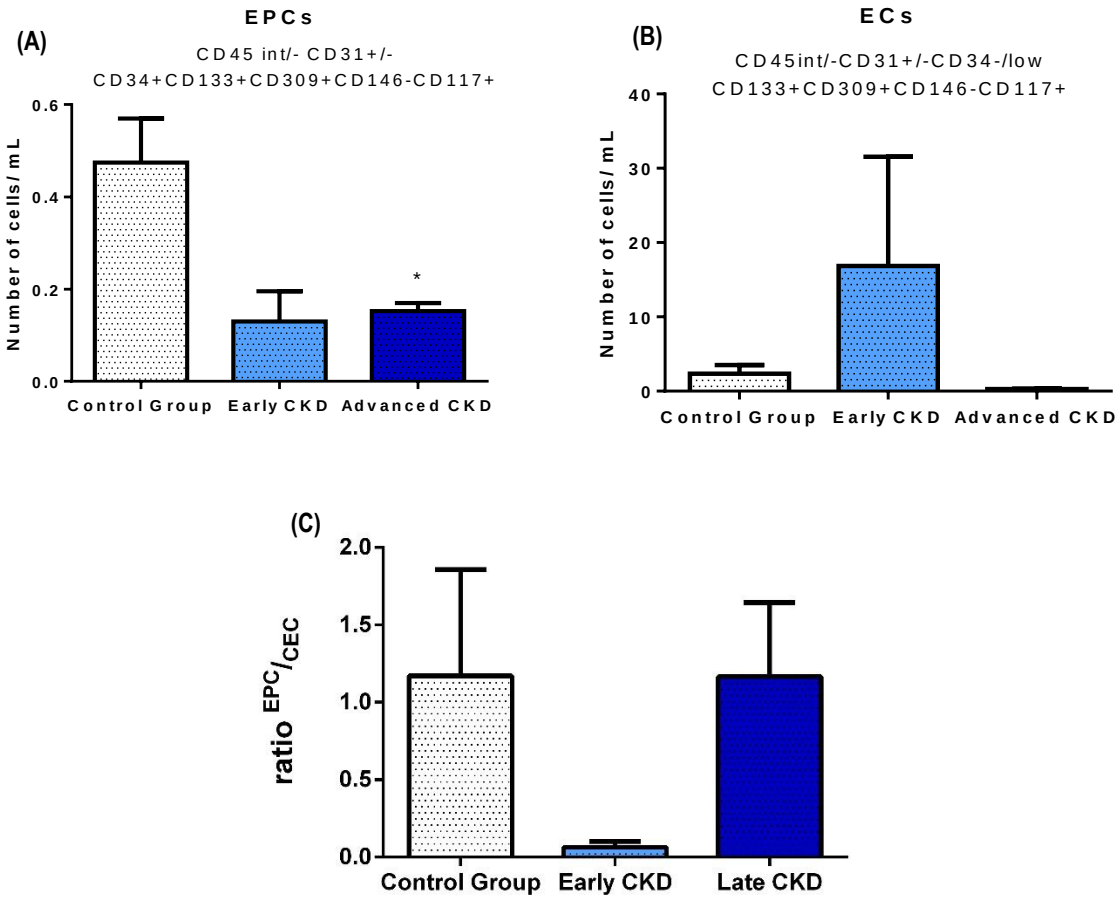

Figure 4 – Levels of EPCs (A), ECs (B) and of Ratio EPC/CEC (C) among early CKD (n=3) and late CKD (n=5) patients and control subjects (n=7). *P<0.05 compared with control group. †P<0.05 compared with early CKD group.

The results obtained with this strategy shows that EPCs are significantly lower in advanced CKD (0.25±0.07 cell/mL) compared with control group (0.47±0.08 cell/mL), p=0.035. The levels of EPCs were also lower in early CKD (0.13±0.05 cell/mL) compared with control group, although it is not significant (p=0.06) (Fig.4.A). The levels of ECs were lower in Advanced CKD (0.27±0.06 cell/mL) in comparison with early CKD (16.86±11.31 cell/mL) (p=0.17), and in control group (2.35±0.92 cell/mL) (p=0.16) (Fig.4.B) The index of regenerative capacity was significantly higher in advanced CKD (1.16±0.33 cell/mL) than in early CKD patients (0.06±0.02 cell/mL) (p=0.13) but was lower than control group (1.17±0.50 cell/mL) (p=0.99) (Fig.4.C).

- **Strategy 3**

For early EPCs identification were selected CD45$^{-/int}$CD31$^{-/+}$, CD117^{-}CD34$^{-/+}$, CD34^{+}CD133^{+} and CD309^{-}CD146^{-} (Fig.5.II). For late EPCs identification were selected CD45$^{-/int}$CD31$^{-/+}$, CD117^{-}CD34$^{-/+}$, CD34^{+}CD133^{-} and CD309^{+}CD146^{-} (Fig.5.III). For ECs identification were selected CD45$^{-/int}$CD31$^{-/+}$, CD117^{-}CD34$^{-/+}$, CD34^{-}CD133^{-} and CD309^{+}CD146^{+} (Fig.5.IV). Taking into account that Hematopoietic progenitor cells express CD34 and CD117 markers in their surface, this subpopulation is identified from CD45$^{-/int}$CD31$^{-/+}$ gate (Fig. 5.I).

