ALMA MATER STUDIORUM

UNIVERSITA' DI BOLOGNA

Dottorato di Ricerca

Metodologie di ricerca nelle malattie vascolari e toraciche

XX ciclo

BIO/10

Peripheral Arterial Disease and Diabetes: Effects on Endothelial Progenitor Cell Differentiation and NO Metabolism

Presentata da:

Dott.ssa Ilaria Basile

Coordinatore Dottorato:

Chiar.mo Prof. Andrea Stella

Relatore:

Chiar.mo Prof. Carlo Guarnieri

Esame finale anno 2008

Contents

List of Abbreviations

ABI	Ankle branchial index
AcLDL	Acetylated LDL
AGE	Advanced glycosilation end products
Ang II	Angiotensin II
b-FGF	Basic fibroblast growth factor
BM	Bone marrow
CAD	Coronary artery disease
CEC	Circulating endothelial cell
CFU-EC	Colony forming units endothelial cell
CLI	Critical limb ischemia
CRP	C-Reactive Protein
CVD	Cardiovascular disease
DAF-2DA	Diaminofluorescein 4,5 diacetated
Dil	1,1-dioctadecyl-3,3,3,3-tetramethilindocarbocyanine
DM	Diabetes mellitus
DR	Diabetic retinopathy
eNOS	Endothelial NOS
EPC	Endothelial Progenitor Cell
EPCAD	Endothelial Progenitor Cells in Coronary Artery Disease
EPO	Erythropoietin
ET-1	Endothelin-1
FFA	Free fatty acids
G-CSF	Granulocyte-colony stimulating factor
GLP-1	Glucagon like peptide-1
GM-CSF	Granulocyte–Macrophage Colony-Stimulating Factor Interleukin
HUVEC	Human umbilical vein endothelial cell
IC	Intermittent claudication
ICAM	Intracellular adhesion molecules
IGF-I	Insuline-like growth factor-I
IL	Interleukin
INF-γ	Interferon Gamma
iNOS	Inducible NOS

IP	Ischemic Period
IP-10	Inducible Protein-10
I-TAC	IFN-inducible T-cell a-Chemoattractant
IR	Insulin resistance
L-Arg	L-Arginine
L-NAME	N^G-nitro-L-arginine methyl ester hydrocloride
L-NIL	N^6-(1-Iminoethyl)-L-lysine dihydrocloride
L-NIO	N^5-(1-Iminoethyl)-L-ornithine dihydrocloride
LDL	Low density lipoprotein
LOX-1	Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1
MAP	mitogen-activating protein kinase
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
Mig	Monokine Induced by IFN-g
MMP	Matrix Metalloproteinase
MNC	Mononuclear Cell
mRNA	Messenger RNA
MTT	3-[4,5-dimethylthiazol-2-]-2,5-dipheniltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal NOS
NO	Nitric Oxide
NOS	Nitric oxide synthase
Nox	NADPH oxidase
OEC	Outgrowth endothelial cells
oxLDL	Oxidized Low-Density Lipoprotein
PAD	Peripheral arterial disease
PAOD	Peripheral arterial occlusive disease
PAI-1	plasminogen activator inhibitor-1
PAR	Protease-Activated Receptor
PECAM-1	Platelet endothelial cellular adhesion molecule 1
PSGL-1	P-selectin glycoprotein ligand-1
PTCA	Percutanueous Transluminal Coronary Angioplasty
RNA	Ribonucleic acid

ROS	Reactive Oxygen Species
Sca-1	Stem cell antigen-1
SDF-1	stromal-derived factor-1
sKitL	soluble Kit ligand
SMC	Smooth muscle cell
SRA	Scavenger Receptor A
TNF-α	Tumor Necrosis Factor-alpha
UEA-1	*Ulex europaeus* agglutinin-1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF receptor
VLA-4	Very late antigen-4
vWF	von Willebrand factor

Introduction

In the recent years it is emerged that peripheral arterial disease (PAD) has become a growing health problem in Western countries. This is a progressive manifestation of atherothrombotic vascular disease, which results into the narrowing of the blood vessels of the lower limbs and, as final consequence, in critical leg ischemia. PAD often occurs along with other cardiovascular risk factors, including diabetes mellitus (DM), low-grade inflammation, hypertension, and lipid disorders. Patients with DM have an increased risk of developing PAD, and that risk increases with the duration of DM.

Diabetes mellitus is a metabolic disorder characterised by elevated glucose levels. People with type 1 diabetes have inadequate insulin production by pancreas islets, while type 2 diabetes is characterized by relative insulin deficiency and or insulin resistance. In particular, type 2 diabetes is becoming more common, maybe because of an ageing population and the increasing prevalence of obesity and sedentary lifestyles. Moreover, there is a growing population of patients identified with insulin resistance (IR), impaired glucose tolerance, and obesity, a pathological condition known as “metabolic syndrome”, which presents increased cardiovascular risk.

Atherosclerosis, which is the earliest symptom, is a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. Endothelial dysfunction is a broad term that implies diminished production or availability of nitric oxide (NO) and/or an imbalance in the relative contribution of endothelium-derived relaxing factors, in particular prostacyclin. The secretion of both these agents is considerably reduced in association with the major risks of atherosclerosis, especially hyperglycaemia and diabetes. Moreover, it has been observed a reduced vascular repair in response to wound healing and reduced collateral formation in response to ischemia.

Neovascularization does not only rely on proliferation of local endothelial cells but also involves bone marrow-derived stem cells, referred to as endothelial progenitor cells, since they exhibit endothelial surface markers and properties. They can promote postnatal vasculogenesis by homing to, differentiating, proliferating and incorporating into new vessels. Consequently, EPCs are critical to endothelium maintenance and repair and their dysfunction contribute to vascular disease.

Nitric oxide itself is involved in neovascularization. In fact, NO produced by eNOS, the endothelial isoform of nitric oxide synthase (NOS), effects endothelial migration and

proliferation.

The aim of this research has been the study of EPCs and their functionality under physiologic condition, considering their role in therapeutic vasculogenesis. Furthermore, it has been analyzed their function under pathological conditions. Thus, EPCs isolated from atherosclerotic and diabetic patients have been examined with respect to proliferation, differentiation and function.

Given the importance of NO in angiogenesis and vasculogenesis, it has been studied the expression of NOS isoforms, inducible NOS (iNOS), eNOS (endothelial NOS), and neuronal NOS (nNOS), comparing normal NOS pattern to that of patients affected with PAD.

In addition, it has to be considered the correlation between endothelial dysfunction and enhanced levels of reactive oxidative species (ROS). In fact, the principal mechanism of ROS-dependent endothelial impairment is the inactivation of NO. As a result, the expression of NADPH oxidase isoforms (Nox1, Nox2 and Nox4) was investigated.

Chapter 1

Peripheral Arterial Disease

Peripheral arterial disease (PAD) is a common progressive manifestation of atherothrombotic vascular disease, resulting into the narrowing of the blood vessels of the lower limbs (1). This atherosclerotic condition is very common in the western world, affecting more than 27 million people in North America and Europe, and this number is expected to increase with the aging population (2). It affects at least 10% of individuals, men and women equally, aged over 65 years and 20% of those aged over 80 years (3).

Albeit it is a common clinical malady, PAD is relatively unknown to the public, compared to heart disease, which is taught widely in health education programs and raises many public health initiatives. Even in the absence of a history of myocardial infarction or stroke, the relative risk of death from cardiovascular causes in patients with PAD is about the same as in patients with coronary or cerebrovascular disease. Patients with critical leg ischemia, the most severe form of PAD, face an annual mortality of 25%, which is predominantly due to myocardial infarction and ischemic stroke (4).

In their study, Caro et al estimated the burden of cardiovascular risk in term of mortality, morbidity and associated risk factors in patients with PAD (5). They compared the PAD cohort to those of people with a first diagnosis of myocardial infarction and stroke. It was evident that the mortality rate of PAD patients was higher compared to those who had an index myocardial infarction, but lower than those who had suffered from stroke. At the end of the follow-up period, half of the patients with PAD were alive, compared to about 60% of those belonging to the myocardial infarction group and 51% of those with stroke.

As expected, risk factors analyses revealed that men with PAD were more likely to have atherothrombotic complications, angina, myocardial infarction, stroke and death (6). Moreover, the risk of myocardial infarction was significantly increased in people aged over 65 years, especially with concomitant angina, diabetes mellitus, heart failure and hypertension. Cardiovascular risk increase in PAD patients depended on the number of additional risk factors, resulting in a “cumulative” cardiovascular risk, similarly to cardiac disease.

However, all epidemiologic cross-sectional studies show that in the general population asymptomatic peripheral arterial occlusive disease (PAOD) is more common than symptomatic PAOD (7). As PAD progresses, patients start to feel pain due to the impaired blood flow, which causes decreased oxygen and nutrient supply in the distal tissue. This can further develop into

tissue ischemia, and consequently to skin ulceration and muscle damage. In fact, the most evident symptom in PAD patients is intermittent claudication (IC), indicating a blood supply deficiency in muscle during exercise. The prevalence of IC increases with age, affecting >5% of patients over 70, and its incidence in diabetic patients doubles or even triples (7).

The asymptomatic PAD subjects remain however a high risk population, and those who are diagnosed PAD are less frequently under lipid-lowering therapy and antiplatelet therapy in comparison to those with cardiovascular disease. This could be explained because of symptom variability. Approximately 36% of patients with PAD have symptoms, however, only one third of them reports symptoms to their physician and receives appropriate treatment (8). It becomes essential to screen patients, being symptom self-reporting unreliable.

Diagnosis of PAD

Diagnosis of PAD is made by medical history, physical examination, and the measurement of ankle blood pressures using a Doppler ultrasound device. The ankle branchial index (ABI) is defined as the ratio of ankle pressure to the branchial systolic pressure. The ABI has been proven to be an effective for recognizing the disease in patients with or without symptoms. The reference range of ABI is 0,91 to 1,4. An ABI ≤0.9 corresponds to a diagnosis of PAD, and it is associated with increased fatal and nonfatal coronary artery disease, and stroke. The lower the ABI is, the disease gets more hemodinamically severe (9).

ABI rate	Clinical
0.9>	Normal
0.7-0.9	Mild claudication
0.5-0.7	Moderate claudication
0.4-0.5	Severe claudication
<0.4	Rest pain, nonhealing ulcers

Table 1. Ankle-brachial index and corresponding symptomatology

It has also been found that individuals with an ABI >1.40 have mortality rates similar to those who have an ABI ≤0.9. These patients have noncompliant vessels secondary to pathological processes involving vascular fibrosis and calcification; they tend to have greater coronary artery calcium (10). Some have suggested a tighter range for normal ABI, since individuals with an ABI between 0.9 and 1.00 manifest more ischemic leg pain (11), subclinical atherosclerosis, and are in greater risk for cardiovascular disease (12). Although the ABI is an excellent screening test, there is only a modest correlation between its value and the functional

limitation. For instance, an ABI of <0.5 is typically observed in patients with resting pain or tissue loss. On the other hand, a similar ABI can be discovered in an individual with only a modest symptomatology (13). These differences are probably due to the heterogeneity in collateral formations or skeletal muscle adaptation.

As described above, there is a substantial overlap between the pathogenesis of PAD and other forms of atherosclerotic disease, like coronary artery disease (CAD) and carotid disease. PAD and CAD share several risk factors, including diabetes, smoking, hypertension, insulin resistance, low high-density lipoprotein levels, elevated low-density lipoprotein levels, and advanced age. Approximately 70% of PAD cases (as defined by ABI) can be attributed to these risk factors (14), somewhat higher than the corresponding estimates for CAD. There are evidences that some of these risk factors have proportionately greater effects on the development of PAD than CAD. For instance, diabetes mellitus (DM) and smoking are particular strong risk factors (15).

In a study conducted by a Dutch group (16), the Limburg PAOD Study, a study population of more than 2,000 people selected from several practice centers in the Netherlands was examined. After a 7 year follow-up, the high incidence of intermittent claudication in patients who were asymptomatic suggests that cessation of smoking and adequate treatment of hypertension and diabetes are the principal preventive objectives in PAOD patients.

Number and combination of risk factors at baseline	Probability (%) of overall PAOD	Probability (%) of asymptomatic PAOD	Probability (%) of symptomatic PAOD
Reference probability	3	2	3
Age ≥65 years	8	5	1
Age ≥65 years and hypertension	15	9	2
Age ≥65 years and smoking	18	10	6
Age ≥65 years and diabetes	18	10	5
Age ≥65 years, hypertension, and diabetes	28	16	9
Age ≥65 years, smoking, and hypertension	29	17	11
Age ≥65 years, smoking, and diabetes	33	18	19
Age ≥65 years, smoking, hypertension, and diabetes	47	27	31

Table 2. Probability (%) of asymptomatic and symptomatic peripheral arterial occlusive disease (PAOD) according to the number and combination of the four most significant risk factors present at baseline*: Limburg PAOD Longitudinal Study, 1988–1997 (*n* = 2,327).

However, despite the evaluation of multiple risk factors, the progression of PAD seems to be highly variable, suggesting the presence of other determinants of the disease, and, in particular, genetic ones.

Classification of Peripheral Arterial Disease and Clinical Manifestations

Various classification have been used to define the severity of PAD, of which the Fontaine and Rutherford classifications are widely used:

Fontaine		Rutherford		
stage	Clinical	Grade	Category	Clinical
I	Asymptomatic	0	0	Asymptomatic
IIa	Mild Claudication		1	Mild Claudication
IIb	Moderate	I	2	Moderate claudication
	Severe claudication	I	3	Severe claudication
III	Ischemic rest pain	II	4	Rest pain
		III	5	Minor tissue loss
IV	Ulceration/gangrene	III	6	Major tissue loss

Tab. 3 Classification of peripheral arterial occlusive disease.

Claudication, rest pain, and/or skin ulceration are the usual symptoms of PAD. The diagnosis of intermittent claudication includes pseudoclaudication and venous claudication. Pseudoclaudication has a nonarterial etiology, its origin can be neurogenic or muscoloskeletal. It may lead to discomfort from spinal stenosis, compartment syndromes, venous congestion, or arthritis. It is symptomatic only in certain positional changes, such as standing. On the contrary, venus claudication causes a burning sensation while standing or walking.

Firstly, therapy in patients with PAD implies modifying risk factors and attempting to relieve symptoms of claudication with exercise or medicine or both. An antiplatelet and cholesterol lowering therapy is considered a basic approach to PAD treatment. Nevertheless, all patients who have rest pain, tissue loss, or significant lifestyle limiting disability, despite aggressive medical treatment should be considered eligible to revascularization.

Invasive diagnostic angiography is reserved for patients in whom revascularization is planned or for those situations in which the results from noninvasive imaging are ambiguous (17). Complications associated with catheter-based angiography are related to vascular access, catheter trauma (atheroembolism, vessel dissection, or perforation), or systemic complications associated with contrast reactions or renal toxicity. Complications associate with

percutanueous transluminal coronary angioplasty (PTCA) are access-site events, amputation, and major bleeding.

Endovascular procedures are indicated for individuals with a lifestyle limiting disability due to intermittent claudication, when clinical features suggest a reasonable likelihood of symptomatic improvement with endovascular intervention. Atherosclerotic occlusive disease can affect different vascular beds, and in particular, the most affected ones in lower limbs are iliac arteries, femoropopliteal arteries, infrapopliteal arteries. Atherosclerotic occlusive disease is two to five times more frequent in femoropopliteal arteries than in iliac arteries. Percutaneous angioplasty, however, is not always effective in treating femoropopliteal arteries. There are many factors that adversely affect the possible advantages of this technique, like occlusion degree (especially if it is bigger than 10 cm), calcification, multiple-lesion segments, rest ischemia and a poor distal runoff.

Infrapopliteal disease usually coexists with more proximal inflow disease and for patients who have claudication, revascularization of proximal inflow vessels is often sufficient to relieve symptoms. In contrast, patients with ischemic ulceration generally need to have flow restored to the foot to heal the lesion. The immediate and long-term results of arterial reconstruction for infrapopliteal disease are better in patients who have claudication than in those who have critical limb ischemia (CLI). Diabetic patients with CLI will more often have 3-vessel below-knee disease and may also have ulceration related to small-vessel disease that will not generally improve with revascularization.

For those patients who are not candidates for revascularization due to critical limb ischemia and for those who can be successfully revascularized but will have compromised limb function, amputation keeps to be considered as a primary therapeutic option (18).

Nevertheless, a comparative study between patients undergoing angioplasty and those undergoing open surgery showed that, firstly, there is no difference in life quality of the two groups, and secondly, costs associated with a surgery-first strategy were higher than for angioplasty. a percutaneous-intervention–first strategy was the treatment of choice in patients who are candidates for either surgery or endovascular intervention.

Moreover, there has been much interest in developing an agent that would stimulate angiogenesis in patients who have CLI but do not have the option for revascularization. In order to achieve this goal, initial work has progressed in the areas of genes that code for various growth factors and the use of endothelial progenitor cells to stimulate angiogenesis.

Chapter 2

Atherosclerosis and Endothelial Dysfunction

Atherosclerosis is a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. It develops similarly in the coronary, cerebrovascular, and peripheral vasculature systems; these vascular beds are interdependent, and subsequent disease progression are manifestations of the same process (2). Therefore, they are associated with the same clinical events as the disease process overlap.

The endothelium is a complex endocrine and paracrine organ, located between the wall of blood vessels and the blood stream (19). It senses mechanical stimuli, such as pressure and shear stress, and hormonal stimuli, such as vasoactive substances. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes, and affect hemostasis. Vascular homeostasis is maintained by a balance between endothelium-derived relaxing and contracting factors. Once this balance is disrupted, because of inflammatory and traditional cardiovascular risk factors, the vasculature becomes susceptible to atheroma formation.

Initiation of Inflammation and Atherosclerosis

It is now well accepted that atherosclerosis is a specific form of the chronic inflammatory process resulting from interactions between plasma lipoproteins, cellular components (monocytes/macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the arterial wall (20). The accumulation of lipid-loaded cells underlying the endothelium of large arteries, namely, fatty streaks or dots, is a marker of early-stage atherosclerotic lesions. Numerous studies of either human atherosclerotic lesions or cholesterol-fed animals have shown that the lipid-loaded cells mainly originate from monocytes subsequently differentiated macrophages (21). These macrophages engulf a large amount of lipids deposited in the subintimal space and take the appearance of foamy structures, designated as foam cells. In this process it is involved the up-regulation of pattern recognition receptors, including scavenger receptors, cell surface membrane proteins capable of binding and internalization of modified lipoproteins (22), and toll-like receptors.

Three major lipoproteins are often observed in the lesions and are considered to be atherogenic, at elevated plasma concentrations: low density lipoprotein (LDL); especially small dense LDL, remmant lipoproteins and lipoprotein (a) [Lp (a)] (23).

These atherogenic lipoproteins, once deposited in the intima, are subjected to chemical modifications, such as oxidation. While oxLDL is invariably associated with foam cells in the lesions Lp (a), though it is present in the lesions, it is seldom associated with foam cell formation, tending to be associated with the extracellular matrix.