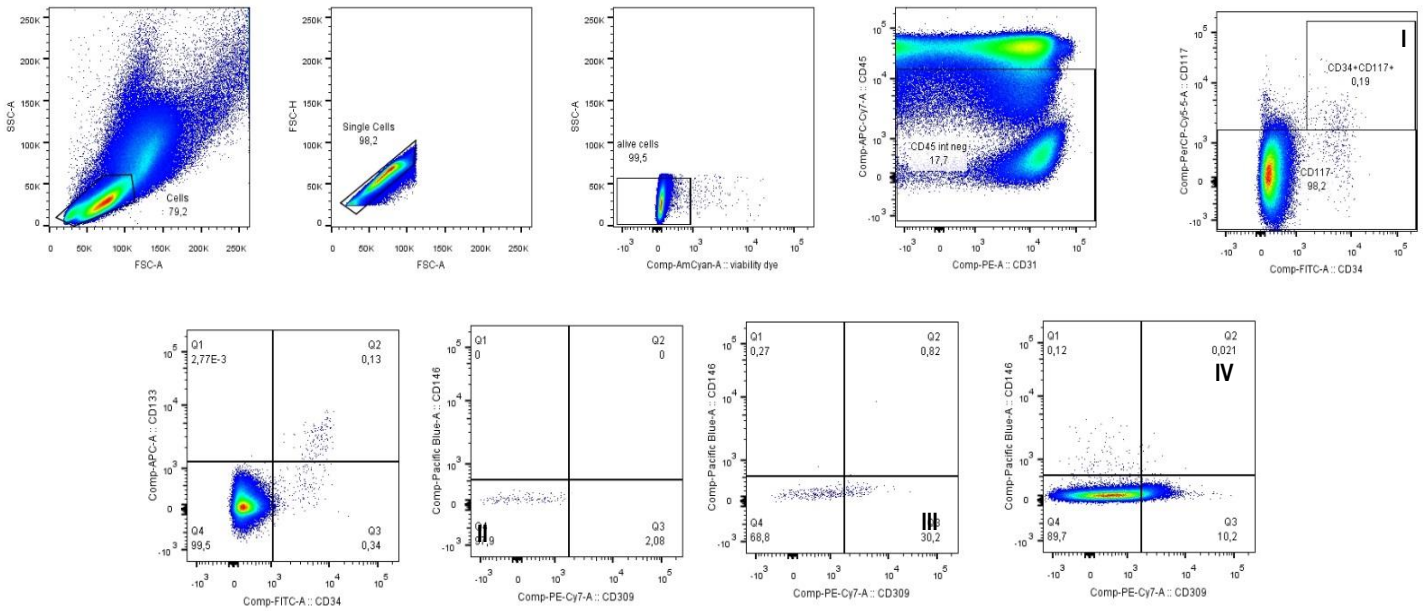

Figure 5 – Representation of Strategy 3 of analysis. **I** – Hematopoietic progenitor cells were identified by CD45$^{-/int}$ CD31$^{-/+}$ cells, CD34^{+}CD117+. II- Early EPCs were identified by CD45$^{-/int}$ CD31$^{-/+}$ cells, CD34-/+ CD117-, CD34+CD133+, CD309^{-}CD146^{-}. **III** – Late EPCs were identified by CD45$^{-/int}$ CD31$^{-/+}$ cells, CD34$^{-/+}$CD117^{-}, CD34^{+}CD133^{-}, CD309^{+}CD146^{-} cells. **IV** – ECs were identified by CD45$^{-/int}$ CD31$^{-/+}$ cells, CD34$^{-/+}$CD117^{-}, CD34^{-}CD133^{-}, CD309^{+}CD146^{+}.