In addition to foam cells, T lymphocytes are scattered around macrophages and foam cells. T cells are predominant in the incipient phase, afterwards they are gradually surpassed by monocyte infiltration in later stages (24). They actively participate to lesion progression, by releasing different cytokines, in addition to those derived from vascular cells. One of the most notable cytokines derived from T lymphocytes is interferon gamma (INF-γ), which has been shown to play diverse role in mediating foam cell formation, smooth muscle cell proliferation, and in regulating the production of matrix metalloproteinases, subsequently influencing plaque stability. As seen in cholesterol-fed animals, while under ordinary circumstances the endothelial monolayer in contact with flowing blood resists firm adhesion of leukocytes, soon after initiating an atherogenic diet electron microscopy reveals attachment of mononuclear cells to the endothelial cells that line the intima, the innermost layer of arteries (25). Mononuclear cell adherence is triggered by a number of adhesion molecules on endothelial cells that are highly up-regulated by the elevation of the levels of atherogenic lipoproteins and cytokines in vitro. One endothelial–leukocyte adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), has emerged as a particularly attractive candidate for the early adhesion of mononuclear leukocytes to arterial endothelium at sites of atheroma initiation. VCAM-1 particularly binds those classes of leukocytes which are present in the nascent

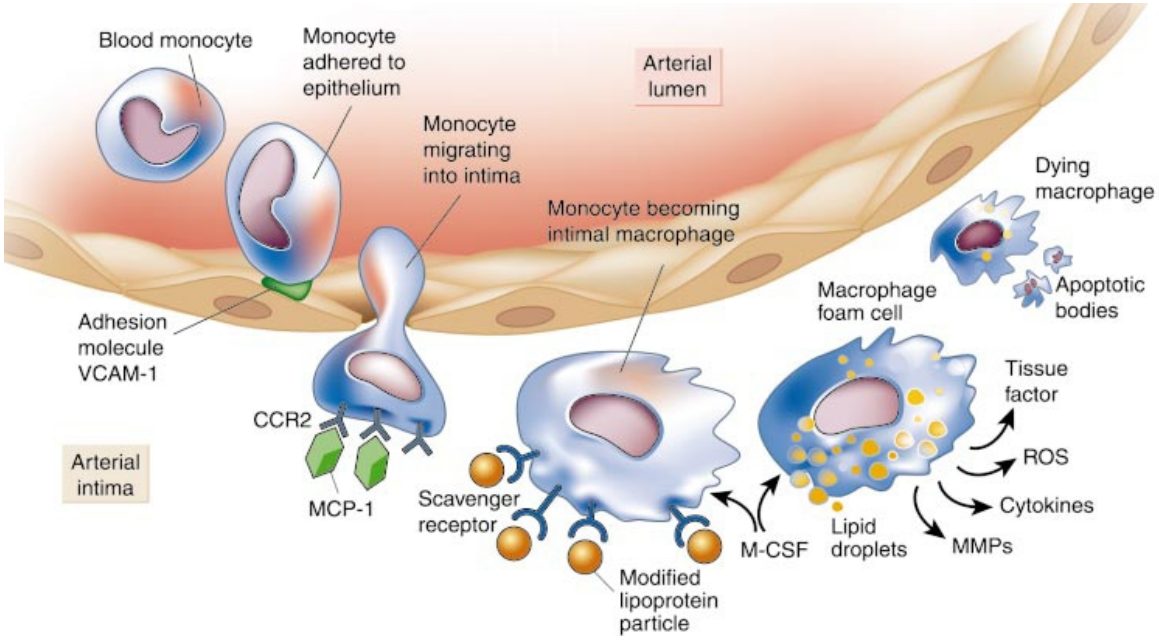


Fig. 1 Recruitment of mononuclear phagocytes to the nascent atherosclerotic plaque and functions of these cells in the mature atheroma (Libby P, 2002).

atheroma, namely, monocytes and T lymphocytes. Endothelial cells express it in response to cholesterol feeding (26), maybe because of the inflammation due to modified lipoprotein particles accumulating in the intima in response to hyperlipidaemia, but VCAM-1 can also be expressed by other cell types like macrophages, myoblasts, dendritic cell. Pro-inflammatory cytokines such as interleukin (IL)-1b or tumour-necrosis factor-a (TNF-a) induce VCAM-1 expression in endothelial cells by this pathway. Human atherosclerotic lesions contain these cytokines. Thus, pro-inflammatory cytokines may link hypercholesterolaemia to VCAM-1 expression. In addition to VCAM-1, other adhesion molecules are involved in leukocyte adherence, such as the intracellular adhesion molecules (ICAM-1,2,3), P- and E-selectin (27). Platelet endothelial cellular adhesion molecule 1 (PECAM-1) is another member of the Ig family expressed by leukocytes, platelets and endothelial cells. PECAM-1 molecules are particularly dense at the junctions between endothelial cells where they mainly participate to homophilic binding between adjacent cells. Therefore, they are involved in endothelium integrity and extravasation of cells from the blood compartment into the vessel and underlying tissue.

VCAM-1 appeared as an indicator of severity of atherosclerosis since it significantly correlated with the extent of peripheral atherosclerosis. However, if adjusted for smoking, the VCAM-1 lost significance whereas sICAM-l appeared as a better marker of the presence and progression of disease.

Mechanisms of Leukocyte Chemoattraction and Activation

After monocytes and T lymphocytes bind the surface of the arterial wall, they migrate into the subendothelial space, where they differentiate into macrophages and foam cells. This migration is induced by chemoattractants in the intima, such as oxLDL, Lp(a), chemoattractant cytokines (chemokines), like monocyte chemoattractant protein (MCP-1), produced by endothelial cells and macrophages, interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), and degraded collagens and elastins (28). Among all the mediators, MCP-1 and Lyso-PC (a component of oxLDL) are the most important and best characterized chemoattractants in the lesions.

MCP-1 is already present at an early stage of lesion development and binds to the specific receptor CCR-2 at the surface of monocytes. In addition to acting as a chemoattractant, oxLDL can also inhibit macrophage mobility. This dual function of oxLDL could explain why macrophages or foam cells fail to return circulation as they are supposed to do.

Once resident in the arterial intima, monocytes acquire the morphological characteristics of macrophages, undergoing a series of changes that lead ultimately to foam cell formation. The monocytes increase the expression of several scavenger receptors capable of taking up modified lipoproteins, i.e. the scavenger receptor A (SRA), and then internalize modified lipoproteins transforming themselves into foam cells. By secreting a number of growth factors and cytokines, they amplify the inflammatory response in the lesion. The macrophage colony-stimulating factor (M-CSF) has been individuated as a candidate activator of several of the steps that stimulate transition of the monocyte to the lipid-laden macrophage. M-CSF augments SRA expression, increases production of cytokines and growth factors by these cells, and also serves as a survival and co-mitogenic stimulus. Moreover, also granulocyte–macrophage colony-stimulating factor (GM-CSF) may promote inflammation in the atheroma.

Atheroma progression

After formation of the fatty streak, the nascent atheroma typically evolves into a more complex lesion, which eventually lead to clinical manifestations. The typical atherosclerotic plaque (also called atheroma) contains a lipid or necrotic core covered by a fibrotic layer. This cap is due to smooth muscle cells, that, once adhered to the fatty streaks, begin to multiply and lay down an abundant extracellular matrix. As the lesion becomes bigger, the arterial lumen narrows until it hampers flow and leads to clinical manifestations. According to the classical view, this process occurred in an inevitable and progressive fashion gradually during time. However, clinical observations evidenced a discontinus progression. Data that emerged from serial angiographic studies suggest that many coronary arterial lesions in humans develop stenoses discontinuously (29).

Two types of plaque are commonly distinguished: stable plaque and instable plaque. Stable plaque is usually composed of a small lipid core and covered by a thick fibromuscular layer with more smooth muscle and extracellular matrix. Unstable plaque has a large pool of lipid core, a thin cap and a large number of inflammatory cells can be present. Stable plaques are the major risk for stenosis or occlusion, since they cause a significant reduction of the vascular lumen. Otherwise, vulnerable plaques are fatal, leading to acute coronary syndrome, if they rupture, and are associated to thrombosis.

Different mechanisms are supposed to be involved in plaque rupture. Superficial erosion, or the formation of microscopic areas of desquamation of endothelial cells that form the monolayer covering the intima, frequently occur in both humans and animals with experimentally induced

atherosclerosis. Such areas often trigger platelet thrombus formations. The inflammatory mediators and oxLDL are responsible of the activation of matrix metalloproteinases (MMPs) that degrade the endothelial basement membrane. Meanwhile, when the inflammation prevails in the intima, smooth muscle cell production of new collagen required for repair and maintenance of the fibrous cap decreases. The result is endothelial desquamation (30).

Secondly, disruption of the microvessels that form in atherosclerotic plaques furnishes another explanation for sudden plaque progression (31). Atheromas develop microvascular channels as a result of neoangiogenesis. These microvessels could have a nutritive function promoting plaque growth and, like those that form in the diabetic retina, the new blood vessels in the plaque may be particularly fragile and prone to micro-haemorrhage.

The third and most common mechanism of plaque disruption, a fracture of the plaque's fibrous cap, also involves inflammation. Certain pro-inflammatory cytokines, such as IFN-g, can limit the synthesis of new collagen by smooth muscle cells. These changes can thin the fibrous cap and render it friable and susceptible to rupture. When the plaque ruptures, blood comes into contact with the tissue factor in the plaque and coagulates. Fissure of the fibrous cap allows the coagulation factors contact with tissue factor, the main pro-thrombotic stimulus found in the lesion's lipid core.

If the thrombus occludes the vessel persistently, an acute myocardial infarction can result. The thrombus may eventually resorb as a result of endogenous or therapeutic thrombolysis. However, a wound healing response triggered by thrombin generated during blood coagulation can stimulate smooth muscle proliferation. The increased migration, proliferation and extracellular matrix synthesis by smooth muscle cells thickens the fibrous cap and causes further expansion of the intima, often now in an inward direction, yielding constriction of the lumen. Stenotic lesions produced by the lumenal encroachment of the fibrose plaque may restrict blood flow, particularly under situations of increased cardiac demand, leading to ischaemia, commonly provoking symptoms such as angina pectoris.

Endothelial Dysfunction: Setting the Stage for Inflammation

Endothelial dysfunction is a broad term that implies diminished production or availability of nitric oxide (NO) and/or an imbalance in the relative contribution of endothelium-derived relaxing factors, such as prostacyclin, different endothelium-derived hyperpolarizing factors, and C-type natriuretic peptide, and contracting factors, such as endothelin-1 (ET-1), angiotensin II (Ang II), tromboxane A_2 and reactive oxygen species (ROS). Endothelial

dysfunction was initially identified as impaired vasodilation to specific stimuli. It was first described in human hypertension in the forearm vasculature in 1990 (32). Impairment of vasodilation was also described in type I and type II diabetes, coronary artery disease, congestive heart failure and chronic renal failure.

Endothelial dysfunction, as assessed in terms of vasomotor dysfunction, can occur long before the structural manifestation of atherosclerosis and thus can serve as a predictor of future cardiovascular events.

NO, about whom it will be attentively discussed further, is the key endothelium-derived relaxing factor that plays a pivotal role in the regulation of vascular tone and vasomotor function (33). In addition to its vasodilatatory effect, NO also protects against vascula injury, inflammation and thrombosis.

ROS are known to quench NO with formation of peroxynitrite, which is a cytotoxic oxidant, and through nitration of proteins, thereby affecting protein function and endothelial function. ROS upregulate adhesion (VCAM-1 and ICAM-1) and chemotactic molecules (MCP-1).

OxLDL causes endothelial activation and changes its biological characteristic partially by reducing the intracellular NO concentration. Angiotensin II, opposes NO action. A recently studied risk factor, the C-reactive protein (CRP) can promote endothelial dysfunction by quenching the production of NO and diminishing its activity.

The latter factor has raised a great interest, as an acute phase reactant. Several evidences suggest that circulating high-sensitivity CRP represents one of strongest independent predictors of vascular death, even stronger than LDL cholesterol. CRP has a direct effect on promoting atherosclerosis and endothelial cell dysfunction. Human recombinant CRP elicits a multitude of effects on endothelial biology favoring a proinflammatory and proatherosclerotic phenotype. It downregulates endothelial NO synthase, stimulates ET-1 and IL-6, up-regulates adhesion molecules and stimulates MCP-1 while facilitating macrophage LDL uptake. Recently, CRP has been shown to facilitate endothelial cell apoptosis and inhibit angiogenesis. In addition to the effect on endothelial cells, CRP inhibits bone-marrow derived endothelial progenitor cell survival and differentiation (34). Direct proatherogenic effects of CRP extend beyond the endothelium to the vascular smooth muscle. It stimulates vascular smooth muscle migration, proliferation, neointimal formation ad ROS production.

The endothelial injury, activation, and dysfunction caused by oxLDL are exerted via lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) activation. LOX-1 was initially identified as the principal oxLDL receptor in endothelial cells. Later, it was evidenced its expression in

macrophages and smooth muscle cells too. In addition to ox-LDL binding, LOX-1 can bind damaged or apoptotic cells, activated platelets, advanced glycation end products, and pathogenic orgaisms. Under physiological conditions, LOX-1 may play a role in host defense or serve to scavenger cellular debris. On the contrary, in pathological state, it can be involved in proatherogenic material binding, playing a role in initiating endothelial dysfunction.

As previously said, oxLDL, via upregulation of LOX-1, induces monocyte adhesion to endothelium, increased production of ROS, apoptosis of SMCs and modulation of MMP activity. Further, on plaque rupture, activated platelets may interact with the surrounding endothelium via LOX-1. This interaction promotes the release of endothelin-1 from endothelial cells and stimulates the generation of ROS that inactivate NO.

In response to endothelium injury, the expression of another family of receptors, the protease-activated receptors (PARs), is increased. PARs are expressed by a variety of cell types in and around blood vessels, including endothelial cells, SMCs, and platelets. PAR activation appears to promote the inflammatory response within the intimal tissue, enhancing the initiation and progression of atherosclerotic plaque. However, the vascular effects of PARs seem to differ when compared to the endothelial dysfunction carried on by other inflammatory markers. In fact, activation of PAR-1 and PAR-2 in human blood vessels has been demonstrated to produce NO, resulting in vasodilation (35). PAR-2 induces SMC migration, and platelet activation, permitting their attachment to the atherosclerotic lesion. Thrombin too can induce the PAR-mediated platelet activation, and recent studies confirm thrombin-PAR-1 connection. PAR-1 has emerged to be the primary receptor that mediates the prothrombotic actions of thrombins.

Another class of enzymes, the matrix metalloproteinases (MMPs), plays an important role in vascular remodelling, which permits changes in size and composition of adult blood vessels, allowing adaption and repair. However, excessive MMP expression may cause inappropriate vascular remodelling, resulting in atherosclerosis. Among MMPs, MMP-9 plays a pivotal role in cardiovascular disease, being its variation related to the presence and severity of atherosclerosis. For instance, MMP-9 levels are increased in type 2 diabetic patients, and patients with acute myocardial infarction and unstable angina. Finally, MMP-9 has also been demonstrated to promote the mobilization of stem cells from bone marrow, the so-called endothelial progenitor cells, which appear to home preferentially to sites of vascular or tissue injury, contributing significantly to both reendothelialization and neovascularization (36).

Chapter 3

Diabetes as a Vascular Disease

Diabetes mellitus is a multifactorial disease, characterized by high blood glucose levels, associated with a number of microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (ischaemic heart disease, cerebrovascular disease and peripheral vascular diseases) complications (37). Type 1 diabetes is characterized by a near-total reliance on exogenous insulin for survival, caused by an autoimmune-mediated destruction of pancreatic islet cell. Type 2 diabetes is more prelevant, and characterized by relative insulin deficiency and or insulin resistance. Some type 2 DM patients require exogenous insulin, but many can achieve glucose control with diet alone or the addition of oral hypoglycemic agent. This disease is becoming more and more common, because of aging population and the increasing prevalence of obesity and sedentary lifestyle (38).

Recent attention has also focused on the prediabetic spectrum of impaired glucose metabolism, with a growing population of patients identified with insulin resistance (IR), impaired glucose tolerance, and obesity. Hypertriglyceridemia, IR, decreased high-density lipoprotein cholesterol, hypertension, and central obesity are clustered factors known as “metabolic syndrome,” which portends increased cardiovascular risk (39).

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in people with diabetes, and coronary heart disease is the most common cause of death among people with type 2 diabetes. People with diabetes are two to four times more likely to develop CVD compared with people without this condition. Diabetes is the leading cause of end-stage renal failure, visual impairment, and blindness in many populations in both developed and developing countries. Lower extremity amputations are at least 10 times more common in people with diabetes than in non-diabetic individuals in developed countries and more than half of all non-traumatic lower limb amputations are due to diabetes.

Diabetic macrovasculopathy is associated with structural and functional changes in large arteries: structural changes result mainly from glycation of wall components and functional changes originate in endothelial dysfunction. Moreover, metabolic [advanced glycation end production (AGE), cytokines], humoral (renin–angiotensin system, endothelin, sympathetic nervous system) and haemodynamic (arterial hypertension and mechanical factors contribute to the characteristic dysfunction in diabetic vasculopathy.

Endothelial Dysfunction and Diabetes

Endothelial dysfunction is a common finding in type 2 diabetic patients. Several independent factors, e.g. insulin resistance, hyperglycemia, hypertension, dyslipidaemia, abdominal obesity and low-grade inflammation, have all been associated with this condition in subjects with type 2 diabetes (40).

Insulin resistance. A number of different altered metabolic states as exemplified by glucose, lipid and cytokine metabolism can lead to peripheral insulin resistance. The ensuing metabolic dysregulation that occurs as a consequence of insulin resistance further exacerbates its progression. Obesity and insulin resistance, independently of other risk factors, are associated with endothelial dysfunction. In the insulin resistant state the normal suppression of free fatty acids (FFA) release from adipose tissue is impaired contributing to diabetic dyslipidaemia i.e. VLDL-hypertriglyceridemia, low HDL-cholesterol concentrations and elevation of FFA. In insulin resistance and type 2 diabetes a state of pro-atherogenesis and low-grade-inflammation occurs (e.g. reflected by plasminogen activator inhibitor-1 (PAI-1), hematocrit, family history of type 2 diabetes, glucagon like peptide-1 (GLP-1), increased TNF-alpha, IL-6, and CRP-levels), all of which have been associated with endothelial dysfunction.

At molecular level, insulin has also a profound stimulatory effect on eNOS gene expression and on the activity of this important enzyme (41). These effects of insulin on eNOS expression and activity and, consequently, on NO production are mediated via thePI3-kinase-dependent signalling pathway. Under normal circumstances, NO, in addition to its vasodilatory function, counteracts the influence of vascular endothelial growth factor (VEGF) on expression of adhesion molecules on the surface of the ECs. In the state of metabolic insulin resistance,

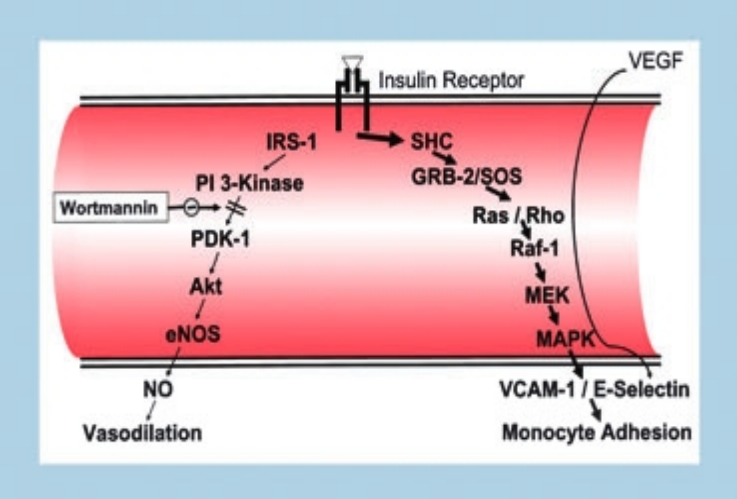


Fig. 2 Mechanisms for the contribution of insulin resistance to atherosclerosis. In vitro model of metabolic insulin resistance with compensatory hyperinsulinemia in vascular endothelium. Kim et al. Circulation 2006; 113: 1888

insulin action via the PI 3-kinase-dependent pathway is diminished (42). As a result, the influence of insulin on eNOS activity and NO production declines significantly. Inadequate NO production fails to prevent VEGF-induced increases in the expression of adhesion molecules and interactions of monocytes with ECs (43). The latter can have strong proatherosclerotic consequences.