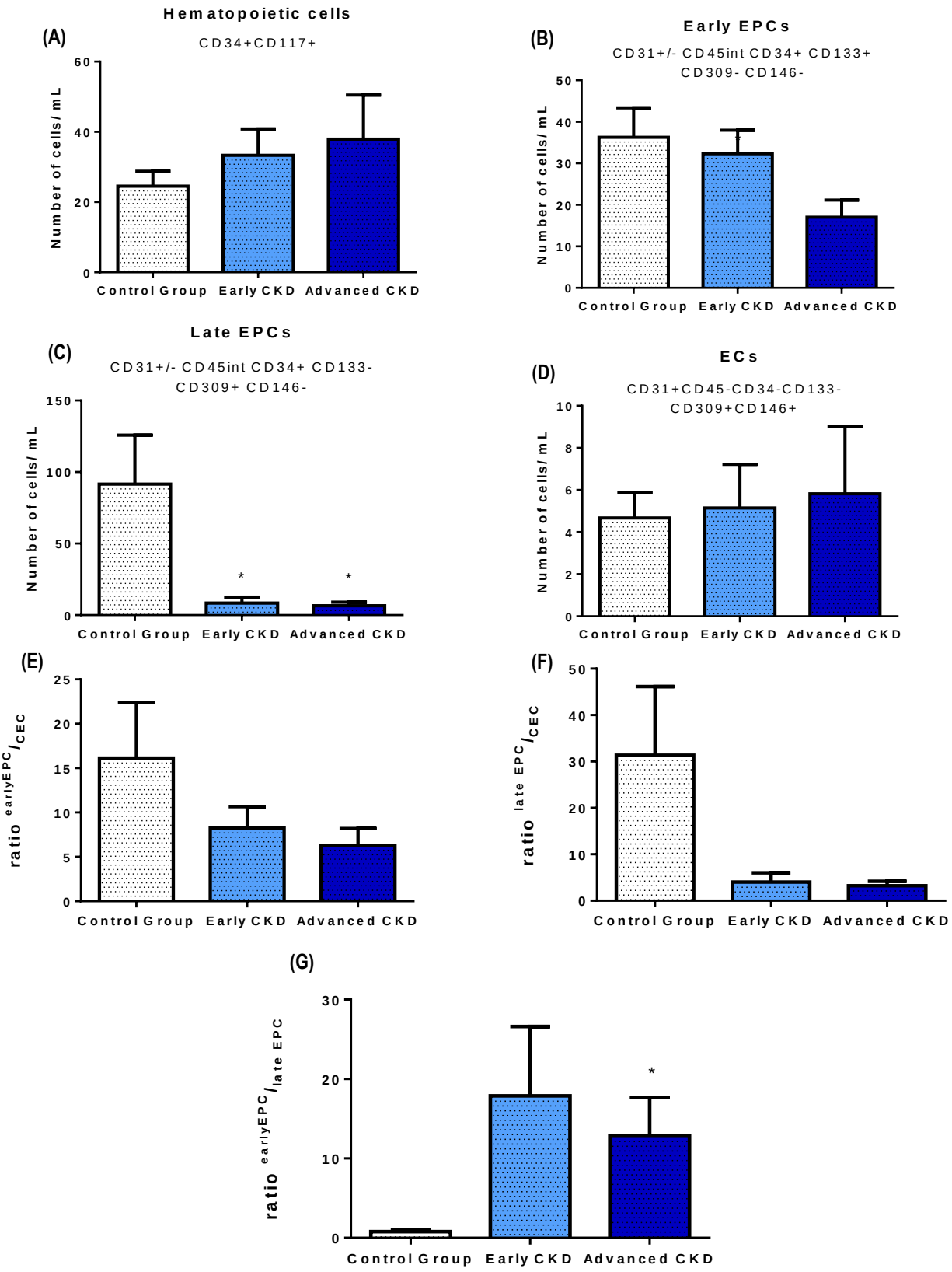

Figure 6 – Levels of Hematopoietic progenitor cells (A), early (B) and late EPCs (C), ECs (D), Ratio early EPCs/CECs (E), Ratio late EPCs/CECs (F) and Ratio early EPCs/late EPCs (G), among early CKD (n=10) and Advanced CKD (n=10) patients and control group (n=10). *P<0.05 compared with control group. †P<0.05 compared with Early CKD group.

The results shows that the levels of hematopoietic progenitor cells were higher in advanced CKD (37.94 ± 8.73 cell/mL) and in early CKD (33.32 ± 6.39 cell/mL) than in control group (24.52 ± 3.28 cell/mL) (Fig.6.A). The levels of early EPCs were significantly lower in advanced CKD (17.03±3.23) compared with early CKD (32.31±4.97 cell/mL), p=0.04, and Control group (36.25±6.16 cell/mL), p=0.03 (Fig.6.B). The numbers of late EPCs were significantly lower in advanced CKD (6.60±1.89 cell/mL), p=0.01, and in early CKD (8.42±2.58 cell/mL), p=0.01, than control group (91.54±29.06 cell/mL) (Fig.6.C). The numbers of ECs were higher in advanced CKD (5.82±1.99 cell/mL) than early CKD (4.65±1.34 cell/mL) and control group (4.67± 0.94 cell/mL) (Fig.6.D).

The ratio among early EPCs and ECs was decreased in advanced CKD (6.82 ± 1.49 cell/mL) and early CKD (9.06 ± 2.42 cell/mL) in comparison with control group (13.20 ± 3.56 cell/mL) (Fig.6.E) and the ratio among late EPCs and ECs was also decreased in advanced CKD (2.02±0.66 cell/mL) and in early CKD (2.68±1.15 cell/mL) in comparison with control group (33.23 cell/mL) (Fig.6.F). In turn, the ratio early EPCs/late EPCs was significantly increased in advanced CKD (12.82 ± 3.94 cell/mL) in comparison with control group (0.78 ± 0.16 cell/mL), the levels of early CKD (17.88± 5.82 cell/mL) were also increased in comparison with Control group, but it is not significant (Fig.6.G).

Annex 2: Informed consent process and study protocols approved by the ethics committee of "Centro Hospitalar de São João, EPE".

Direcção Clínica
10/3/15 CES 251.14

AUTORIZADO
CONSELHO DE ADMINISTRAÇÃO EM REUNIÃO DE 20 MAR 2015

Exmo. Senhor
Presidente do Conselho de Administração do
Centro Hospitalar de S. João – EPE

Assunto: Pedido de autorização para realização de estudo/projecto de investigação

Nome do Investigador Principal: Ana Alexandra Briga Cerqueira

Título do projecto de investigação: "Pesquisa de novos biomarcadores de disfunção endotelial com importância clinica na doença renal crónica"

Pretendendo realizar no(s) Serviço(s) de Nefrologia do Centro Hospitalar de S. João – EPE o estudo/projecto de investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efectivação.

Para o efeito, anexa toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de S. João respeitante a estudos/projectos de investigação, à qual endereçou pedido de apreciação e parecer.

Com os melhores cumprimentos.

Porto, 13 / Outubro / 2014

O INVESTIGADOR/PROMOTOR

Comissão de Ética para a Saúde do Centro Hospitalar de S. João – EPE
Modelo CES 01

Comissão de Ética para a Saúde – Centro Hospitalar São João / FMUP

Parecer

Título do Projecto: Pesquisa de novos biomarcadores de disfunção endotelial com importância clínica na doença renal crónica.

Nome do Investigador Principal: Dra. Ana Alexandra Briga Cerqueira

Local onde sera realizado o estudo: Serviço de Nefrologia, – CHSJ, havendo autorização do respectivo Diretor de Serviço para a realização do mesmo.

Objectivo do estudo: Pesquisa de novos biomarcadores de disfunção endotelial, bioquímicos e celulares, associados ao aumento do risco cardiovascular na doença renal crónica. O principal objectivo é identificar possíveis novos marcadores de estratificação de risco cardiovascular com valor preditivo nesta população. Pretende, também, avaliar a influência de uma dieta de restrição de sódio e fósforo na regeneração endotelial avaliada pelos níveis das células progenitoras endoteliais circulantes nestes doentes.

Período previsto de conclusão: 2017

Benefícios: N/A

Risco: Estão referidos a duração do exame (aproximadamente 20 min), e a colheita de uma amostra de sangue e urina.

Respeito pela liberdade e autonomia do sujeito do ensaio: Prevê-se a obtenção do consentimento informado, complementado por um suporte de informação escrita para os participantes, que refere os objectivos do estudo, os riscos/benefícios, bem como a liberdade em participar.

Confidencialidade dos dados: está garantida a confidencialidade dos dados e esta informação será restrita aos investigadores.

A Investigadora Principal dispõe de competência técnica e científica para a realização do estudo.

O estudo não prevê a realização de questionários.

Custos: O estudo não prevê custos acrescidos para os participantes nem para a instituição. O estudo será financiado pela Sociedade Portuguesa de Nefrologia.

Parecer: Em face da análise do protocolo de estudo, proponho a sua aprovação pela CES do CHSJ.

Porto, CHSJ, 24 de Novembro de 2014

O Relator

Dr. John Preto

7. SEGURO

a. *Este estudo/projecto de investigação prevê intervenção clínica que implique a existência de um seguro para os participantes?*

SIM ☐ (Se sim, junte, por favor, cópia da Apólice de Seguro respectiva)

NÃO ☑

NÃO APLICÁVEL ☐

8. TERMO DE RESPONSABILIDADE

Eu, Ana Alexandra Briga Cerqueira,

abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsinquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 13 / Outubro / 2014

Ana Cerqueira

O Investigador Principal

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO

emitido na reunião plenária da CES de 25 / Novembro / 2014

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

Annex 3: Scientific Production

- Poster presentation in *Congress I3S 4th Annual Meeting 2014*, realized in "Hotel AXIS VERMAR" on 30-31 October 2014.

Evaluation of circulating Endothelial Progenitors Cells by multicolor flow cytometry as a prognostic and predictive microvasculature regeneration biomarker in patients with Chronic Kidney Disease

S. Martins [1,2,3], C. Leitão [4], H. Oliveira [4], A. Cerqueira [1,2,3,4], M. Pestana [1,2,3,5], J. Quelhas-Santos [1,2]

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Introduction

Endothelial progenitor cells (EPC) are small immature precursor cells, detectable in plasma and bone marrow, with a physiologic role in the vascular and tissue regeneration. To date, EPC numbers have been correlated with the numbers of cardiovascular risk factors, extent of coronary disease, and future cardiovascular events. The absolute number, or functional capacity of EPCs, was suggested to be reduced in several disease states including chronic kidney disease (CKD). EPCs may be a potential mechanism by which vascular integrity is compromised, increasing cardiovascular disease risk and contributing to renal disease progression in CKD.

Circulating EPCs play important roles in accelerating endothelialization at areas of vascular damage (Fig. 1), and EPC enumeration is a viable strategy for assessing reparative capacity. However, there is no standardized methodology of EPCs analysis or consensus on their phenotype. Moreover, published data on this subject is often conflicting and obtained from heterogeneous studies. Based on the previous considerations, we hypothesized that the assessment of EPCs levels could represent an important prognostic and predictive biomarker of endothelial dysfunction in CKD population.

Figure 1 – Representation of endothelial senescence (I) and repair of vascular damage by bone marrow-derived progenitor cells (EPCs).

Objective

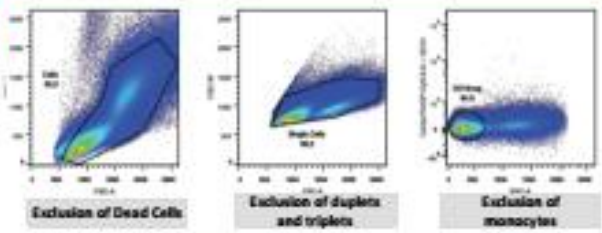
Because there are no specific markers for EPCs, the main objective of this study is the establishment of a standardized protocol to EPCs identification in peripheral blood using multicolor flow cytometry technique.

Materials and Methods

Figure 2 – Representation of the methodological steps.

A 26-ml sample of venous blood will be used for the isolation of endothelial cells (ECs) and endothelial progenitor cells (EPCs), identified by flow cytometry (BD FACSCanto™ II system, BD Biosciences) using a combination of fluorochrome-conjugated primary antibodies for their specific cell surface markers: CD31, CD34, CD45, CD133, CD117, CD146, and CD309. Due to the rare nature of EC and EPCs, a large number of events will be collected, at least 500,000 to 1 million. Exclusion of dead cells will be done according to a fixable viability dye staining. CD34 is a cell surface markers express in hematopoietic stem cells and both ECs and EPCs. Identification of ECs will be done by double CD31+/CD45-/CD146+ labeling. Identification of EPCs will be performed by CD34+/CD45-/CD117+/CD133+/CD309+ multiple labeling.

Preliminary Results



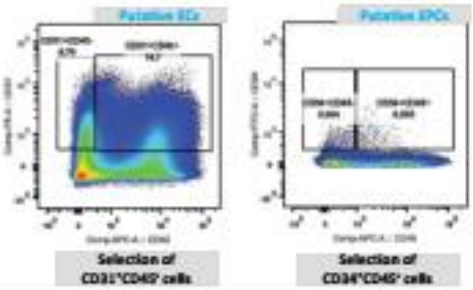


Figure 3 - Analysis data by Flow Jo 10 software.