Hyperglycaemia. Hyperglycaemia is a hallmark of diabetes and of both impaired fasting and postprandial glucose tolerance (44). High glucose concentration exerts its detrimental action on the cardiovascular system in multiple ways. Through increased formation of advance glycosylation end products (AGE), it generates reactive oxygen species (ROS), activates proinflammatory transcription and alters the structural and biochemical function of numerous proteins. Through protein kinase C (PKC) activation, it can lead to a variety of phosphorylation events that alter intracellular signalling. Hyperglycaemia and AGE and PKC have been shown to activate mitogen-activating protein kinase (MAP) that can lead to proliferative events. Excess of glucose flux into the cells can enter the glucosamine pathway where it leads to abnormal glycosylation of critical proteins such as the transcription factor SP-1, resulting in abnormal gene expression.

Hyperglycaemia rapidly activates a series of stress pathways in the ECs, leading to disruption of the antiatherogenic properties of healthy endothelium. ECs exposed to high glucose concentration (25 mM) acutely generate increased ROS and ultimately undergo apoptotic cell death (44). This injurious effect of hyperglycaemia is likely mediated via induction of PKC, accumulation of AGE, shunting glucose molecule into the polyol and glucosamine pathways and overwhelming mitochondrial oxidative capacity, leading to generation of mitochondrial ROS and poly(ADP-ribose) polymerase activation. Hyperglycaemia acutely activates and later inhibits the PI 3-kinase/Akt pathway that regulates endothelial NO synthase (eNOS) and cell survival and proliferation (45). AGE-receptor activation and PKC activation induce expression of adhesion molecules, cytokines, CD 36 and PAI-1 (46).

Moreover, hyperglycaemia has effects on ECs, by induction of matrix protein and metalloproteinase expression in ECs, and interfering with re-endothelialization by disrupting the proliferation and migration of ECs. By this series of events, hyperglycaemia promotes a vasoconstrictive, proinflammatory and prothrombotic vascular response to injury, which may be compounded by compensatory hyperinsulinaemia of insulin resistance.

Inflammation. The association of the inflammatory state with obesity and insulin resistance was described in 1993 by Hotamisligil et al. (47). In this study, adipocyte expression of the

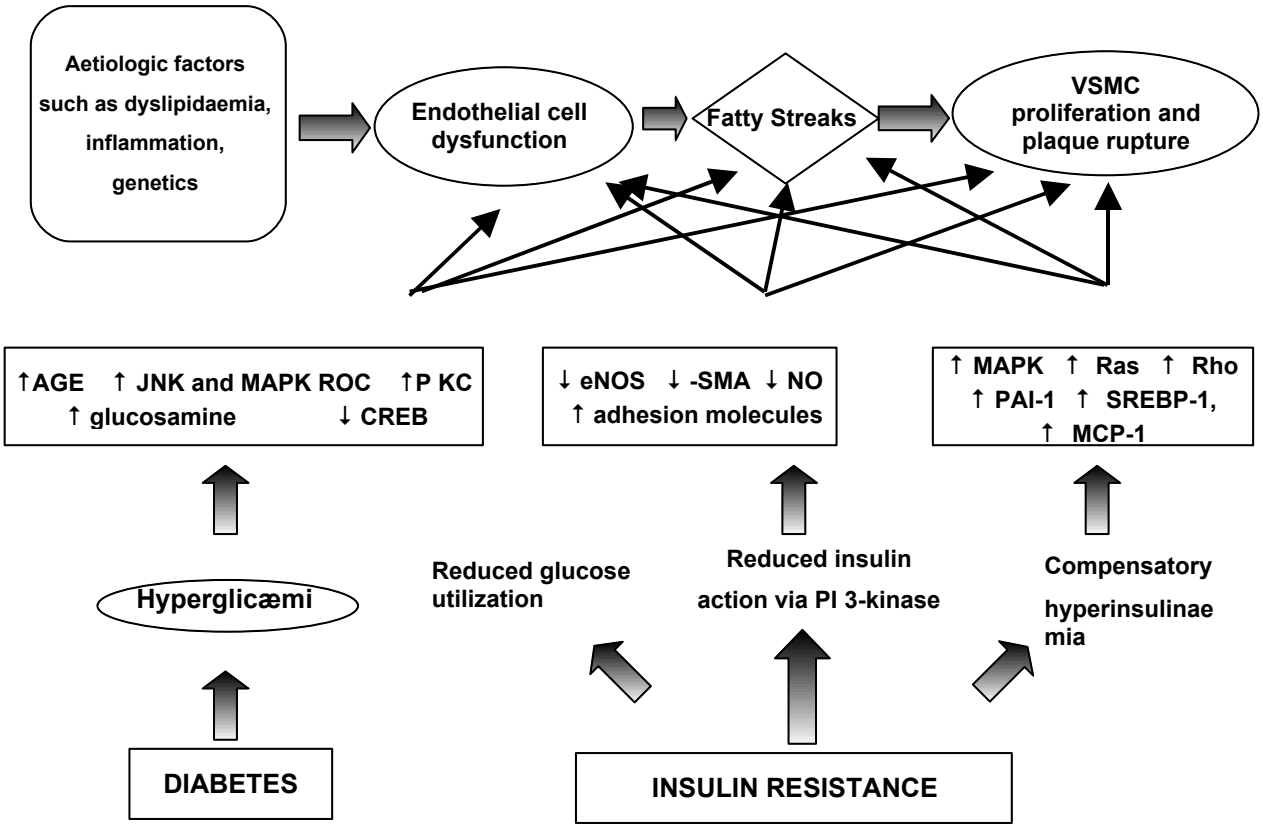


Fig. 3 Influence of diabetes and insulin resistance on progression of atherosclerosis. (AGE, advance glycosylation end products; CREB, cAMP response element–binding protein; eNOS, endothelial NO synthase; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PKC, protein kinase C; ROC, reactive oxygen species; a-SMA, SM-actin; SREBP-1, sterol regulatory element–binding protein-1; VSMC, vascular smooth muscle cells.

pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) was observed to be markedly increased in obese mice, and neutralisation of TNFα led to an improvement in insulin resistance. The enhancement of inflammation by a diminished endothelial insulin response could in itself be one possible explanation, since insulin exerts anti-inflammatory effects at the cellular and molecular level both *in vitro* and *in vivo.* A low-dose infusion of insulin has been shown to suppress NADPH oxidase expression and ICAM-1 and MCP-1 concentrations. Conversely, a longer-term insulin infusion (over four hours) in normal subjects was associated with induction of endothelial dysfunction. Type 2 diabetes may in part be precipitated or accelerated by an acute phase reaction as part of the innate immune response, in which large amounts of cytokines are released from adipose tissue. These cytokines (TNF-alpha, IL-6, PAI-1) and adipokines (adiponectin and leptin) have been suggested to be associated with inflammation and insulin resistance.

Finally, the metabolic syndrome and type 2 diabetes are characterised by several haemodynamic and metabolic abnormalities. In the increased CVD risk found in persons with diabetes and hypertension, endothelial dysfunction would play an important role. Alterations in the vascular endothelium linked to diabetes include elevated expression and plasma levels of

vasoconstrictors, such as angiotensin II and endothelin-1, increased expression of adhesion molecules and associated enhanced adhesion of platelets and monocytes to vascular endothelium, plus impairment of NO release and reduced NO responsiveness. In conclusion, endothelial dysfunction seems to be the trigger in atherogenesis and diabetes-associated vascular disease and explains, at least in part, the enhanced progression of CVD in type 2 diabetes.

Chapter 4

Endothelial Progenitor Cells

In 1997 it was first observed that cells present in the adult circulation of human healthy volunteers acquired an endothelial cell-like phenotype in vitro and incorporate into capillaries in vivo (48). These bone-marrow derived cells were defined endothelial progenitor cells (EPCs)

Until then, only resident endothelial cells were thought to participate to vessel formation and remodelling, processes referred to as vasculogenesis, angiogenesis and atherogenesis. Vasculogenesis denotes de novo blood vessel formation during embryogenesis, in which angiogenic progenitor cells migrate to vascularization sites, differentiating into endothelial cells. The formation of new capillary-like vessels from an existing blood vessel is termed angiogenesis, while arteriogenesis refers to existing artery remodelling (49). Angiogenesis leads to the creation of small, high-resistance capillaries and arteriogenesis to large interconnetting arterioles. Both angiogenesis and arteriogenesis appear to be necessary for the resolution of tissue ischemia. Clinical angiogenesis trials using angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) failed to reverse ischemic condition. The formation of small peri-ischemic capillaries is insufficient.

Thereafter, findings on EPCs have drastically changed the understanding of new vessel formation, since endothelial progenitor cells showed having a potential that relies to their ability to mature into endothelial cells: re-endothelization and neovascularization. It is now

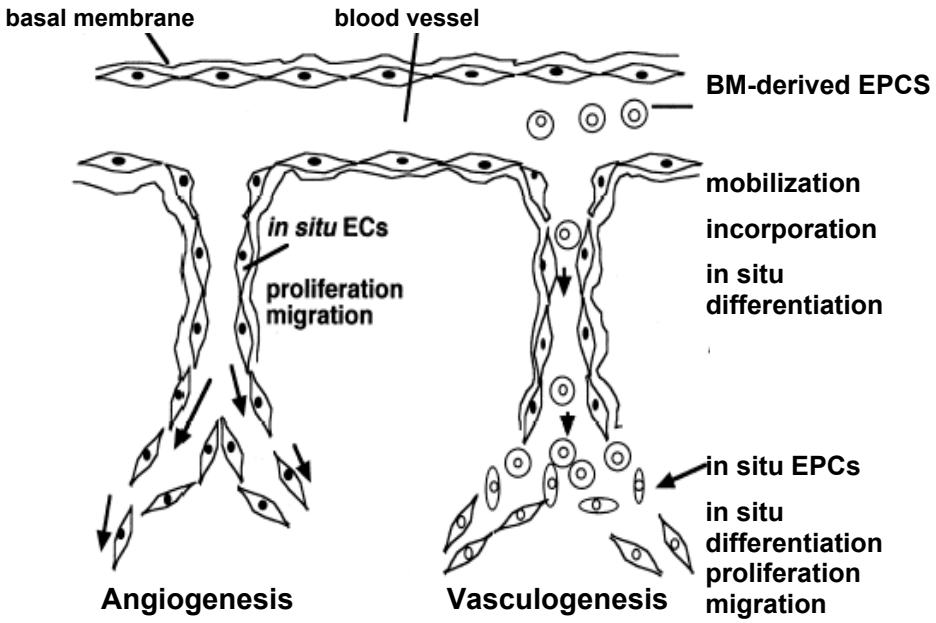


Fig. 4 Postnatal neovascularization in physiological or pathological condition via angiogenesis and vasculogenesis (Iwaguro H et al. 2005).

recognised that a portion of normal endothelium derives from circulating EPCs, that are incorporated in situ with subsequent differentiation (50). Moreover, studies evidenced the EPCs should reside in vessel walls (51).

Vasculogenesis is in response to endothelial injury, due to wound healing (52), limb ischemia (48), burns, coronary bypass and myocardial infarction (53), or in response to physiological processes, as for endometrium organogenesis (54).

In vivo studies, in which donor bone-marrow derived EPCs had been marked before injection, showed that EPC contribution to new vessel formation vary from 5 to 25% in response to "exogenous" stimula, i.e. granulocyte-colony stimulating factor (G-CSF) pretreatment (55) or angiogenic growth factor pretreatment (56). Moreover, in tumor vasculogenesis, this rate can raise to 35-45% (57).

EPCs Isolation

EPCs can be isolated from peripheral blood (48), cord blood and bone marrow. Recently, EPCs have been isolated in different tissues, such as the endothelium itself (51), skeletal muscle tissue (58), cardiac tissue (59), adipose tissue (60) and finally, nervous tissue (61).

Bone Marrow	Peripheral blood	Tissue-resident cells
hematopoietic stem cells	CD133+/KDR* cells	Endothelial tissue
SP-cells	Myeloid intermediates	Adipose Tissue (HSC, MSC)
Mesenchymal stem cells		Cardiac progenitor cells
		Neuroprogenitor cells

Tab.4. Various cell types which have been differentiated into endothelial cells.

Asahara and coworkers first isolated EPCs from human peripheral blood by magnetic-bead immunoselection of mononuclear cells (MNCs) expressing the hematopoietic antigen CD34 ($MNCs^{CD34+}$) (48). They first described that these bone-marrow derived hematopoietic stem cells (HSCs) can give rise to ECs and contribute to endothelial recovery. Isolated cells were cultured for 7-10 days under endothelial differentiating conditions and gave origin to adherent, spindle-shaped cells: the so-called AT cells (spindle-shaped adherent and attaching cells). Once injected in mouse ischemic limb, these cells contributed to neovascularization.

Because CD34 is not exclusively expressed in hematopoietic stem cells but, at lower level, in ECs too, EPC linage was characterized via the expression of another hematopoietic marker, a

120 kDa glycosilated polypeptide, with 5 transmembrane-domains, CD133 (initially called AC133) (62). CD133 expression defines a very early population of progenitor cells, immature HSCs, in which the two sub-fractions of adult bone-marrow stem cells (the hematopoietic and the mesenchymal subset) seem to be represented.

Because of MNC population heterogeneity, several other studies have tried to better define the EPC characteristics. EPCs should express CD34, CD133 and VEGF receptor-2 (VEGFR-2), also known as flk-1 or KDR.

Recent studies have shown that also cells expressing CD14 antigen (monocytes/macrophages) (63), can effectively participate to neovasculogenesis, either CD34 negative or CD133 negative (64). Despite the different subpopulation characteristics, also these cells show analogue vasculogenic abilities (65), suggesting that EPCs represent a heterogeneous group at various differentiation stages.

EPC origin and differentiation

Evidences suggest that, in embryonic development, hematopoietic stem cells and EPCs share some markers and possibly derive from a common precursor: the hemangioblast (66).

The contribution of HSCs to neovascularization improvement has been documented by using bone marrow transplantation with tagged cells to replenish HSCs after irradiation. These tagged cells home to sites of ischemia and contribute to vessel formation. Therefore, hematopoietic stem cells were suggested as candidates of EPC precursor cell population. The first studies supporting this concept were performed with isolated cells expressing the classic HSC marker protein CD34 or the more immature one CD133. Both cell population differentiated in ECs in vitro under appropriate endothelial differentiating conditions.

HSCs and EPCs share some surface proteins, such as CD34, CD133, VEGFR-2, Tie-2 (angiopoietin 1 receptor), CD117 and Sca-1 (stem cell antigen-1). Once EPCs are mobilized from bone-marrow, they begin to loose stem cell markers (initially CD133 and later CD34) and to express endothelial-specific markers, such as von Willebrand factor (vWF), vascular endothelial-cadherin (VE-cadherin) and E-selectin (67). Gulati and coworkers suggested the classification of cultured EPCs in two types: early EPCs, which at 7 day culture present the monocyte marker CD34, and late outgrowth endothelial cells (OECs) (68). These cells appear from the third week of culture on and are developed exclusively from the CD14- fraction. Human OECs, but not EPCs, expressed the key regulatory proteins endothelial nitric oxide synthase (eNOS) and caveolin-1. The CD14- population express endothelial markers and have

great expansion capacity. Successive studies confirmed the expression of monocyte markers (CD14) or macrophage ones (CD11 e CD45) in 4-7 day cultured EPCs (63), that is associated to a lower proliferation capacity than HSCs or cord-blood derived EPCs.

Finally, these monocyte derived cells present a CD133+/-, CD34+, VEGFR-2+, CD14+, VE-cadherin-, and eNOS- phenotype. From the CD14- subpopulation it is possible to obtain late EPCs with a more endothelial-like phenotype (CD133-, CD34+/-, VEGFR-2+, CD14-, VE-cadherin+, eNOS+, and vWF+).

However, the heterogeneity of EPC population make it difficult to have a precise characterization of effective EPCs.

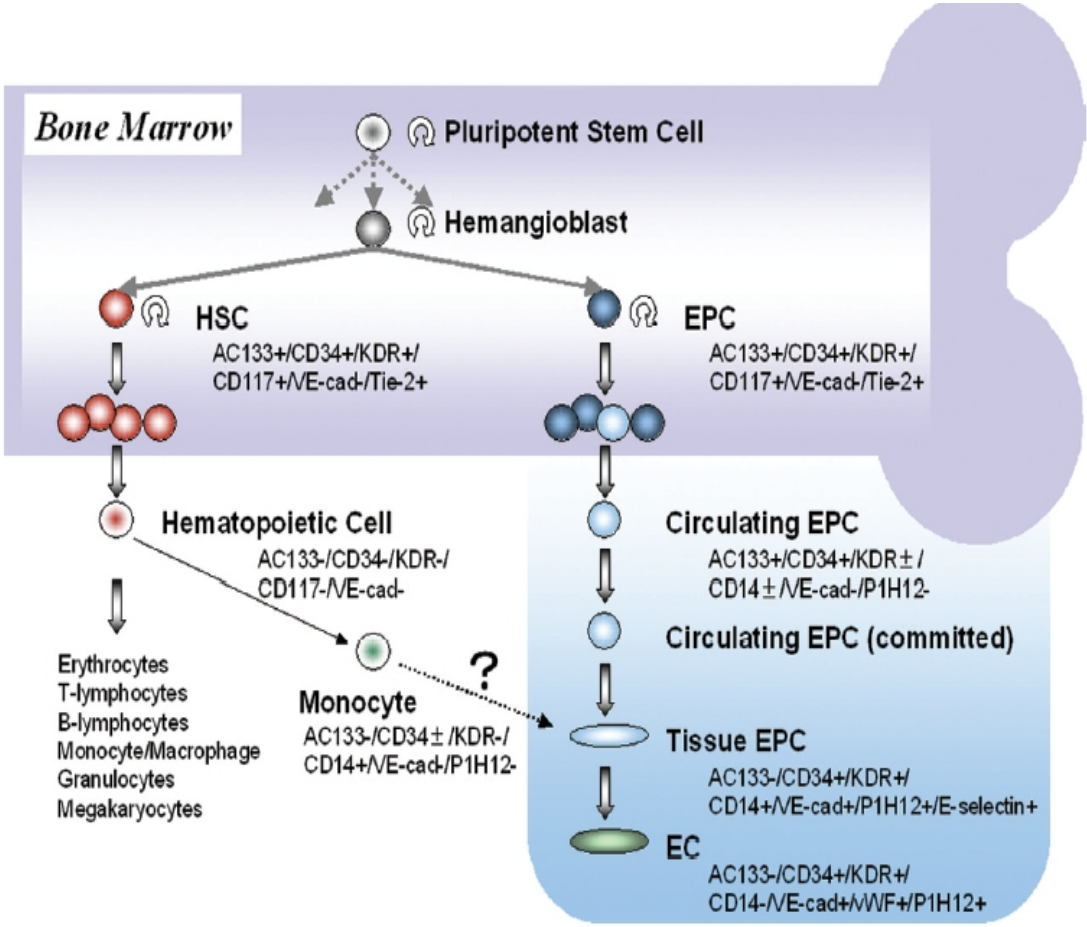


Fig. 5 Putative cascade and expressional profiles of human bone marrow-derived endothelial progenitor cell differentiation. (+: positive, –: negative).

A comparative study evidenced that, just after isolation, MNCs in toto, both CD14- and CD14-, are ineffective for neovascularization. Nevertheless, after 7 day culture in the same endothelial-differentiating conditions, both cell subpopulations had analogue ability to incorporate in newly blood vessels and improved neovascularization (65).

EPC characterization

As we previously said, it does not exist a way to unequivocally define EPCs, excepting the capacity of a non-EC to adapt an endothelial phenotype, and the stemness characteristics. However, a series of parameters can be adopted in order to better characterize EPCs.

a. Morphological characteristics

Whichever the EPC subtype is, the cell has a spindle shape and is adherent to the substratum. Asahara first defined them as spindle-shaped adherent and attaching cells (AT cells).

Others defined hematopoietic cell progenitors by their ability to form colonies or small clusters of cells. Colonies are defined as a cell mass composed of a central cord of round cells with elongated spindle-shaped cells sprouting at the periphery of the colony, namely colony-forming unit endothelial cells (CFU-ECs). The cluster of round cells sits on top of spindle shaped adherent cells that emigrate from the base of the cell cluster. Over time, the cluster of cells disappears, leaving the adherent spindle-shaped cells that display features of endothelial cells. Some authors consider only the CFU cells the real endothelial progenitor (69). After a long-term culture (3-4 weeks), colonies disappear and late progenitor cells, OECs, are observed. These cells are disposed in a monolayer, with a typical cobblestone aspect of mature endothelial cells (68). Moreover, thanks to electronic microscopy, it has been possible to observe cylindric structures, containing microtubules, which are characteristic of vascular endothelium (70)**.**

b. Antigenic characterization

One of the most important endothelial markers is the VEGF receptor-2, that is the only marker that is expressed in the hemangioblast, and keeps to be expressed during cell differentiation (67). VEGF, an endothelial-specific mitogen and survival factor, is one of the most potent angiogenic factors. It is crucial for both angiogenesis and vasculogenesis, promoting, for instance, EPC recruitment (54) and is used in all endothelial differentiating cocktails. VEGF contribute to cell mobilization also via VEGF receptor 1 (VEGFR1 of flt-1).

Moreover, other markers whose expression is studied for EPC characterization are vWF, some endothelial adesion molecules, such as platelet endothelial cell adhesion molecule (PECAM-1 o CD31), VE-cadherin, and E-selectin. In addition, being the endothelial NOS constitutively expressed by vascular endothelial cells, its expression is eventually considered a marker of endothelial differentiation.

c. Functional Characteristics

As previously said, some authors think that the colony forming ability can be considered as a typical characteristic of hematopoietic cells. According to Ingram and coworkers, by plating hematopoietic cells in special double-layer agar cultures with multiple recombinant cytokines, it also possible the identification of two types of clonal hematopoietic progenitors: the highly proliferative (high-proliferative potential–colony-forming cells or HPP-CFCs) and the more differentiated low-proliferative potential–colony-forming cell (LPP-CFC), HPP-CFC colonies are visible in the culture dishes without need for magnification. HPP-CFC clones could be replated in CFC assays with emergence of secondary HPP-CFCs, as well as committed erythroid and myeloid and multipotent progenitors. On the contrary, LPP-ECFCs form smaller colonies but do not form secondary LPP-ECFC colonies upon replating; this EPC stage represents the most proliferative population of EPCs that can be isolated from healthy adult peripheral blood. They do give rise to endothelial cell clusters and these endothelial cluster-forming cells give rise only to mature non-dividing ECs.

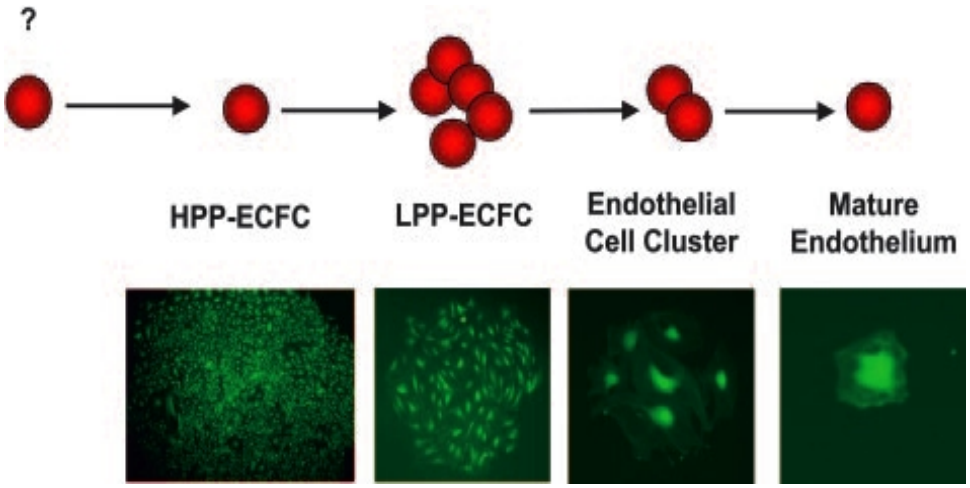


Fig. 6. Endopoiesis: the process of endothelial cell development suggested by Ingram DA et al. 2005.

Another important functional characteristic is the capacity to form cord- and tubular-like structures in three-dimensional gels, like Matrigel (71), as mature endothelial cells do. Additionally, migratory capacity of EPCs is tested. It can be assessed by their ability to cross the membrane of a modified Boyden chamber, in presence of chemotactic stimuli. Migrated cells are collected in the transwell support, then are counted by light microscopy. NO production is also considered an index of endothelial functionality.

A commonly used characterization of endothelial differentiation is based on EPC functions of uptaking acetylated low density lipoproteins (AcLDL) and binding lectin, *Ulex europaeus*

agglutinin-1 (UEA-1). While ac-LDL uptake is dependent on scavenger receptors common to monocytes, UEA-1 binding is considered more specific for endothelial cells.

Mobilization and homing of EPCs

The mobilization of EPCs from the bone marrow is a complex process, regulated by a variety of enzymes, growth factors and surface receptors (72). An initial step in this recruitment is represented by the activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane bound Kit ligand to a soluble Kit ligand (sKitL). This activation is followed by detachment of early cKit-positive progenitor cells from the bone marrow stromal niche and their subsequent movement to the vascular zone of the bone marrow. Growth factors such as VEGF, PIGF, Ang-1 and stromal-derived factor-1 (SDF-1) induce the mobilization and the expansion of EPC and HSC populations in the bone marrow. VEGF, which binds to its receptor VEGFR-2 (termed also Flk-1 or KDR), mediates the further maturation of the axis "bone marrow angioblast-early EPC-late EPC". VEGF may also activate MMP-9, and that would confirm its key role in the process of recruitment and release of EPCs. In addition, eNOS expressed by bone marrow stromal cells may also influence the recruitment of angiogenic progenitor cells and thus play an essential role for their mobilization, since recent data have demonstrated impaired mobilization of EPCs in eNOS-deficient mice (73). Furthermore, this eNOS deficiency was related to a reduced activity of MMP-9.

Physiologically, ischemia is believed to be the predominant signal to induce mobilization of EPCs from the bone marrow (74). Thereby, ischemia is believed to upregulate VEGF or SDF-1, which in turn are released to the circulation and induce mobilization of progenitor cells from the bone marrow via a MMP-9-dependent mechanism. Additional factors inducing mobilization of progenitor cells from the bone marrow have been initially discovered in hematology to harvest hematopoietic stem cells from the peripheral blood for bone marrow transplantation. For instance, granulocyte-colony stimulating factor (G-CSF), a cytokine, which is typically used for mobilization of $CD34^+$ cells in patients, also increased the levels of circulating endothelial progenitor cells. A related cytokine, the granulocyte monocyte-colony stimulating factor (GM-CSF), augments EPC levels. Moreover, erythropoietin (EPO), which stimulates proliferation and maturation of erythroid precursors, also increased peripheral blood endothelial progenitor cells in mice and in men. The correlation between EPO serum levels and the number of $CD34^+$ or $CD133^+$ hematopoietic stem cells in the bone marrow in patients with ischemic coronary artery disease further supports an important role of endogenous EPO levels

as a physiologic determinant of EPC mobilization. At present, it is not clear which of the mobilizing factors most potently elevates the levels of EPCs. Whereas a similar increase in white blood cell counts was achieved by G-CSF application, the number of endothelial colonies (CFU-EC) was significantly lower in G-CSF- compared to VEGF- or SDF-1-treated mice. In addition, the extent of increasing neutrophil and lymphocyte levels, which may provoke proinflammatory responses, has to be considered for a potential therapeutic application.

It has also been observed a pharmacological modulation of systemic EPC levels. Statins were shown to increase the number and the functional activity of EPCs in vitro, in mice, and in patients with stable coronary artery disease. The increase in EPC numbers was associated with increased bone marrow-derived cells after balloon injury and accelerated endothelial regeneration. Recent studies demonstrated that estrogen increased the levels of circulating EPCs.

Once mobilized, EPCs were released from the vascular zone of the bone marrow into the circulation. To date, no clear definition exists as to when a circulating EPC turns into a mature, fully differentiated endothelial cell *in vivo*. An initiation of this differentiation may by the migration of EPCs from the bone marrow into the peripheral circulation and after adhesion and insertion into the monolayer of surrounding mature endothelial cells this differentiation process

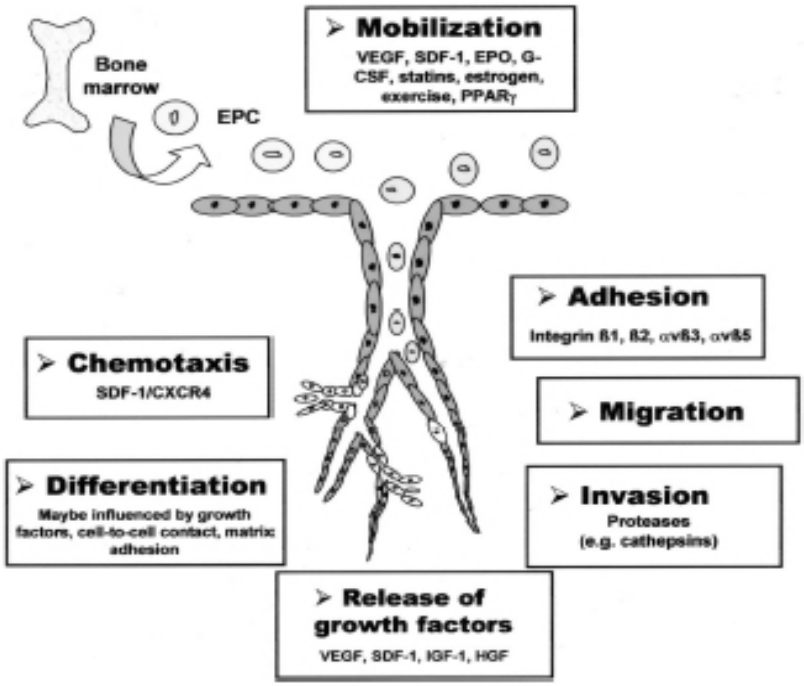


Fig. 7. Mechanism of EPC homing and differentiation. Factors that are proposed to regulate the distinct steps are indicated (Urbich C. et al. 2004)

may be completed. However, the exact differentiation cascade of EPCs *in vivo* is hardly comprehensible.

Adhesion molecules of the selectin and integrin family are also essential for EPC arrest to endothelial cells (75). EPCs have been found to exhibit surface expression of the P-selectin glycoprotein ligand-1 (PSGL-1) and to be recruited on activated endothelial cells in a P- and E-selectin-dependent manner. Moreover, chemokines have been shown to trigger integrin activation to mediate arrest of rolling leukocytes. Isolated human EPCs demonstrated significantly increased and constitutive adhesion on the β2-integrin ligands fibrinogen and ICAM-1 under physiological flow conditions *in vitro*. Functionally active β2-integrins (LFA-1, Mac-1) further mediated arrest of EPCs to mature endothelial cells and their subsequent emigration into tissues, implying the importance of this integrin subclass not only after arterial injury but also during angiogenesis in ischemic regions, where sprouting of newly formed capillary structures is required. The association of β2-integrins with the homing of hematopoietic progenitor cells (including also EPC populations) during neovascularization was demonstrated in a mouse model of hind limb ischemia (76). In addition to β2-integrins, β1-integrins have also been involved in the homing of bone marrow-derived progenitor cells to the remodeling vasculature. The α4β1 integrin (also known as very late antigen-4, VLA-4) plays an important role in EPC homing. Blockade of VLA-4 significantly reduces the incorporation of CD34+ cells to areas of active tumor vascularization, and, recent data revealed a critical involvement of VLA-4 in the homing of human EPCs to ischemic tissue (77). Vascular cell adhesion molecule (VCAM) but also fibronectin are well known ligands for VLA-4. While VCAM is primarily up regulated in ischemic tissue, interaction of VLA-4 with fibronectin occurs mainly when denuded vascular ECM is exposed to the blood flow, e.g. after endothelial injury.

It is conceivable that coating of biomaterials by EPC attracting compounds may be helpful for tissue engineering. Similar in-vivo effects on accelerated reendothelialization were observed after short-term treatment with statins, when a significant up-regulation of α5, β1 and αvβ5 integrins on the EPC surface was also noted. Local delivery of VEGF-A within a region of endothelial denudation, e.g. after stent implantation, may be helpful to selectively attract EPCs and to achieve stent endothelialization, but has to be scrutinized with caution in the context of advanced atherosclerosis due to the risk of plaque destabilization. Finally, coating strategies with antibodies against progenitor cell antigens (e.g. CD34) were reported to effectively accelerate endothelialization by capturing circulating EPCs after stent placement or vascular graft implantation.

Conditions and factors influencing the number and the recruitment of EPCs

Physiological variations in the number of EPCs have been recently described. For example, estrogens and physical training enhance the number of circulating EPCs. Several studies have further described the influence of various pathological conditions and some drugs and growth factors on the number of EPCs *in vivo* (see Table 5). In this context, the number of circulating EPCs and their migratory activity have been reported to be reduced in patients with risk factors for ischemic cardiovascular disease, such as genetic predisposition or smoking.

The functional capacity of EPCs to form CFUs is also negatively correlated with the Framingham risk score. Furthermore, EPCs from patients with diabetes mellitus type 2 are characterized by a decreased proliferative capacity, impaired homing and reduced ability for formation of capillary tubes *in vitro* (78). A similar EPC-dysfunction was found in patients with type 1 diabetes. On the other hand, acute myocardial infarction is associated with mobilization and a rapid increase of circulating EPCs. Similarly to acute cardiovascular events, vascular trauma such as coronary bypass grafting or burn injury induces a rapid but transient mobilization of VEGFR2+/AC133+ EPCs. Additionally, a biphasic variation in the EPC-number has been oberved in patients with chronic heart failure, namely elevation in the early and reduction in the advanced phases (79).

Drug intake can also influence the number and the migratory activity of EPCs. For example, treatment with HMG-CoA reductase inhibitors increase the number of circulating EPCs, which may contribute to endothelial repair after balloon catheter injury or to angiogenesis, by activation of the PI3-kinase/Akt pathway in EPCs. The number of EPCs can also be increased after application of several chemokines and cytokines, such as VEGF, placental growth factor, erythropoietin or G-CSF. Some chemokines regulating the recruitment of mononuclear cells during the development of atherosclerotic vascular lesions may influence also the recruitment of EPCs. For example, early EPCs can be mobilized by stromal cell-derived factor-1 (SDF-1), that has been shown further to protect EPCs from apoptosis.

EPCs and atherosclerosis

If EPCs contribute to an enhanced endothelial cell repair after focal endothelial cell damage, EPCs may have a pivotal role in maintaining the integrity of the endothelium in conditions of a dissiminating endothelial cell damage as seen for example in endothelial dysfunction, the earliest manifestation of atherosclerotic disease.

Condition or factor	Changes in number/function of EPCs or CD34+ cells	Investigators
Physiological:		
Gender (eg, estrogens)	↑ CD34+/VEGFR2+ cells	Strehlow et al., *Circulation* (2003)
Physical training	↑ EPC number	Adams et al., *ATVB* (2004)
Pathological:		
Coronary artery disease/number of risk factors	↓ EPC number and migration	Vasa et al., *Circ Res* (2001)
	↓ CD34+/KDR+ cells	
Smoking	↓ EPCs or CD34+/KDR+ cells	
Family history	↓ EPCs or CD34+/KDR+ cells	
Hypertension	↓ EPC migration	
Cumulative cardiovascular risk factor score	↓ EPC CFUs	Hill et al., *N Engl J Med* (2003)
Myocardial infarction	↑ CD34+ cells	Shintani et al., *Circulation* (2001)
	↑ CD34+/AC133+/VEGFR2+ cells	
Vascular injury	↑ AC133+/VEGFR2+ cells	Gill et al., *Circ Res* (2001)
Congestive heart failure (class I-II)	↑ CD34+ cells	Valgimigli et al., *Circulation* (2004)
	↑ CD34+/AC133+/VEGFR2+ cells	
	↑ EPC CFUs	
Congestive heart failure (class III-IV)	↓ CD34+ cells	
	↓ CD34+/AC133+/VEGFR2+ cells	
	↓ EPC CFUs	
In-stent restenosis	↓ EPC CFUs	George et al., *ATVB* (2003)
	↓ EPC adhesion	
Drugs and cytokines:		
HMG-CoA reductase inhibitors	↑ EPC number	Dimmeler et al., *J Clin Invest* (2001)
G-CSF	↑ CD133+/VEGFR2+ cells	Peichev et al., *Blood* (2000)
Erythropoietin	↑ CD34+/CD45+ cells	Bahlmann et al., *Blood* (2004)

Table 5. Conditions, drugs and cytokines that may affect number and function of human EPCs.

In a key publication, Rauscher and colleagues demonstrated that the systemic transfusion of stem and progenitor cells derived from young nonatherosclerotic ApoE-/- mice prevents atherosclerotic lesion progression in ApoE-/- recipients despite persistent hypercholesterolemia (80). Treatment with bone marrow stem cells from aged ApoE-/- mice with manifest atherosclerosis did not effectively prevent atherosclerotic lesion progression. Apparently, endothelial cell repair capacity depends on the age of stem cells, underlining the important influence of cardiovascular risk factors on the bone marrow. First evidence that EPCs have a vasculoprotective action in patients with atherosclerotic disease comes from the *Endothelial Progenitor Cells in Coronary Artery Disease* (EPCAD) study. The number of CD34+/KDR+ EPCs was measured in 519 patients with angiographically documented CAD and correlated with cardiovascular outcomes (81). Primary endpoints included cardiovascular mortality, the occurrence of a first major cardiovascular event (myocardial infarction, hospitalization,

revascularization, and cardiovascular death), revascularization, hospitalization, and all-cause mortality after 12 months. After adjustment for vascular risk factors, drug therapy, and concomitant disease, increased EPC levels were independently associated with a lower risk for cardiovascular events. In a subgroup of patients the number of colony forming units endothelial cells (CFU-EC), considered as a marker of the clonogenic potential of formerly circulating EPCs, was determined. It resulted that the number of circulating cells the functional capacity is closely correlated with cardiovascular event rates. In a study by Hu *et al.* neointimal lesions in allografts contained endothelial cells derived from circulating progenitor cells, suggesting a role of EPCs in plaque angiogenesis (82).

EPCs and Diabetes

Numerous studies have demonstrated an effect of hyperglycaemia on EPC biology. Culturing EPCs derived from healthy subjects under high glucose conditions impaires EPC number and function (83). Glucose effect on EPCs is because of a dose and time-dependent activation of the p38 mitogen-activated protein (MAP) kinase. If cultured in high glucose conditions, these cells present impaired function and decreased nitric oxide and matrix metalloproteinase (MMP)-9 production (84). BM-MNCs derived from streptozotocin-induced diabetic mice differentiate less efficiently into EPCs. Albeit, they have a lower expression of eNOS and VEGF. Injecting non-diabetic EPCs in diabetic and non-diabetic recipients, no real difference is observed in cell recruitment, suggesting that short term exposure barely affects EPC function in vivo. The impaired neovascularization associated with diabetes may be explained by the inability of diabetic EPCs to respond to SDF-1, an important factor in EPC homing.