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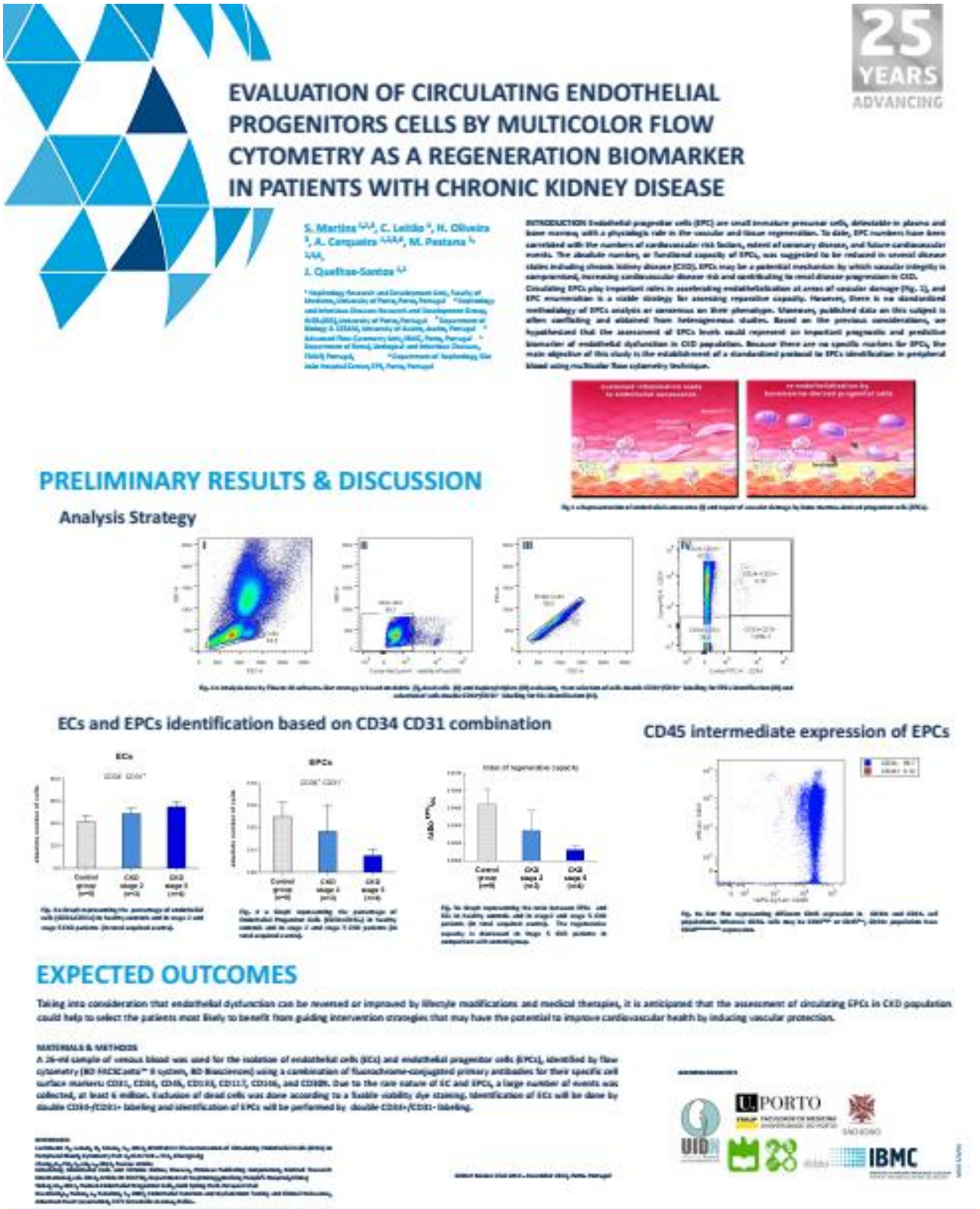
Deanfield,J., Halcox, J., Rabelink, T., 2007, Endothelial Function and Dysfunction: Testing and Clinical Relevance, American Heart Association, 7272 Greenville Avenue, Dallas.

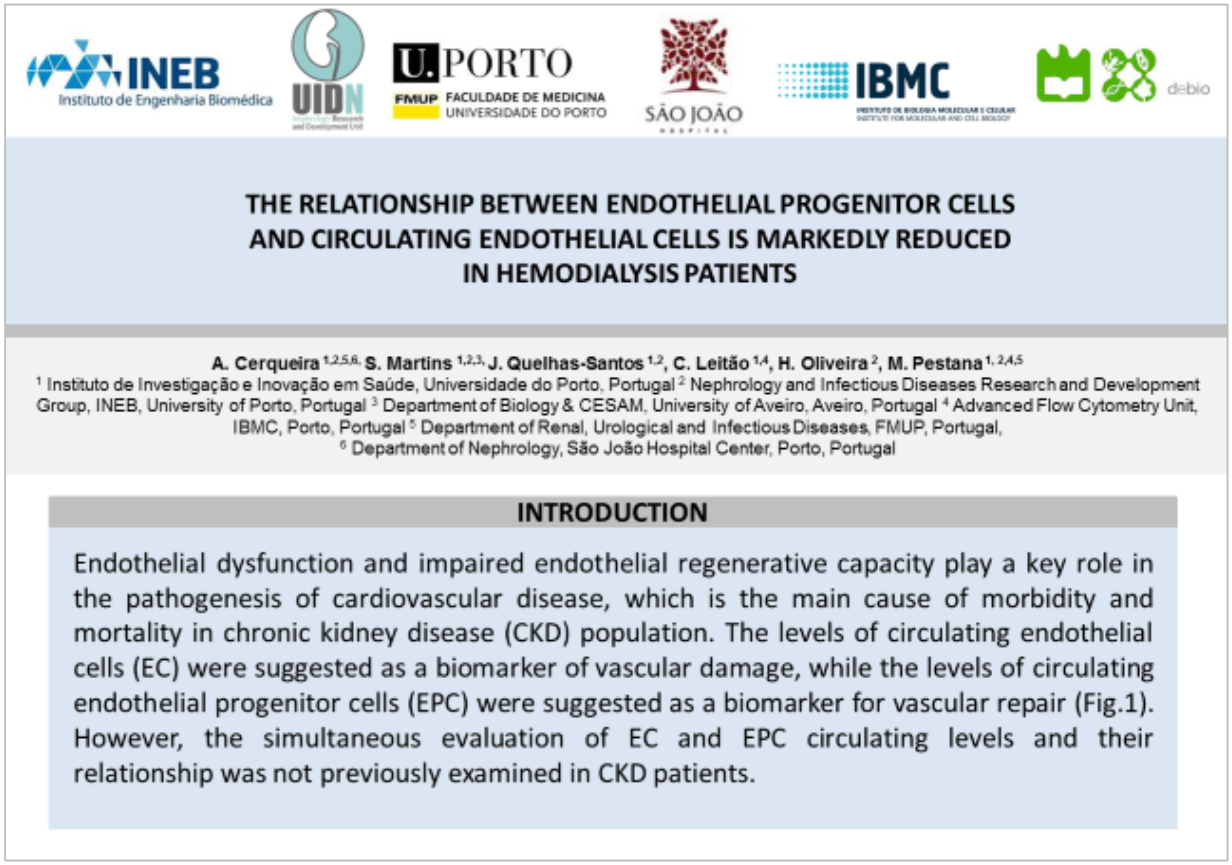
Expected Outcomes

Taking into consideration that endothelial dysfunction can be reversed or improved by lifestyle modifications and medical therapies, it is anticipated that the assessment of circulating EPCs in CKD population could help to select the patients most likely to benefit from guiding intervention strategies that may have the potential to improve cardiovascular health by inducing vascular protection.

- Poster presentation in symposium *Winter Science Club 2014*, realized in "Biblioteca Almeida Garret" on 19 December 2014.



- ePoster presentation in *Encontro Renal 2015 XXIX Congresso Português de Nefrologia, XXIX Congresso APEDT, VII Congresso Luso-Brasileiro de Nefrologia,* realized in Vilamoura on 15-18 de Março.



THE RELATIONSHIP BETWEEN ENDOTHELIAL PROGENITOR CELLS AND CIRCULATING ENDOTHELIAL CELLS IS MARKEDLY REDUCED IN HEMODIALYSIS PATIENTS

A. Cerqueira [1,2,5,6], S. Martins [1,2,3], J. Quelhas-Santos [1,2], C. Leitão [1,4], H. Oliveira [2], M. Pestana [1, 2,4,5]
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INTRODUCTION

Endothelial dysfunction and impaired endothelial regenerative capacity play a key role in the pathogenesis of cardiovascular disease, which is the main cause of morbidity and mortality in chronic kidney disease (CKD) population. The levels of circulating endothelial cells (EC) were suggested as a biomarker of vascular damage, while the levels of circulating endothelial progenitor cells (EPC) were suggested as a biomarker for vascular repair (Fig.1). However, the simultaneous evaluation of EC and EPC circulating levels and their relationship was not previously examined in CKD patients.

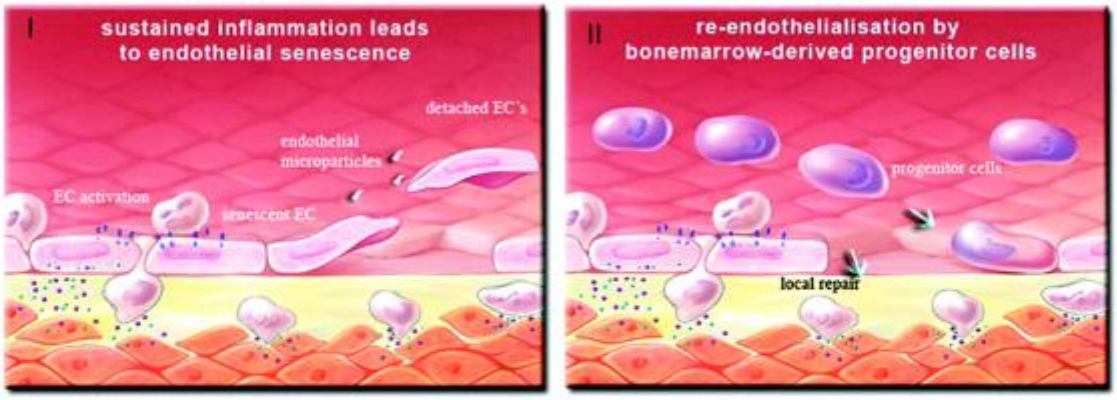

Figure 1 - Representation of endothelial cells senescence (I) and repair of vascular damage by bone marrow-derived progenitor cells (EPCs).

METHODS

Here we present the preliminary findings of circulating levels of both ECs and EPCs in blood samples from ESRD patients on hemodialysis (n=5) and healthy subjects (n=5), assessed by flow cytometry (BD FACSCanto™ II system) using a combination of fluorochrome-conjugated primary antibodies for their specific cell surface markers: CD45, CD133, CD146, and CD309. Due to the rare nature of ECs and EPCs, a large number of events was collected, at least 500,000. Exclusion of dead cells was done according to a fixable viability dye staining. Identification of ECs and EPCs was performed by $CD45^{-}/CD309^{-}/CD133^{-}/CD146^{+}$ and $CD45^{low}/CD309^{+}/CD133^{+}/CD146$ multiple labeling, respectively (Fig. 2).