Several studies in type 1 and type 2 diabetes confirmed these preliminar findings. In fact, type 1 diabetic patients present a reduced number of EPCs and angiogenic capacity.

A reduced number of EPCs is observed in type 2 diabetes, and a further reduction has been seen in those patients affected by peripheral vascular disease. Cells isolated from patients with type 2 diabetes demonstrated a decrease in the adhesion to activated endothelial cells, but normal adhesion to quiescent endothelial cells, and matrix molecules (78).

However, in patients with diabetic retinopathy (DR) it has been observed a different EPC concentration. These patients have a higher number of EPCs, and higher plasma levels of VEGF. This contrast has been termed “diabetic paradox”. The enhanced endothelial differentiation of circulating progenitor cells characterizes DR patients, as shown by the high $CD34^{+}KDR^{+}$ proportion and the better EPC efficiency (85).

Many therapeutic treatments, such as the use of ACE inhibitors and statins, glycaemic control by use of insulin or oral hypoglycamic agents, have been shown to augment EPC number and function. Further approaches, such as EPC administration, may represent a novel treatment for diabetic vasculopathy.

Possible Clinical Application of EPCs as Diagnostic and Therapeutic Tools

The number and the functional characteristics (eg, formation of CFUs, adhesion potential) of EPCs may be successfully used as diagnostic tool and/or surrogate prognostic marker for ischemic heart disease.

The possible clinical application of EPCs consist of two directions: neo-vascularization of ischemic tissue or endothelialization of denuded endothelium (eg, after balloon angioplasty) and of vascular grafts. However, an important limitation for a therapeutic use of autologous post-natal EPCs is their low number in the circulation. This low cell number is further reduced in patients with risk factors for ischemic coronary heart disease. Approaches to overcome this problem include three strategies: (I) the *ex vivo* transfection with different genes, (II) the mobilization of EPCs *in vivo*, or III) the local infusion of autologous bone marrow cell suspensions without preselection. Recent studies demonstrated a significant increase in the number of isolated human EPCs after *ex vivo* transfection with adenovirus encoding VEGF164 (86). Gene transfer of the target ischemic tissue with angiogenic chemokines might be another effective strategy for therapeutic neo-vascularization. Thus, intramuscular or intramyocardial VEGF gene transfer has been shown to mobilize EPCs in patients with limb ischemia or inoperable coronary disease. Similar results were observed in a very recent animal study, employing an intramuscular gene transfer of SDF-1 for mobilization of EPCs (87). An alternative for mobilization of EPCs may be represented by the HMG-CoA reductase inhibitors. Several studies in animals have shown a therapeutic potential of EPCs for improvement of the cardiac function after myocardial infarction (88). Furthermore, transplanted bone marrow-derived angioblasts protect cardiomyocytes from apoptosis and reduce the negative myocardial remodeling after infarction. Consequently, the possibility for autologous transplantation of bone marrow-derived mononuclear cells, including also the EPCs fraction, could represent a new strategy to obtain therapeutic angiogenesis also in humans. This was explored for the first time in patients with chronic limb ischemia, which showed a significant improvement of the function of the ischemic limbs during a 6-month follow-up period after transplantation of mononuclear cells (89). Further clinical studies evidenced the

capacity of autologous bone marrow-derived cells or isolated and *ex vivo* expanded autologous EPCs for partial repair of infarcted myocardium in humans. However, it has not to be excluded the possibility of side-effects, such as uncontrolled neovascularization (*i.e.*, tumor-, plaque or neointimal-growth).

In order to overcome this problem, a possible solution could be the local application of EPCs close to the ischemic/injured area (eg, intra-coronary or intra-myocardial after myocardial infarction).

Another intriguing direction of therapeutic application for EPCs is the construction of endothelial-coated vascular grafts in the context of tissue engineering or the use of EPCs for reendothelialization after balloon catheter injury. In this case, a cell-based gene therapy strategy may also be used. For example, an interesting study in rabbits demonstrated that transplantation of autologous EPCs overexpressing eNOS in injured vessels clearly improved the function of reconstituted endothelium, leading to prevention of thrombosis and to inhibition of the neointimal hyperplasia (90).

One critical point is the definition itself of these endothelial progenitor cells. As mentioned above, it does not exist a single phenotype which uniquely characterizes EPCs. Endothelial progenitor cells from various sources and with dramatically different expression patterns of surface markers have been used successfully to improve neovascularization (91). Moreover, early spindle-like cells and late outgrowing EPCs showed comparable in vivo vasculogenic capacity. One interpretation could be that different cells share a similar capacity. Alternatively, another explanation could be the lack of appropriate marker(s) to define the effective population of cells. Given that only a minor percentage of these cells are incorporated into the capillaries, a contaminating population common in the different cell sources may account for the biological effects in vivo.

The second critical point is how these endothelial progenitor cells enhance neovascularization. Various studies showed that bone marrow–derived cells incorporate into the newly formed capillaries and express endothelial markers, suggesting that endothelial progenitor cells enhance neovascularization by physically contributing to the newly formed capillaries. However, the absolute number of incorporated endothelial progenitor cells dramatically varies between 0% and 90% in the different studies. The experimental animal model (tumor angiogenesis versus limb ischemia), the time point assessed after ischemia, and the use of the endothelial marker proteins, which are more or less specific for endothelial cells, may have contributed to these dramatically different numbers. To achieve a functional improvement,

EPCs need to be ex-vivo cultured to enrich an active subpopulation (which is approximately 0.5% of total monocytic cells) out of peripheral mononuclear cells.

Another possible mechanism for EPC enhanced neovascularization could be a paracrine activity of these cells, by the release of proangiogenic factors (74). EPCs may act similar to

Target	Donor	Recipient	Type and Source of Cells	Method of Delivery	Therapeutic Effects
Preclinical					
Myocardial ischemia	Swine	Autologous	$CD31^+$, peripheral blood	Transendocardial with NOGA mapping	↑Rentrop score, ↑EF, ↑capillary density
Myocardial ischemia	Swine	Autologous	MNC, bone marrow	Transendocardial	↑capillary density, ↑collateral flow, ↑ myocardial contractility
Hibernating myocardium	Swine	Autologous	MNC, peripheral blood	Transendocardial	↑EF, ↑capillary density, ↑flow
			MNC, bone marrow		↑EF, ↑collateral flow
Myocardial ischemia	Rat	Autologous	MNC, bone marrow	Intramyocardial	↑capillary density
Myocardial infarction	Human	Nude rat	$CD34^+$, peripheral blood	Intramyocardial	↑EF, ↑capillary density, ↓fibrosis
			MNC, peripheral blood	Intravenous	↑EF, ↑capillary density, ↓fibrosis
Myocardial infarction	Human	Nude rat	$CD34^+$, bone marrow	Tail vein injection	↑EF, ↑capillary density, ↓fibrosis, ↓apoptosis, ↓infarct size
Myocardial infarction	GFP mouse	Syngenic mouse	$Lin^-c\text{-}kit^+$, bone marrow	Peri-infarct region	↑LVDP, ↑capillary density, ↓infarct,
Myocardial infarction	Mouse	Autologous	bone marrow mobilization	Homing	↑EF, ↑capillary density, ↓ remodelling,
Hind limb ischemia	Rat	Autologous	MNC, bone marrow	Intramuscular injection in Gastrocnemius	↑capillary density, ↑blood flow, ↓$AVDO_2$, ↑exercise capacity
Hind limb ischemia	Rabbit	Autologous	MNC, bone marrow	Intramuscular injection in thigh	↑capillary density, ↑blood flow
Hindlimb ischemia	Human	Athymic mice	MNC, peripheral blood	Intracardiac injection	↑capillary density, ↑blood flow
Hind limb ischemia	Human	Athymic mice	MNC, peripheral blood overexpressing VEGF	Tail vein injection	↓autoamputation, ↑capillary density, ↑blood
Hind limb ischemia	Human	Nude rat	MNC, peripheral blood	Intramuscular injection in thigh	↑capillary density, ↑blood flow
			MNC, chord blood		
Clinical					
Myocardial infarction	Human	Autologous	$CD133^+$, bone marrow	Infarct border	↑EF, ↑collateral flow (SPECT)
Myocardial infarction	Human	Autologous	MNC, bone marrow	Intracoronary balloon catheter	↓infarct size, ↑wall motion,
Myocardial infarction (TOPCARE-AMI trial)	Human	Autologous	MNC, bone marrow, peripheral blood	Intracoronary balloon catheter	↑contractility, ↑myocardial perfusion ↓remodeling ↑EF
Myocardial infarction (BOOST trial)	Human	Autologous	$CD34^+$, bone marrow	Intracoronary during PCA	
Myocardial infarction	Human	Autologous	$CD34^+/CD117^+/AC133^+$	Intracoronary with PCA	↑EF, ↑LV wall thickness, ↓ESV
Myocardial ischemia (Unstable angina)	Human	Autologous	MNC, bone marrow	Transendocardial with NOGA mapping	↓anginal episodes, ↑wall thickening, ↑wall motion, ↑EF
Myocardial infarction	Human	Autologous	$CD34^+$	1: intracoronary, 2: G-CSF	↑EF, ↑exercise time, ↑myocardial
(MAGIC trial)				mobilization	perfusion, ↑angiogenesis
Hind limb ischemia	Human	Autologous	MNC, bone marrow	Intramuscular injection in Gastrocnemius	ankle-brachial index, ↑pain-free walking, ↑transcutaneous PO_2

Table 6. Preclinical and Clinical Studies of Neovascularization Therapies (Dzau VJ et al, 2005).

$AVDO_2$ indicates arteriovenous oxygen difference; EF, ejection fraction; PO_2, partial pressure of oxigen.

monocytes/macrophages, which can increase arteriogenesis by providing cytokines and growth factors. EPCs cultivated from different sources showed a marked expression of many growth factors, such as VEGF, HGF, IGF-1, IL-8 and G-CSF (92), that could increment the angiogenic process carried on by the surrounding mature endothelial cells. Moreover, adherent monocytic cells, which were cultivated under similar conditions, but did not express endothelial marker proteins, also release VEGF, HGF, and G-CSF. In contrast, infusion of macrophages, which are known to release growth factors, but were not incorporated into vessel-like structures, induced only a slight increase in neovascularization after ischemia, indicating -but not proving- that the capacity of EPCs to physically contribute to vessel-like structures may contribute to their potent capacity to improve neovascularization.

Finally, it has to be considered that most of the preclinical studies are performed with healthy and young animals. In order to transfer the experimental findings into the clinic, we must consider that the patients usually have an established coronary artery disease, are of advanced age, and have various risk factors such as diabetes, hypercholesterolemia, or hypertension. These circumstances may limit the effect of cell or gene therapy. Evidences suggest that the fundamental mechanism by which therapeutic neovascularization augments collateral development is to provide cytokine supplements to individuals who, because of advanced age, diabetes, hypercholesterolemia, or other as yet undefined circumstances, are unable to appropriately upregulate cytokine expression in response to tissue ischemia. Resident endothelial cells may also be a limiting factor, having minor vasculogenic potential. Thus, delivery of endothelial progenitor cells would be one strategy to overcome this problem. However, age, risks factors for cardiovascular disease, and diabetes are associated with reduced number and functional activity of endothelial progenitor cells in the peripheral blood of patients. Novel strategies to counteract stem/progenitor cell dysfunction in aged patients with coronary artery disease or diabetes may include expression of protective and/or antiaging genes.

Chapter 5

Nitric Oxide and Endothelial Progenitor Cells

Nitric oxide ($NO^{\cdot}$) is a gaseous second messenger with a wide range of physiological and pathophysiological activities. This molecule is a free radical, and can be oxidized, reduced or complexed with other biomolecules, depending on the microenvironment. However, NO is almost unreactive as free radical as compared to other oxygen radicals. Indeed, NO decays within seconds after its synthesis if left unbound in solution because it reacts with either molecular oxygen or superoxide. Moreover, NO is freely diffusible across membranes. The molecule possesses a small dipole moment because of the similar electronegativity of oxygen and nitrogen, making it essentially hydrophobic.

NO does not remain as $NO\cdot$ -radical moiety in biological environment. In aqueous systems and at air-liquid interfaces, $NO\cdot$ -generation yields nitrite ($NO2^{-}$) and nitrate ($NO3^{-}$) as end products. NO generates a chemiluminescent product upon reaction with ozone. The $NO\cdot$ -radical reacts rapidly with the superoxide radical, forming highly reactive peroxynitrite anion ($ONOO^{-}$) that in turn gives origin to a dangerous hydroxyl radical (93) .

NO forms complexes with transition metal ions and predominantly binds to heme group of proteins (94). It combines with oxyhemoglobin, which represents the principal carrier for NO in blood, to produce methemoglobin and nitrate. Thus, methemoglobin levels are a useful index of NO production. Likewise, NO binds to the haem prosthetic group of guanylyl cyclase, inducing conformational modifications that lead to enzyme activation. Guanylyl cyclase increases the production of cGMP, modulating endothelium-dependent relaxation, platelet function and inhibitory transmission. Other NO-sensitive metalloproteins are cytochrome P450, NADH-ubiquinone oxidoreductase (mithocondrial complex I), FADH2-succinate-Q reductase complex (complex II) and cytochrome C oxydase (complex III), ferritin, myoglobin, cyclo-oxygenase, catalase and ribonucleotide reductase, subsequently inhibiting DNA synthesis.

Nitric Oxide Synthesis

Nitric oxide is produced by nitric oxide synthase (NOS), a haem containing enzyme that is linked to NADPH-derived electron transport (95). NOS catalyzes the oxidation of L-arginine to L-citrulline and NO, using tetrahydrobiopterin as an essential co-factor.

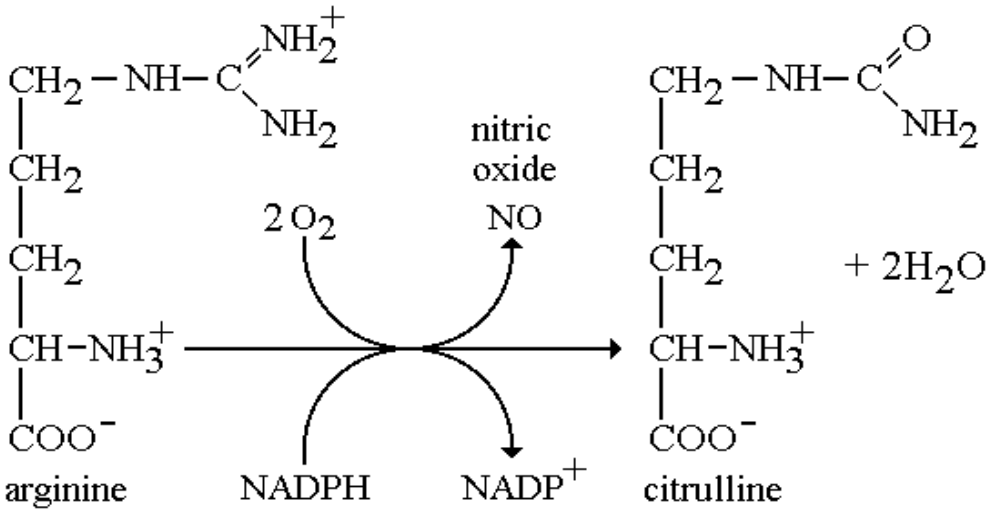


Fig. 8. Substrates, co-factors and products of the enzymatic reaction catalyzed by NOS.

There are three isoforms of NOS: endothelial NOS (eNOS, also referred to as type III NOS), so called since it was initially identified in bovine vascular endothelial cells, it is constitutively expressed by vascular endothelial cells. It has a calcium dependent activiy and generates relatively low levels of NO. The other costitutive isoform, neuronal NOS (nNOS, type I NOS), first isolated in rat neuronal tissue, which mediates the transmission of neuronal signals. And the inducible NOS (iNOS, type II NOS), that is transcriptionally regulated by inflammatory cytokines and other stimuli. It is calcium-independent and generates higher levels of NO. Although nNOS and eNOS are considered to be constitutive, eNOS expression can be induced by different stimuli: estrogens, shear stress, hypoxia, exercise, catecholamines, vasopressin, bradykinin, histamin and serotonin. While nNOS expression is stimulated by pro-convulsive neuronal stimuli. Likewise, iNOS is induced by several stimuli, but also constitutively expressed in certain conditions in adult and neonatal cardiomyocites, and human myocardium affected by dilatative cardiomyopathy (96). Moreover, the three isoforms are differently localized at the subcellular level. The eNOS isoform is localized in caveolae, uncoated plasmalemmal invaginations, where caveolin, the principal structural protein, interacts with endothelial NOS leading to enzyme inhibition in a reversible process modulated by Ca^{++}-calmodulin. nNOS is associated to sarcolemmal membranes and in the central nervous system. On the contrary, iNOS do not have an aminoacidic sequence suitable for linkage to cell membranes, and it is normally present in solution in cytosol.

All NOS isoforms are modular enzymes (97). In intact NOS, a C-terminal reductase domain (which binds NADPH, FMN, and FAD) is linked to the N-terminal oxygenase domain of the

other monomer. The oxygenase domain carries a prosthetic heme group and can bind (6*R*-) 5,6,7,8-tetrahydrobiopterin (BH4), molecular oxygen, and the substrate L-arginine.

Definition	Regulation	Localization
1. neuronal (type I)	**≥constitutive ++ Ca /CaM dipendent**	**Nervous terminations, ECs and muscle cells**
2. macrophagic (type II)	**≥inducible ++ Ca /CaM indipendent**	**Macrophages, ECs, SMCs and cardiomyocites**
3. endothelial (type III)	**≥constitutive ++ Ca /CaM dipendent**	**ECs and other cell types**

Table. 7. Nitric Oxide Synthase Isoforms.

Sequences located near the cysteine ligand of the heme are apparently also involved in L-arginine and BH4 binding. All 3 NOS isoforms possess a zinc-thiolate cluster formed by a zinc ion that is tetrahedrally coordinated to 2 CXXXXC motifs (1 contributed by each monomer) at the NOS dimer interface. Chemical removal of zinc from NOS or the possibility of expressing a zinc-deficient NOS that remained catalytically active demonstrated that the zinc in NOS is structural rather than catalytic. All NOS isozymes catalyze flavin-mediated electron transfer from the C-terminally bound NADPH to the heme on the N terminus. Calmodulin (on calcium-induced binding) increases the rate of electron transfer from NADPH via the reductase domain flavins to the heme center. In order to synthesize NO·, the enzyme needs to cycle twice. In a first step, NOS hydroxylates L-arginine to *N*- hydroxy-L-arginine (which remains largely bound to the enzyme). In a second step, NOS oxidizes *N*-hydroxy-Larginine to L-citrulline and NO·. The flow of electrons within NOS is tightly regulated. If disturbed, the ferrous-dioxygen complex dissociates, and O_2 is generated from the oxygenase domain instead of NO·. This is referred to as NOS uncoupling.

Pathophysiological Effects of Nitric Oxide

Nitric oxide released from the endothelium, following the stimulation of endothelial NOS, mediates vasodilation and inhibits platelet aggregation. Moreover, it prevents adhesion of neutrophils, and expression of macrophage chemotactic protein, thereby limiting inflammation (98).

In nervous system, NO effects are GMP-mediated. It modulates grief perception and neuronal plasticity, it is involved in cerebral development and short and long-term learning. Additionally, most non-adrenergic, non-cholinergic (NANC) neurons are NO-activated via the same cGMP-dependent mechanism. NANC nerves subserving important roles in gastrointestinal motility and penile erection are among those functioning through NO signals (99).

The immune system seems to have harnessed the toxic properties of NO to kill invading organisms, pathogens and tumor cells. The elevated NO concentrations generated by activated immune cells results in widespread disruption of other biologically important iron-complexed proteins. NO also reacts extremely rapidly with superoxide ($O2^-$) to form peroxynitrite, a potent oxidant with the potential to disrupt protein structures through the nitration of protein tyrosine residues. In support of this role, nitro-tyrosine has been identified both immunohistochemically and analytically in the debris of tissue damaged by inflammation.

The role of NO in regulating apoptosis is controversial. Stimulation of the expression of the inducible form of the NO-synthase by lipopolysaccharide or inflammatory cytokines has been shown to induce apoptosis of several cell types, mainly macrophages. In contrast, the majority of the studies demonstrated an anti-apoptotic and cell protective effect of NO in endothelial cells. The mechanisms of NO-mediated inhibiting of endothelial cell apoptosis may include several transcriptional and post-transcriptional events. First, the guanylyl cyclase activation and cGMP release. Second, NO directly interferes with caspases, the key enzymes of apoptosis signaling. Moreover, NO blocks the activation of pro-apoptotic stress activated jun-kinase (JNK). On the contrary, pro-apoptotic effects were mainly observed in macrophages. This could be explained by the the fact that eNOS generates only low concentrations of NO, with an anti-inflammatory and anti-apoptotic effects, whereas higher concentrations iNOS-generated would result in apoptosis.

Role of eNOS in Neovascularization

The NO produced by eNOS mediates a variety of physiological functions in vivo including neovascularization, regulation of blood vessel tone, platelet aggregation, vascular permeability and leukocyte-endothelial interactions. In addition to NO, more than 20 angiogenic factors have been individuated in the recent years, such as vascular endothelial growth factors (VEGF-A, B, C, D and E), placental-derived growth factor (PIGF), platelet-derived growth factors (PDGF-A, B, and C), fibroblast growth factors (FIGF-1 and 2), transforming growth factors

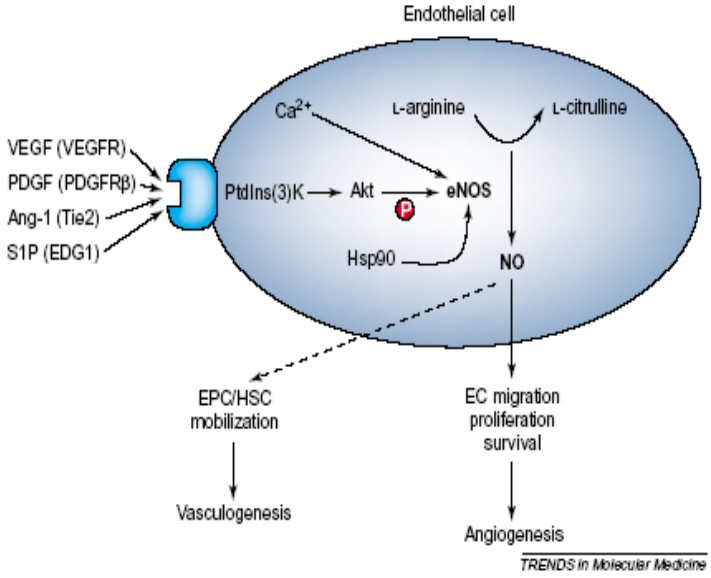


Fig. 9. Upstream signalling events leading to the induction of neovascularization by nitric oxide.

(TGF-α and β), hepatocyte growth factor (HGF), platelet derived endothelial growth factor (PD-EGF), tumour necrosis factor α (TNFα), interleukin-8 (IL-8), angiopoietins (Ang-1 and 2) and sphingosine 1-phosphate (S1P). However, NO does not only induces endothelial migration and proliferation, but also modulates the effects of many of the growth factors mentioned before.

VEGF is one of the most potent angiogenic factors and activates eNOS. The interaction between VEGF and NO has a major role in vascular permeability, vessel tone and angiogenesis during inflammation, wound healing and tumor growth. A selective modulation of eNOS activity would represent an attractive strategy for altering angiogenesis and vascular permeability (100).

eNOS plays an important role also in another type of neovascularization, the postnatal vasculogenesis, which involves EPCs. In their study, Aicher and coworkers examined the effect of eNOS on EPC effectiveness in neovascularization (73). They reported that $NOS3^{-/-}$ mice had defective hematopoietic recovery and progenitor cell mobilization, resulting into increased mortality after myelosuppression and reduced VEGF-induced mobilization of EPCs. Intravenous infusion of wild type EPCs rescued the impaired neovascularization of $NOS3^{-/-}$ mice in a model of hind-limb ischemia. $NOS3^{-/-}$ mice presented a profoundly reduced activity of pro-MMP-9. In fact MMP-9 activation by NO is a crucial event for cell mobilization. Furthermore, the restored neovascularization after infusion of wild-type cells indicates that eNOS is also required at the site of vessel formation.

Role of iNOS in Neovascularization

Recently also the potential importance of iNOS in neovascularization has been investigated. The study by Mayr et al reported a central role for the inducible NOS in vein graft reendothelization by circulating progenitor cells (CPCs) (101). In fact, they observed that deletion of the iNOS gene resulted in enhanced neointima formation, attenuated reendothelialization by CPCs, and attenuated production of the vascular endothelial growth factor (VEGF). Conversely, the application of VEGF in iNOS-/- mice promoted reendothelialization and prevented neointima formation. This demonstrated that the induction of VEGF in the vein graft occurs via a pathway involving NO and that VEGF plays a central role for reendothelialization by CPCs. Having the CPCs and the SMCs the same origin, NO would switch cell differentiation toward an endothelial phenotype, inhibiting the synthesis and the action of smooth cell growth factors.

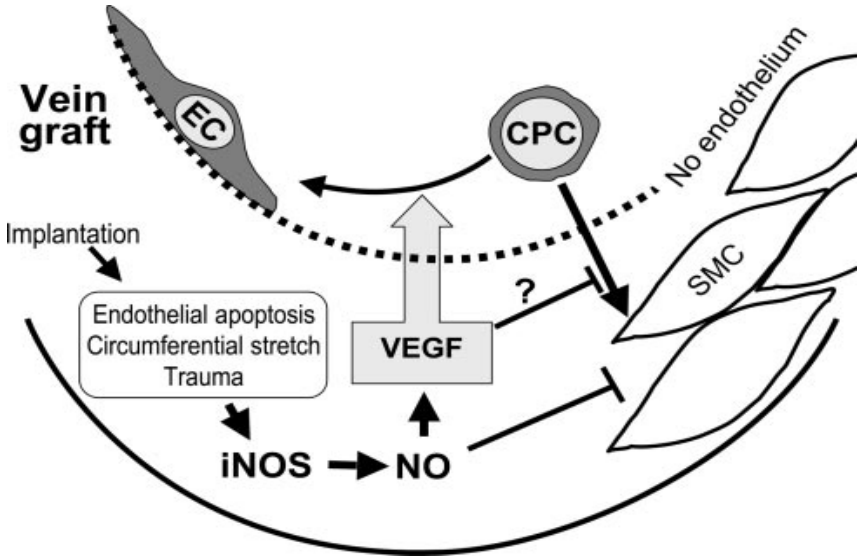


Fig. 10. Putative mechanisms leading to the reendothelization of vein grafts by circulating progenitor cells. (Brandes RP. 2006)

Losordo et al. studied the EPC contribute to cardioprotective effects of ischemic preconditioning, and showed the importance of both endothelial and inducible NOS activity in these cells (91). Precisely, their study pointed out that repeated short ischemic periods (IP) enhanced EPC mobilization, recruitment and homing in the myocardial tissue. Myocardial protection by EPCs is affected by cell incorporation in vascular structures, but also by eNOS, iNOS activity. By using bone-marrow cells originated from eNOS-/- o iNOS-/- donors, it has been shown that cardiac protection depends on eNOS or iNOS according to the ischemic insult used on heart.

Nitric Oxide and Diabetes

Being NO important for the regulation of EPC mobilization and function, it has been studied the role of NOS isoforms in diabetic EPC dysfunction. Impairments in eNOS function have been reported with hyperglycaemia, insulin resistance, and in peripheral tissue from diabetic patients (102). Therefore, it has been studied if eNOS function in diabetic bone marrow cells is altered. It has been observed enhanced eNOS expression in the bone marrow of diabetic rats, whereas eNOS monomerization and superoxide anion production were increased (103). By using NOS inhibitors, superoxide anion was blocked. This suggested that probably it was occurred the eNOS uncoupling, which had previously been linked to the enzyme monomerization. As a consequence, NO bioavailability in bone marrow is reduced.

Moreover, NO seems to be involved in insulin response to arginine. In the past years, accumulating evidences suggested that arginine could influence insulin secretion in a complex way, by exterting a metabolic action based on its monooxygenation by NOS. Indeed, the constitutive neuronal isoform, nNOS, has been characterised in rat pancreatic islets and β-cell lines (104). It is implicated in an inhibitory modulation of insulin secretion in experiments mainly based on the use of pharmacological NOS inhibitors, such as N-ω-nitro-L-arginine methyl ester (L-NAME). In a diabetic model obtained by combined injection of streptozoticin (STZ) and nicotizamide (NA), L-NAME markedly enhanced arginine-induced insulin release. Furthermore, exposure of diabetic islets to the NOS inhibitor failed to modify their response to arginine. This could be due to either a decreased nNOS expression in diabetic islets or a reduced activity of the enzyme (105).

In addition, in their study Bradley at al. demonstrated that patients with prediabetes/type 2 diabetes have 35% to 40% lower skeletal nNOS protein expression (106) and elevated iNOS levels. A higher iNOS expression could the consequence of the inflammatory status set in diabetes. It would be also involved in the blood retinal barrier breakdown in the early stages of retinopathy, through a ICAM-1 upregulation and tight junction downregulation (107).

Chapter 6

ROS and Endothelial Dysfunction

It is now widely recognized that an increase in oxidative stress denotates an imbalance between reactive oxygen species (ROS) production, such as superoxide and H_2O_2, and antioxidant species, and plays an important role in the genesis of the dysfunction in endothelial phenotype. As previously described, the principal mechanism underlying ROS-dependent impairment of endothelium-mediated vasorelaxation is the inactivation of NO, with a reaction that also generates peroxynitrite. Interestingly, oxidative stress leading to endothelial vasodilator dysfunction is also a predictor of adverse outcome in patients with coronary artery disease (108). In addition to the interaction with NO, ROS have important direct effects through the modulation of diverse redox-sensitive signalling pathways in endothelial cells which influence gene and protein expression and may have an impact on many different functions. Among these functions, there are endothelial cell growth, proliferation, migration, survival, cytoskeletal reorganisation, cell shape, adhesion molecule expression, permeability and the secretion of inflammatory cytokines. There are several potential sources of superoxide in endothelial cells, including mitochondria, cytochrome P450-based enzymes, uncoupled eNOS, xanthine oxidase and NADPH oxidases. Among these sources, NADPH oxidases represent the only enzymes whose primary function is ROS generation. Moreover, NADPH oxidases are specifically activated by many of the stimuli that are known to cause endothelial dysfunction and activation and they can be considered the major ROS source in endothelial dysfunction and redox signalling. Recently, these enzymes have even been found to be important in signal transduction pathways involved in angiogenesis and neovascularisation (109).

Furthermore, an important attribute of the NADPH oxidases is their potential to augment ROS generation by other enzymes. For example, NADPH oxidases can cause uncoupling of NOS, secondary to oxidative degradation of the NOS cofactor tetrahydrobiopterin (H4B), thereby leading to superoxide rather than NO generation.

NADPH oxidase

The NADPH oxidase complex was first described in neutrophils where it is involved in non-specific host defence against microbes ingested during phagocytosis, through the generation of large quantities of superoxide and protons (110). The phagocytic oxidase is composed of two membrane-associated subunits, p22phox and gp91phox, which form a flavocytochrome (cyt b558). The process of electron transfer from NADPH to molecular oxygen, which results in

superoxide formation, is catalysed by the oxidase following its activation through the translocation of several cytosolic regulatory subunits (p47phox, p67phox, p40phox and Rac), which associate with cytochrome b558.

Components of the NADPH oxidase were found to be present in many nonphagocytic cells, including VSMC (111), endothelial cells (112), adventitial and cardiac fibroblasts, and cardiomyocytes (113). Because of different biochemical activity of the phagocytic and nonphagocytic oxidase, a whole family of NADPH oxidases has been identified basing on distinct homologues of gp91pho. The Nox family may be classified into two groups, based on predicted domain structures: (i) Nox1-4 all contain six transmembrane domains and have NADPH and FAD-binding domains at the cytoplasmic C-terminus; (ii) Nox5 has a similar basic structure but with an additional N-terminal calmodulin-like Ca2+-binding domain.

In addition, two related oxidases, Duox1 and 2, include a further N-terminal extension with a peroxidase-homology domain that is separated from the Ca2+-binding domain by an additional transmembrane segment. The expression of these different Nox isoforms varies according to cell and tissue. Nox2 and Nox4 are co-expressed in endothelial cells, although there are a few reports that Nox1 and Nox5 might also be present in some settings. In homogenates of human arteries and veins Nox2 mRNA expression was higher than that of Nox4 (114). These two isoforms are probably regulated by different stimuli and may play distinct roles in endothelial cells through expression in different subcellular locations.

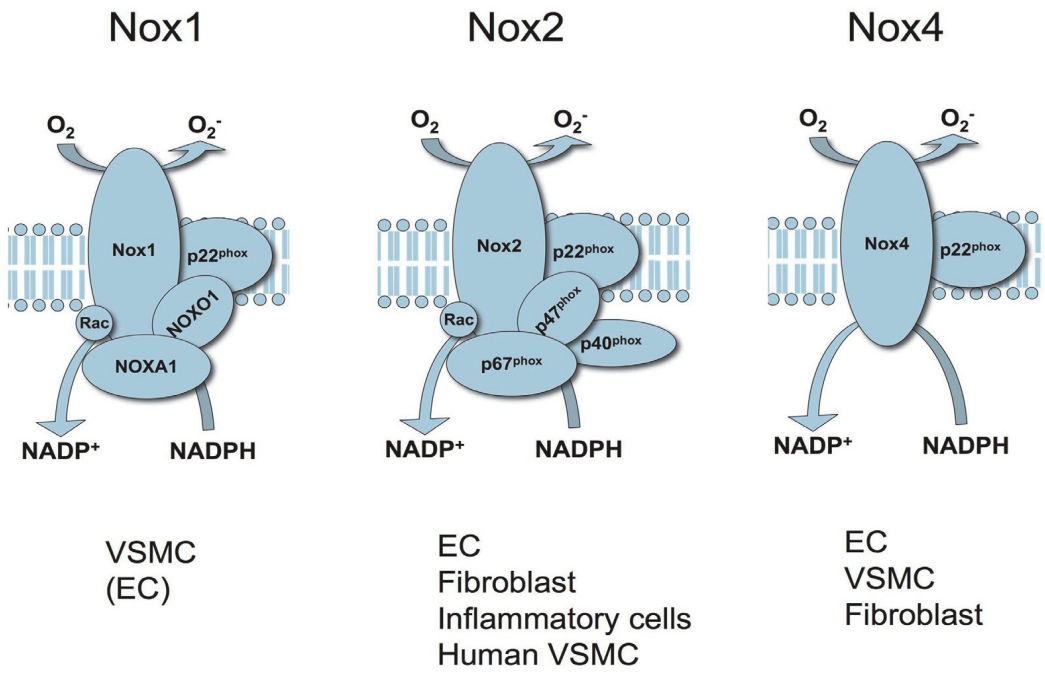


Fig. 11. Schematic diagram of the different NADPH oxidase isoforms (Dworakowski R. et al, 2008).

NADPH oxidases and Endothelial Dysfunction

A significant body of evidence implicates NADPH oxidase activation in the endothelial dysfunction mechanisms, like the up-regulation of cell surface adhesion molecules, chemokines, and an increase in permeability. TNF-α and other pro-inflammatory cytokines increase the expression of ICAM-1, VCAM-1 and MCP-1 in an nuclear factor- B (NF- B) dependent manner *via* the activation of Nox2 (115). Angiotensin II-induced stimulation of the AT1 receptor is a potent agonist for activation of the Nox2 oxidase in endothelial cells.

Shear stress results in several-fold induction of Nox1, Nox2, and Nox4. Nox1 expression increases ~3-fold following balloon injury and precedes restenosis and atherosclerosis. In addition, both tissue hypoxia and VEGF may be important stimuli for Nox2 activation in neovascularization, and an involvement of this isoform in ischemia-induced and VEGF-induced neovascularisation has been confirmed *in vivo* (109).

In diabetes mellitus, chronic hyperglycemia involves biochemical abnormalities including increased polyol pathway flux, increased formation of advanced glycation end products (AGEs), activation of protein kinase C, and increased flux through the hexosamine pathway. All these pathways seem to result from overproduction of ROS (116), and antioxidants are protective against deleterious effects of high glucose levels on vascular endothelial cells. Nox enzymes, particularly the Rac-regulated enzymes Nox1 and Nox2, play a role in endothelial dysfunction in the setting of diabetes mellitus (117). Some of the cardiovascular pathologies seen in diabetes may be mediated by the glycated proteins that result from high glucose levels. An example is the Nox2-dependent ROS production stimulated by glycated BSA.

Moreover, Nox1, Nox2, and Nox4 seems to be important in insulin secretion mechanisms. They had been found in pancreatic islet cells and glucose-stimulated insulin secretion was suppressed by a general Nox inhibitor, supporting the hypothesis of a role for one or more Nox enzymes in normal pancreatic islet function (118). Despite this functional role of Nox, it has been conceived an involvement of these enzymes in diabetes progression. An excessive ROS production may damage pancreatic islets leading to type 1 diabetes. In early type 1 diabetes, systemic markers of oxidative stress correlate with insulin requirements, suggesting that oxidative stress in the pancreatic islet leads to damages in insulin-secreting beta-cells (119). In type II diabetes, increased expression of Nox1 occurs in islets, and may exacerbate disease over time by damaging insulin-producing cells (120).

The Nox2 could have a dual role by promoting endothelial cell activation as well as inhibiting it, although the precise mechanisms underlying these opposing effects remain unknown.

Chapter 7

Aim of the study

Better understanding the mechanisms of impaired vascular repair has clinical relevance in cardiovascular and peripheral arterial disease. The endothelial progenitor cells have an important role in vascular homeostasis and actively participate to vasculogenesis. Thus, an impaired cell function is associated with endothelial dysfunction and vascular disease. The long term goal of this study is a possible improvement of cell-mediated vascular repair in those individuals who present a reduced endothelial progenitor cell availability and activity, in order to improve the effectiveness of vein graft transplantion. To this end, it has been investigated the functional changes underlying the EPC dysfunction in patients with peripheral arterial disease.

The first aim has been the study of the proliferation, differentiation and function of EPCs derived from healthy volunteers. Given the role of NO in endothelium homeostasis, it has been studied the expression of NOS isoforms, and the effect of NOS inhibition. Moreover, due to the ROS dependent NO inactivation, it has been considered the expression of NADPH oxidases.

Secondly, in order to evaluate the effects of diabetic hyperglicaemic conditions on EPCs, isolated cells were cultured at elevated glucose concentration, considering the possible changes in cell proliferation and function.

The third aim has been the study of EPCs derived from patients affected by peripheral arterial disease, whether diabetic or not, in terms of proliferation, differentiation, and function. Furthermore, it has also been assessed if functional changes may subsist in any difference in NOS and NAPDH oxidase expression.

Chapter 8

Materials and Methods

Peripheral Blood Samples

Endothelial progenitor cells were isolated from peripheral blood samples, both from healthy subjects and from patients with PAD.

Blood samples from healthy human adult volunteers of both genders were supplied by the Service of Transfusion Medicine of S. Orsola-Malpighi Hospital of Bologna (Italy).