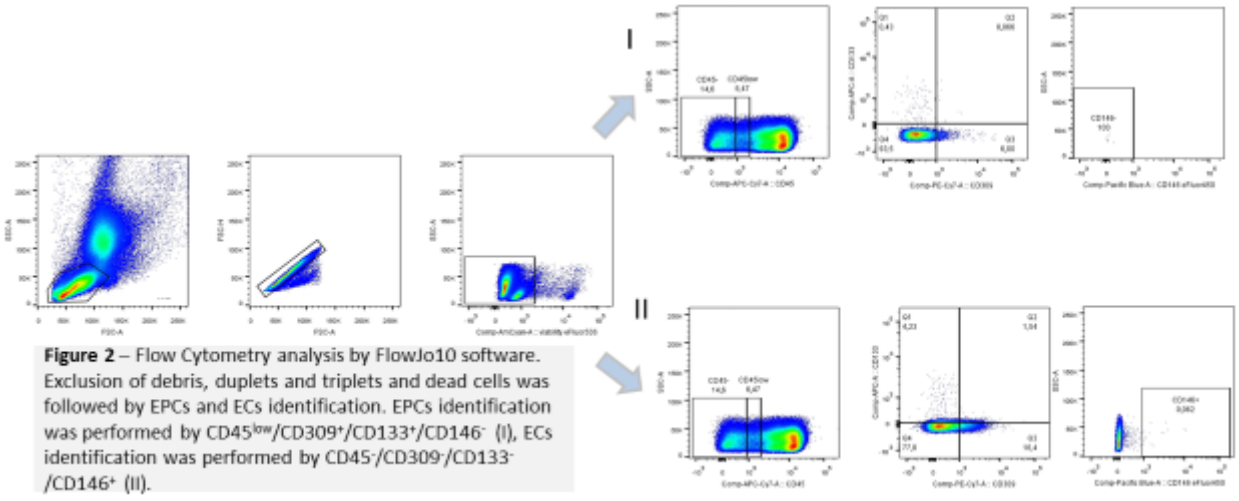

Figure 2 – Flow Cytometry analysis by FlowJo10 software. Exclusion of debris, duplets and triplets and dead cells was followed by EPCs and ECs identification. EPCs identification was performed by $CD45^{low}/CD309^{+}/CD133^{+}/CD146^{-}$ (I), ECs identification was performed by $CD45^{-}/CD309^{-}/CD133^{-}/CD146^{+}$ (II).

RESULTS

The absolute number of circulating ECs (CD45-/CD309-/CD133-/CD146+) in ESRD group was significantly increased in comparison with control group (2.50±0.25 vs 0.51±0.17, p<0.01) (Fig.3). In addition, the absolute number of circulating EPCs (CD45low/CD309+/CD133+/CD146+) was non-significantly reduced in ESRD population (0.03±0.01 vs 0.05±0.01, ns) (Fig.4).

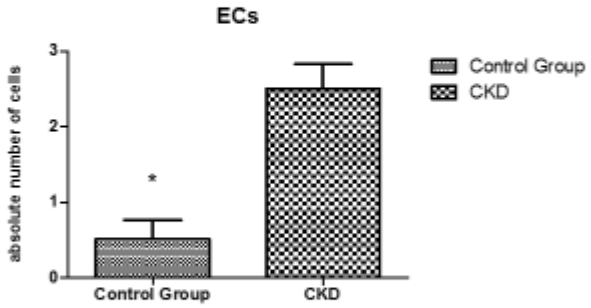

Figure 3 – Graph representing the percentage of endothelial cells (CD45-CD309-CD133-CD146+) in healthy controls and in CKD patients (in total acquired events).

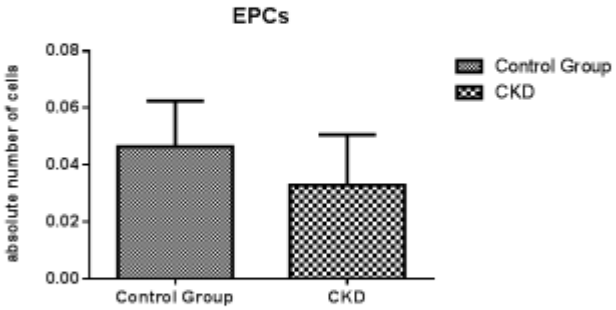

Figure 4 – Graph representing the percentage of Endothelial Progenitor Cells (CD45lowCD309+CD133+CD146+) in healthy controls and in stage 2 and stage 5 CKD patients (in total acquired events).

RESULTS

Moreover, the ratio EPCs/ECs was markedly reduced in ESRD group (0.01±0.01 vs 0.48±0.24, p<0.05).

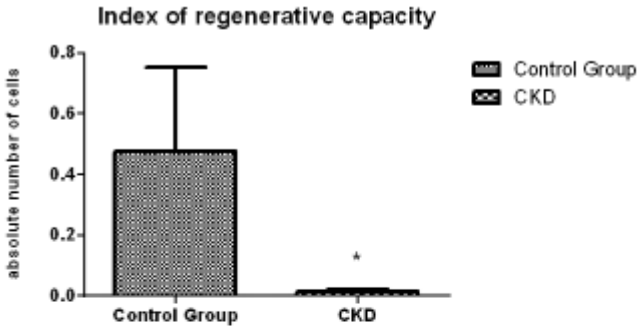

Fig. 5– Graph representing the ratio between EPCs and ECs in healthy controls and in CKD patients (in total acquired events). The regenerative capacity is decreased in CKD patients in comparison with control group.