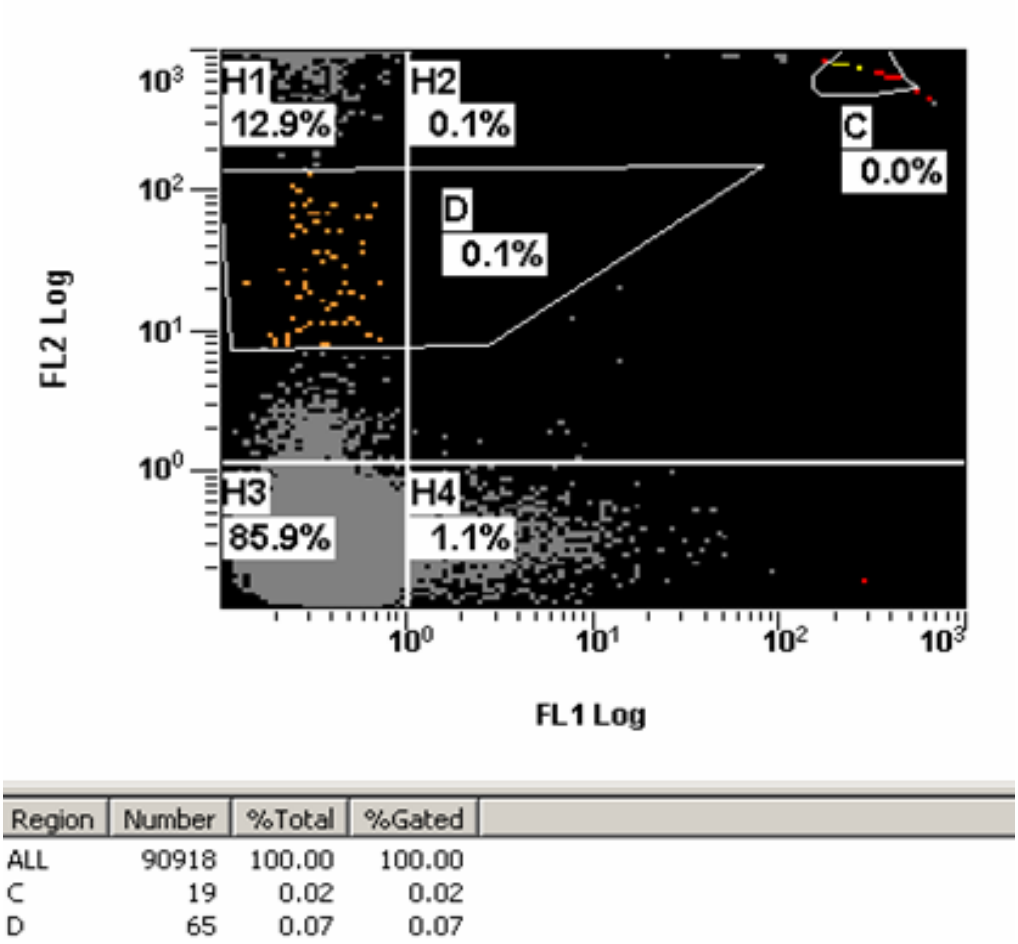
In this study 21 patients were recruited at the Vascular Surgery Unit of the S. Orsola-Malpighi Hospital of Bologna (Italy). All patients were affected by peripheral arterial occlusive disease, and critical ischemia. Most of patients had a IV stage disease, affecting various vascular beds, mainly femoropopliteal artery. 70% of patients recruited for this study suffered from type 2 diabetes.

Determination of Circulating Endothelial Cell Number in Patient Samples

For this analysis it was used CELLQUANT FF-CD146 kit (BIOCYTEX Marseille France). This kit is based on CD146 cell enrichment by immuno-magnetic separation from total blood samples and successive count of circulating endothelial cells (CECs).

Sample Preparation**.** For each sample, 2 mL of total blood were divided into two vials each containing 1 mL of diluent solution and 50µL of CD146-coated magnetic microspheres. After two cycles of magnetization and homogenization, respectively of 5 and 20 min, that allow cells to interact with coated antigenes, supernatant was removed so that to eliminate CD146 negative cells. 1mL lysis reagent was added to the pellet, in order to lyse red blood cells, that could interfere with the analysis. In the control vial, enriched cell suspension was added with CD45 monoclonal antigen conjugated with FITC. In the second one, both CD45 and CD146 conjugated with phicoerythrin (PE) antigens were added. After 10 min incubation, cells were fixed and marked microspheres were added.

Cytofluorimetric Analysis. Analysis were performed by using the FC500 cytofluorimeter (Beckman-coulter). Cells from healthy subjects was used as control.
The number of cells detected in each vial was expressed by the ratio of cells in D region to cells in region C. Number of CD146 positive cells was obtained by the difference between the number of cells detected in the first vial (CD45 cells) and the number of cells detected in the second vial (CD45 and CD146 cells).



Region	Number	%Total	%Gated
ALL	90918	100.00	100.00
C	19	0.02	0.02
D	65	0.07	0.07

Fig. 12. Number of CECs detected by cytofluorimetry.

Cell Culture in Hyperglycaemic Conditions

In order to study the effects of high glucose concentration, cells were cultured in medium additioned with glucose, at a final 22 mM glucose concentration (121). As a control, cells were cultured in a medium with a normal glucose concentration (5,5 mM), but with the same osmolarity of high glucose concentration medium, by adding mannitol 16,5 mM.

Cell Viability

Cell proliferation was evaluated by 3-[4,5-dimethylthiazol-2-]-2,5-diphenyltetrazolium bromide (MTT) viability assay, which is based on the reduction by living and early apoptotic cells of MTT salt into insoluble purple formazan crystals, which are solubilised by the addition of a detergent. The coloured salt production can be quantified by spectrophotometric means, measuring the absorbance of the solution. The number of surviving cells is directly

proportional to the level of the formazan. Cells were plated in 96-well plates coated with human fibronectin, and kept in culture for 10 days. MTT assay was performed at 3, 7 and 10 days of culture. The quantification of formazan crystals was obtained by measuring the absorbance of the cell solution at 500 nm by using a VICTOR^2 1420 Multilabel Counter.

Morphological Characterization

Adherent cells were observed with phase contrast inverted microscope Olympus IX50. Cells which presented a spindle shape were considered as differentiating EPCs.

In order to evaluate cell differentiation, cells were counted at different culture time (3, 7, and 10 days) in five different fields per Petri dish, with a 20X magnification. It was considered the total number of cells and the number of spindle shaped cells.

Cellular Staining and AcLDL Uptake

Adherent cells resulted positive to double-fluorescence staining for Dil-labeled acetylated low density lipoprotein uptake (acLDL, Moleculare Probes) and FITC labeled *Ulex Europaeus* agglutinin binding (FITC UEA-1, Sigma-Aldrich) were identified as early EPCs.

Briefly, direct fluorescent staining was used to detect the binding of fluorescein isothiocyanate (FITC) - labeled *Ulex Europaeus* agglutinin (UEA-1, Sigma, St. Louis, Missouri, USA) and cell uptake of 1,1-dioctadecyl-3,3,3,3-tetramethilindocarbocyanine (Dil) – labelled acetylated low density lipoprotein (acLDL, Molecular Probes, Eugene, Oregon, USA). Cells were incubated with 10 μg/ml acLDL for 4 h, and 10 μg/ml UEA-1, for 2 h, at 37 °C and fixed with 3% paraformaldehyde for 15 min. Cells with double-fluorescent staining were identified as differentiating EPCs and their average number per field was evaluated counting 10 randomly selected high-power fields (20x) under an inverted fluorescent microscope (IX50 Olympus). HUVECs were chosen as positive controls either for direct fluorescence staining (Dil-acLDL, FITC-UEA-1). More than 85 % of HUVECs was positively stained for the above-mentioned endothelial markers.

Vasculogenesis Assay

The vasculogenic capacity was determined by the de novo vessel formation in a three-dimensional gel. 96-well tissue culture plates were coated with *Matrigel Matrix* (Becton

Dickinson) that polymerizes at 37 °C to produce biologically active matrix material resembling the mammalian cellular basement membrane. Therefore, two week cultured EPCs were plated alone (20000 cells/well) or co-cultured with HUVEC (10000 cells/well HUVEC plus 10000 cells/well EPCs). Cells were incubated at 37°C for 24 h and tubule formation was observed under an inverted light microscope IX50 Olympus at 10X magnification.

When plated together with HUVECs, EPCs were previously labelled with Ac-LDL-Dil for 4 h in order to allow the identification of the two populations.

NO Metabolism

NO Production

Intracellular NO production was determined by using Diaminofluorescein –2 Diacetate (DAF-2DA) is a non-fluorescent cell permeable reagent. It can measure free nitric oxide in living cells under physiological conditions. Once inside the cell the diacetate groups on the DFA-2DA reagent are hydrolyzed by cytosolic esterases thus releasing DAF-2 and sequestering the reagent inside the cell. Nitric oxide produced by the cell converts the non-fluorescent dye, DAF-2, to its fluorescent triazole derivative, DAF-2T, that can be observed by exciting the reagent at 488nm and then measuring the emission at 515nm.

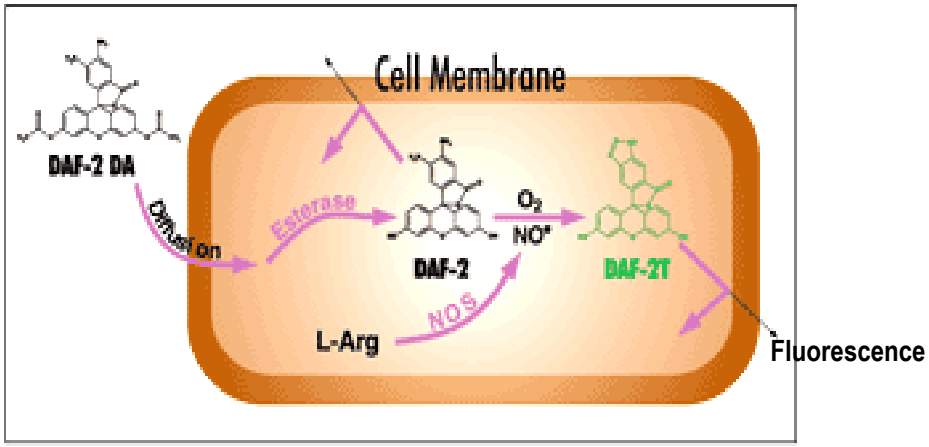


Fig. 13. Mechanism of DAF-2DA conversion to the fluorescent molecule DAF-2T.

EPCs at 14 day culture were plated in 96-well plates and treated with NOS inhibitors: N^G-nitro-L-arginine methyl ester hydrocloride (L-NAME) (Sigma) 100 mM; N^6-(1-Iminoethyl)-L-lysine dihydrocloride (L-NIL) (Alexis Biochemicals) 20 μM, and N^5-(1-Iminoethyl)-L-ornithine dihydrocloride (L-NIO) (Alexis Biochemicals) 10 μM. Thereby, after 48 hours cells were incubated with DAF-2/DA 10 μM (Sigma) diluted in a buffer solution containing [mM] NaCl

142, KCl 2, K_2HPO_4 1.2, $MgSO_4$ 1, HEPES 10, $CaCl_2$ 1.3, and glucose 10, at 37 °C for 30 minutes. Emission fluorescence was measured with a VICTOR^2 1420 Multilabel Counter (Perkin Elmer).

NO and Cell Proliferation

Cells were plated into 96-well plates coated with human fibronectin at a density 300000 cell/cm^2. Cells were treated with NOS inhibitors, N^G-nitro-L-arginine methyl ester hydrocloride (L-NAME) (Sigma) 100 mM, N^6-(1-Iminoethyl)-L-lysine dihydrocloride (L-NIL) (Alexis Biochemicals) 20 μM, and N^5-(1-Iminoethyl)-L-ornithine dihydrocloride (L-NIO) (Alexis Biochemicals) 10 μM and kept in culture for 10 days. Cell proliferation was assessed by MTT assay at 3, 7, and 10 days of culture.

NO and Vasculogenesis

Cells were pretreated with NOS inhibitors, L-NAME (Sigma) 100 mM, L-NIL(Alexis Biochemicals) 20 μM, and L-NIO10 μM and kept in culture for 12 days. At day 13 cells were seeded together with HUVECs in 96-well plates coated with *Matrigel Matrix* (Becton Dickinson), as previously described. Cells were incubated at 37°C for 24 h and tubule formation was observed under an inverted light microscope IX50 Olympus at 10X magnification.

Quantification of NOS mRNA Expression by Real-Time PCR

The assay for mRNA expression of NOS isoforms was optimized for the Light Cycler version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany), with a Real Time-PCR in two steps. RNA extraction was performed by using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and, for smaller samples, RNAqueous-Micro Isolation Kit (Ambion, Texas, USA). The reverse transcription was performed by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. NOS mRNA expression was quantified relative to the β-actin housekeeping gene. The primer sequences were as follows: β-actin, sense 5'-CCA GAG GCG TAC AGG GAT AG-3' and antisense 5'-CCA ACC GCG AGA AGA TGA-3' (122). eNOS, sense 5'-CCG GGT ATC CAG GTC CAT-3'and antisense 5'-GAC CCT CAC CGC TAC AAC AT-3' (123); iNOS, sense 5'-TGG CCA TCC TCA CAG GAG-3' and antisense 5'-CAA AGG

CTG TGA GTC CTG CAC-3' (124). nNOS, sense 5'-CCG CGC TTA CAA ACT TGC-3' and antisense 5'-TGA GTC CAT TGC CTT CAT TG-3'; PCR fragment of 424 bp (125). Real-time PCR was performed in Light Cycler capillaries using a master mix containing TaqMan DNA polymerase and the respetive probes for each NOS type mRNA (LightCycler TaqMan Master, Roche Molecular Biochemicals). DNA fragment amplification was performed after the addition to the master mix of primers (final concentration: 0.5 μM) and template cDNA (10 to 100 ng). The protocol was the following: 1 cycle at 95 °C for 15 min for the initial enzyme activation, then 45 cycles of denaturation (94°C for 10 s), annealing (60 to 63°C for 10 s) and extension (72°C for 6 to 15 s). In order to verify the purity of the products, a melting curve was produced after the completion of each PCR amplification by increasing the temperature of the reaction mixtures up to 95 °C, by 0.1 °C/s, starting at 60 °C for 60 s.

The Roche software used the second derivative maximum method to calculate the fractional cycle numbers where the fluorescence rises above background at the fastest rate (crossing point, C_p). The amount of mRNA was calculated according to Pfaffl (2001) relative to the housekeeping gene β-actin. All the values were normalised to the corresponding mRNA of NOS isoforms expressed by HUVECs at passages between 3 and 6.

NOS Protein Expression

Whole protein extract (80 μg/sample) was prepared from MNCs and EPCs (1 x 10^7 cells/ml) at 7, 14 and 21 days of culture. The samples were electrophoresed by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (1 h, 150 V) and blotted (1 h, 100 V) onto nitrocellulose membranes (Bio-rad, CA, USA). After ishing in TBS-Tween (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05 % Tween-20), the membranes were treated for 1 h with 5 % non-fat dry milk (Bio-rad, CA, USA) to block non-specific binding sites. The membranes were incubated for two hours with rabbit polyclonal antibodies for iNOS (NOS2), eNOS (NOS3), and nNOS (NOS1) (Santa Cruz, Biotechnology, Europe) diluted 1:1000 in TBS-Tween. The immune reaction was revealed by a peroxidase-labelled goat anti-rabbit antibody (Santa Cruz, Biotechnology, Europe) diluted 1:2500 in TBS-Tween. The secondary antibody was added to membranes for 1 h at room temperature, followed by 1 min exposure with the Luminol reagent (Santa Cruz, Biotechnology, Europe) which was revealed by high-sensitivity photographic film. β-actin was used as internal control.

Silencing of iNOS by Small Interfering RNA (siRNA)

To obtain inactivation of iNOS, cells were transiently transfected by siRNA Transfection Reagent (Santa Cruz) with iNOS siRNA. The sequence for iNOS was CCA GAG GAG CTT CTA CTT CAA (125), sense primer was 5'-rArCrArArCrArGrGrArArCrCUrArCrCrArGrC TT and antisense primer 5'-rGrCUrGrGUrArGrGUUrCrCUrGUUrGU TT. Cells were plated 7 x $10^{6/}$mL in 60 mm dish plates and cultured under normal growth conditions for 3 days. At day 4 cells were washed with medium and then incubated with 1500 µL siRNA Transfection Medium added with siRNA Transfection Reagent 10 µL and diluted siRNA 50-100 pmol/µL for 5 h at 37 °C. Then M199 medium with 20% FBS was added to the transfection mixture and cells were incubated for additional 18-24 h before removing it. Then medium was aspirated and replaced with normal M199 with 10% FBS and growth factors.

Quantification of NADPH Oxidase mRNA expression by Real-Time PCR

The assay for mRNA expression of Nox1, Nox2, and Nox4 was performed by the Light Cycler version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany), with a RT-PCR in two steps. RNA extraction was performed (Roche Diagnostics, Mannheim, Germany) and, for smaller samples, RNAqueous-Micro Isolation Kit (Ambion, Texas, USA). The reverse transcription was performed by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. mRNA expression was quantified relative to the β-actin housekeeping gene. The primer sequences were as follows: β-actin, sense 5'-CCA GAG GCG TAC AGG GAT AG-3' and antisense 5'-CCA ACC GCG AGA AGA TGA-3' (122). Nox1, sense 5'-TTTGGATGGGTGCATAACAA-3' and antisense 5'-AAGGATCCTCCGGTTTTACC-3' (127); Nox2, sense 5'-CGATGGTTTTGAAAGGTTGA-3' and antisense 5'-GGTTTTGGCGATCTCAACAG-3' (128). Nox4, sense 5'-CCG CGC TTA CAA ACT TGC-3' and antisense 5'-TGA GTC CAT TGC CTT CAT TG-3' (127). Real-time PCR was performed as described before.

Statistical Analysis

Statistical Analysis was performed with a Student's test and results are expressed as mean± SEM. Probability values of $P<0.05$ were considered statistically significant.

Chapter 9

Results

AIM I: Study of EPCs derived from healthy subjects and the role of NO.

Culture of EPCs

Mononuclear cells from healthy volunteers were plated under differentiating conditions at a concentration 10^6 cells/mL and kept in culture for 14 days. Soon after plating, cells presented a round shape and low attaching ability, but, from the 3rd day on, it was possible to observe spindle-shaped and attaching cells (AT cells). Their number increases throughout the days of culture, and cells begin to have a mature endothelial cell-like aspect, also referred to as a typical cobblestone-aspect.

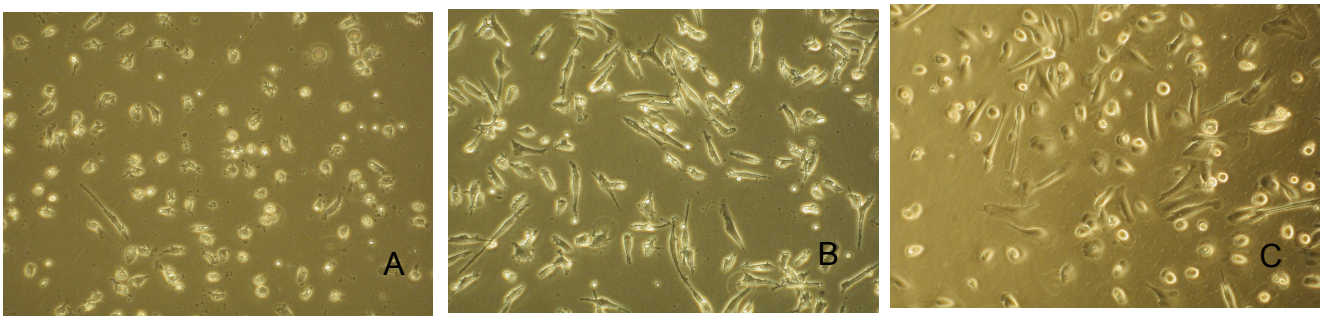


Fig. 14. Morphological changes of EPCs during the differentiation process. (A) cells at the 3rd day of culture: only few AT cells are detectable; (B) cells at the 7th day: AT cell number is considerably increased. (C) day 10: cells begin to have a morphology more similar to mature endothelial cells.