DISCUSSION

Taken together, our results suggested that the EPCs/ECs ratio may represent a better tool to evaluate the balance between endothelial damage and repair than the levels of ECs and EPCs alone.

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- Poster presentation in. 52nd *ERA-EDTA Congress*, realized in London on 28-31 May.

RENALASE AND OTHER BIOMARKERS CARDIOVASCULAR RISK IN CHRONIC KIDNEY DISEASE

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OBJECTIVES

Cardiovascular (CV) events are the leading cause of death in patients with chronic kidney disease (CKD). Endothelial dysfunction (ED) is a predictor of CV events and functional assessment of the endothelium can identify early endothelial damage and predict CV risk. Renalase is a newly-described circulating oxidase endowed with hypotensive and cardioprotective effects, which is mainly expressed in kidney but also in heart and endothelium. The aim of this study was to assess associations between renalase plasma levels, renal function, biomarkers of CV risk and endothelial dysfunction in 64 pre-dialysis CKD patients, stages 1 to 5, followed up in our outpatient clinic.

METHODS

We examined 64 (35F:29M) CKD patients followed in our outpatient clinic, with mean age of 60 ±2 years, and mean eGFR: 49.7±4 ml/min/1,73m2. The most frequent cause of CKD (15.6%) was diabetic nephropathy and mean Charlson index was 4.7±0.4.

Laboratory data: → assessed using standard laboratory methods

Renal function (eGFR),
Phosphate (Pi),
Parathormone (iPTH)
Natriuretic peptide type B (BNP),

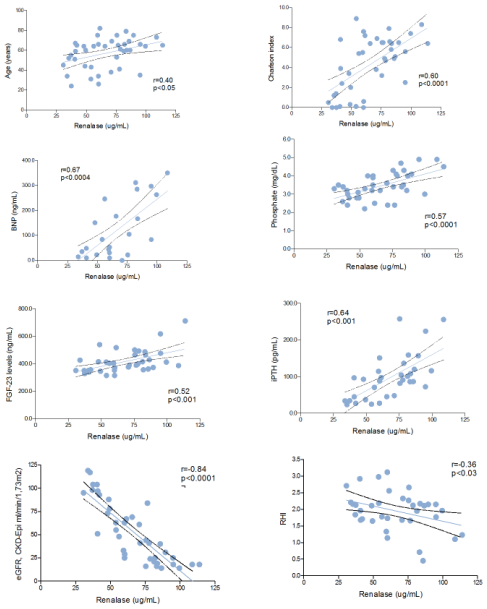
Intact FGF-23 blood levels were measured by ELISA from Immutopics,Inc.,
Plasma renalase levels were assessed using a commercially **ELISA kit from Uscn Life Science Inc.,**

Endothelial function (RHI) → **ENDO-PAT 2000**

RESULTS

	Early CKD (KDIGO G1-G3a) N=32	Advanced CKD (KDIGO G3b to G5) N=32	*p*
eGFR CKD_EPI (ml/min/1,73m²)	**74,79**	**24,87**	**$3x10^{-17}$**
Age	53,22	66,50	**0,001**
Charlson Comorbidity Index	**3,35**	**6,15**	**$1x10^{-5}$**
Body Mass Index (kg/m²)	28,41	27,72	0,62
Systolic BP (mmHg)	130,42	142,85	**0,03**
Diastolic BP (mmHg)	73,05	76,21	0,30
Hemoglobin (g/dL)	13,41	12,00	**0,005**
pCreat (mg/dL)	1,05	2,54	**$3x10^{-13}$**
pUrea (mg/dL)	55,16	113,23	**$2x10^{-10}$**
Sodium (mmol/L)	140,03	140,07	0,96
Potassium (mmol/L)	4,72	4,69	0,82
Bicarbonate (mmol/L)	28,56	26,05	**0,02**
Ratio Prot/Creat	640,54	1552,02	0,05
Total Cholesterol (mg/dL)	197,43	181,83	0,16
LDL (mg/dL)	116,81	104,46	0,17
Triglicerides (mg/dL)	138,45	206,28	0,30
HDL (mg/dL)	54,82	45,93	**0,03**
Uric acid (mg/dL)	6,07	7,46	**0,01**
C reactive protein (mg/L)	8,76	10,09	0,86
Sedimentation rate	28,17	47,59	**0,02**
Albumin (g/L)	37,99	40,73	0,29
Calcium (mEq/L)	4,98	4,84	0,51
Phosphorus (mg/dL)	3,18	3,88	**$2x10^{-5}$**
Vitamin D (ng/mL)	21,76	21,83	0,99
BNP (ng/mL)	72,15	216,03	**0,02**
Parathormone (pg/mL)	53,75	203,91	**0,003**
FGF-23 (ng/mL)	**392,19**	**443,97**	**0,04**
RHI	**2,25**	**1,72**	**0,004**
Renalase (ug/mL)	**49,19**	**83,18**	**$2x10^{-9}$**

In adjusted analysis the increase in renalase plasma levels was only significantly correlated with the decrease of eGFR (r=-0.31, p<0.05). This relationship proved to be independent of changes in age, Charlson Index, RHI, FGF-23, serum Pi and iPTH.



CONCLUSIONS

- The increase in plasma renalase levels observed in CKD patients is primarily dependent on renal function.
- We are at the very beginning to understand whether renalase is a protective factor of hypertension in kidney disease or just an innocent bystander.
- It remains to be established whether or not renalase plasma levels are associated with increased of CV risk in CKD.
- More research is needed to establish whether renalase can become a useful therapeutic target.

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