If plated at higher concentrations, these cells tended to join together, forming the clusters described in literature.

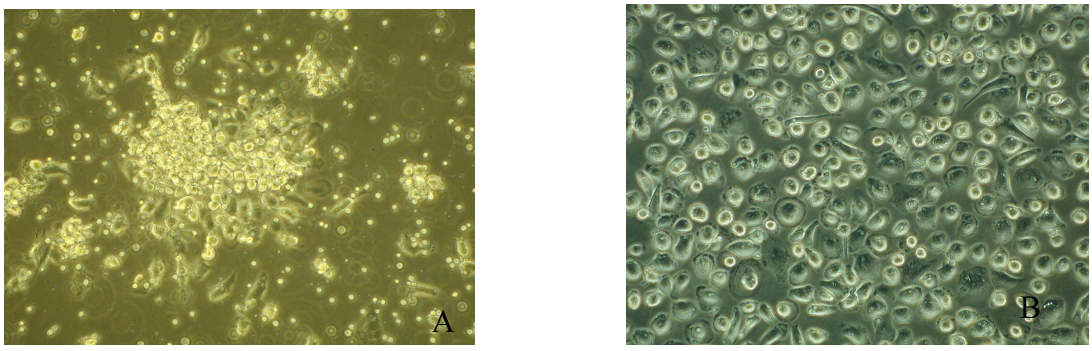


Fig. 15. Evolution in EPC morphology. Already from the 7th day of culture, it is possible to observe cell agglomerates, called clusters (a). The clusters represent the colonies of CFU-cells who give rise to the late endothelial precursors.

Functional characterization

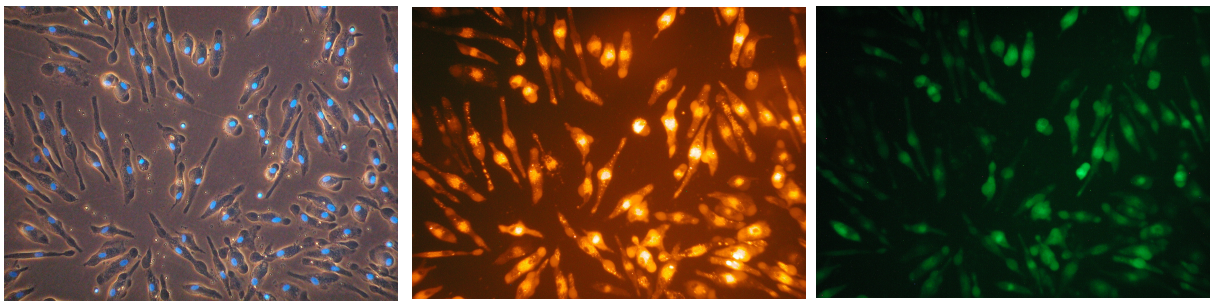


Fig. 16. Characterization of endothelial progenitor cells. Cell nuclei were labelled with 4',6-diamidino-2-phenylindole (a). Therefore, cells were both stained with Dil-AcLDL (b) and UEA-FITC (b).

a b c

In order to confirm the differentiation toward an endothelial phenotype, cells were characterized by Dil-Ac-LDL uptake and UEA-FITC binding. Then, they were observed at inverted light microscopy and it was counted the number of cells who had incorporated Dil-AcLDL, cells who had bound UEA-FITC, and cells who were positive for both characterizations. Only these cells were considered as EPCs differentiating toward an endothelial phenotype.

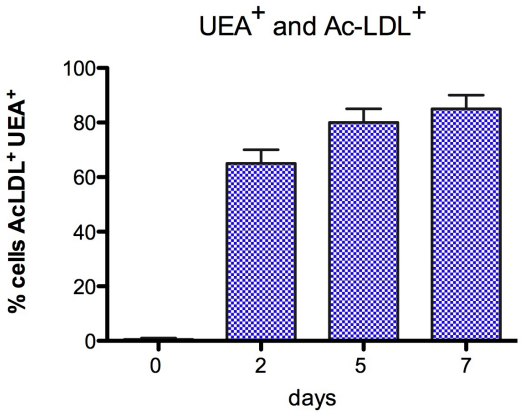


Fig. 17. Trend of EPC characterization. Cells were plated on fibronectin coated plates and the characterization of differentiating cells was assessed by double staining with Dil-acLDL uptake and UEA-FITC binding. Already from the 2 day of culture a high rate of cells was positive to staining, and could be considered as EPCs.

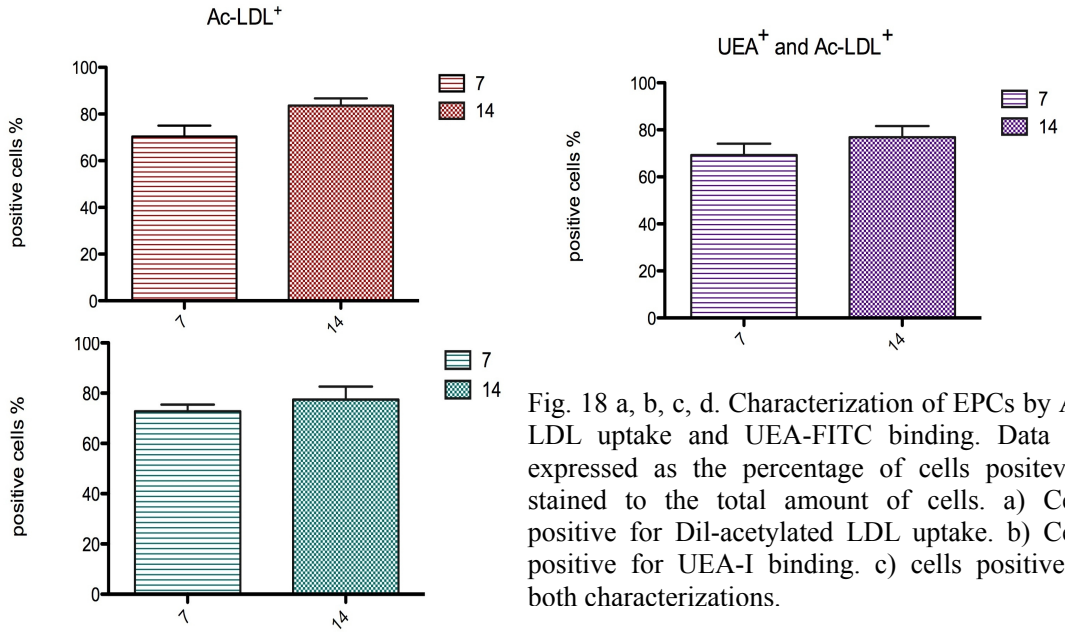


Fig. 18 a, b, c, d. Characterization of EPCs by Ac-LDL uptake and UEA-FITC binding. Data are expressed as the percentage of cells positevely stained to the total amount of cells. a) Cells positive for Dil-acetylated LDL uptake. b) Cells positive for UEA-I binding. c) cells positive to both characterizations.

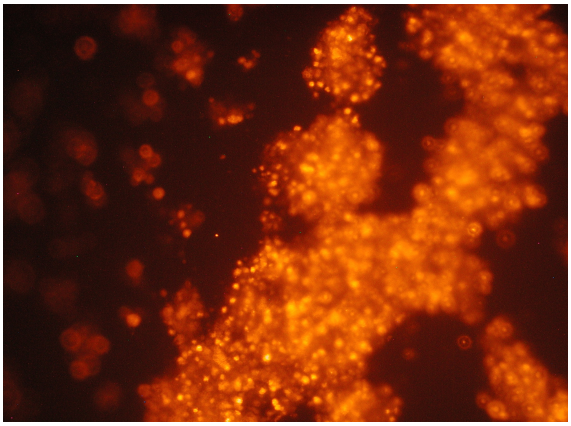


Fig. 19. EPCs vasculogenic ability. EPCs were marked with AcLDL and cultured for 24 hours on a Matrigel layer. Tube formation was observed under fluorescence inverted light microscopy. EPCs were not able to form new vessels ex novo , even if an approssimative capillary-like structure was observed

Already after 2 day culture, MNCs appeared to be positive to EPC characterization. Differentiated mononuclear cell population should have at least 80 % of positively stained cells.

Moreover, another functional characterization so that to verify endothelial differentiation was the vasculogenesis assay. Cells were cultured for two weeks, then plated in Matrigel coated plates and incubated for 24 hours. As observed at inverted light microscopy, EPCs did not present the same vasculogenic ability the mature endothelial cells have. After 24 hours, no tube

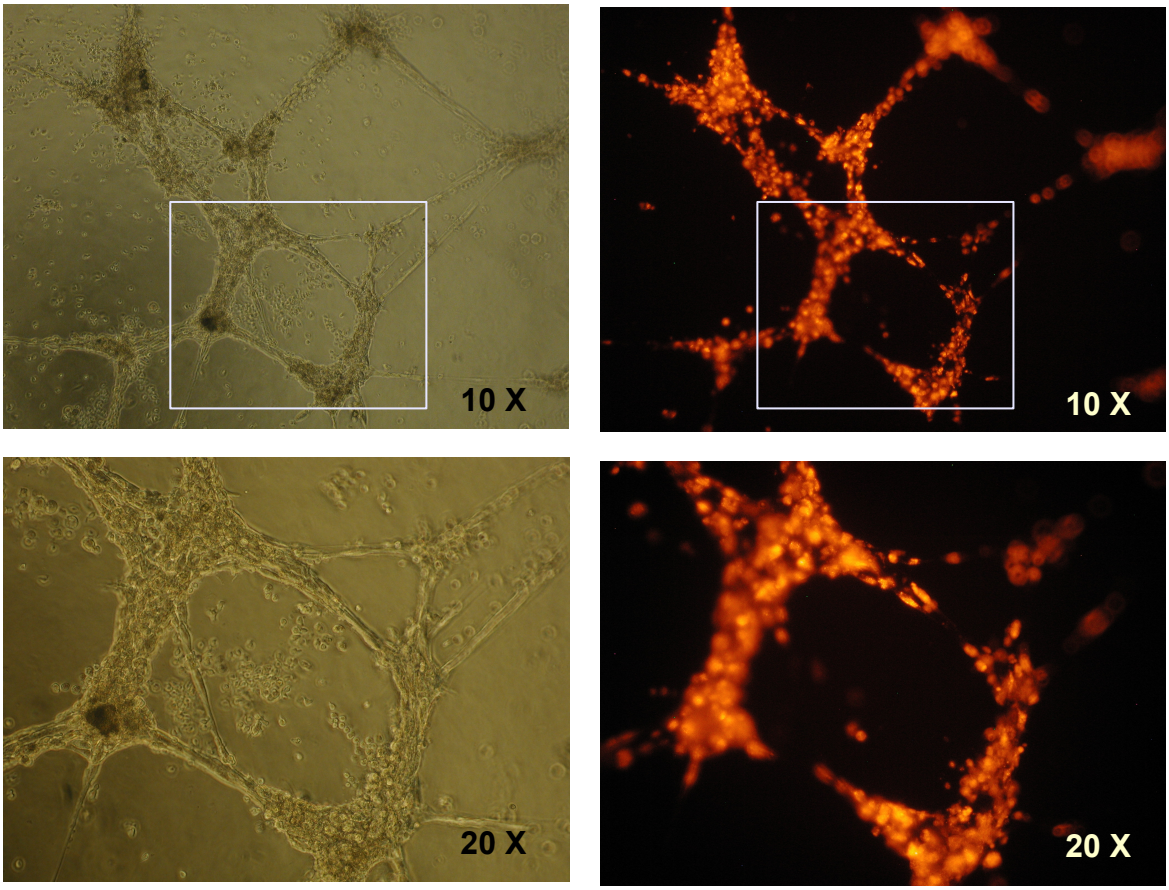


Fig.20. Incorporation of EPCs in capillary-like structures formed by HUVECs. EPCs were previously labelled with Dil-AcLDL and incubated for 24 h in Matrigel coated plates together with HUVECs. Afterward they were observed under inverted light microscope, at 10X and 20X magnification. It was evident that EPCs actively participated to HUVEC tube formation.

formation was observed. Cells tended to form agglomerates, and eventually assume an approssimative capillary-like structure. Albeit EPCs showed little vasculogenesis ability if cultured alone, they were able to participate to vasculogenesis carried out by mature endothelial cells, such as HUVECs. EPCs, previously labelled with AcLDL, were plated together with HUVECs on Matrigel monolayer. Labelled EPCs were visibly inserted in the new capillary like structures created by HUVECs, thereby giving an evidence of EPC involvement in postnatal neovascularization.

Role of NO in EPC function

Being the NO essential for neovascularization, it has been investigated if lower vasculogenic potential of EPCs could be referable to a lower NO synthesis. Firstly, it was assessed if EPCs were able to produce NO. Cells were labelled with the selective probe DAF-2DA and observed under fluorescence light microscopy (fig. 21). Hence, it has been studied the expression of NOS isoforms and the effects of NOS inhibition on EPC differentiation and function.

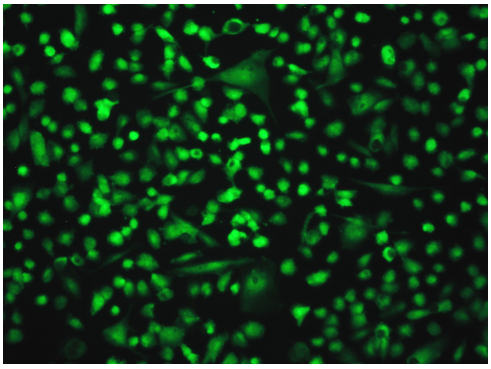


Fig. 21. Production of NO. Cells were incubated with non fluorescent probe DAF- 2DA that in presence of NO gives origin to a fluorescent molecule.

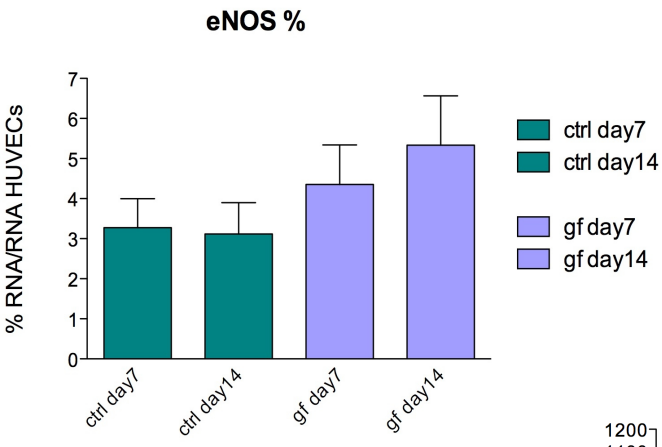
NOS expression

Regarding the protein levels of NOS isoforms, cell lysates were analysed by western blot technique. While HUVECs presented high levels of eNOS and low levels of nNOS, these two isoforms had not been detected in EPCs. Except for iNOS, whose expression in EPCs is detectable by Western blot, the eNOS and nNOS protein concentrations are presumably to low to be detected.

NOS isoforms	MNCs	EPCs	HUVECs
eNOS	-	-	++
nNOS	-	-	+
iNOS	-	+	-

Tab. 10. Expression of NOS proteins. eNOS and nNOS had not been detected by Western Blot in EPCs, probably because of too low concentration of these two enzymes in EPCs. As positive control it was used an extract of HUVECs. (-) no protein detected by Western Blot analysis. (+) protein detected by Western Blot

Being NOS proteins fairly undetectable by Wester Blot, it had been chosen a more sensible method, based on mRNA quantification. It was assessed the expression of eNOS mRNA and iNOS mRNA in MNCs culture under normal condition (control) and under differentiating condition (GF). eNOS mRNA is poorly expressed in EPCs, compared to mature endothelial cells (only 5% of eNOS mRNA in HUVECs). However its expression increases in cells cultured under differentiating conditions, suggesting that cells are differentiating towards an endothelial phenotype. On the contrary, iNOS mRNA levels in differentiated EPCs are 2-3 fold higher than in HUVECs , confirming data obtained thanks to Western Blot analysis.



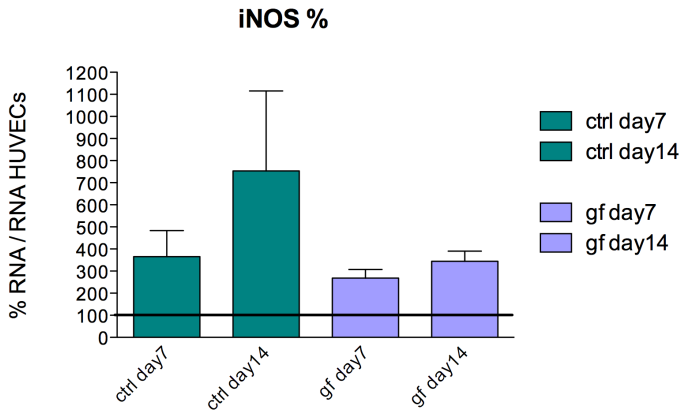


Fig. 22. Levels of mRNA coding for NOS isoforms. Values are expressed as a ratio of mRNA of EPCs to the mRNA of a reference cell type, such as HUVECs, mRNA is expressed via a formula, $2^{(10-\Delta cp)}x10^5$ in which ΔCp is the difference between the Cp of studied gene and the Cp of the housekeeping gene. Ctrl: cells cultured with normal M199, Gf: cells cultured under differentiating growth factors.

An ulterior confirmation of NOS pattern in EPCs was obtained by selectively inhibiting the three isoforms and subsequently measuring NO production with the fluorescent probe. In fact, iNOS inhibition with L-NIL resulted in the lowest NO production.

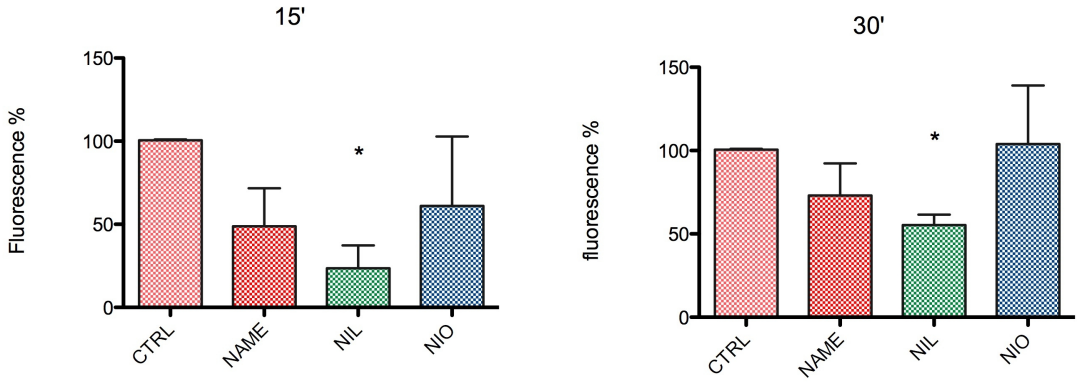


Fig. 23. Measure of NO production with DAF-2DA. 48 h before analysis, cells were treated with NOS inhibitor L-NAME (1 mM), iNOS specific L-NIL (100µM) and eNOS specific L-NIO (10 µM). Then, they were incubated for 30 min at 37 °C with DAF-2DA 10 µM. Fluorescence was measured with a spectophotometer.

NO and proliferation

The second aim was to evaluate how nitric oxide influences EPC function. Hence, the first step was studying its effect on endothelial cell proliferation. HUVECs were cultured with NOS inhibitor for 10 days, and cell viability was measured by MTT assay at different culture time.

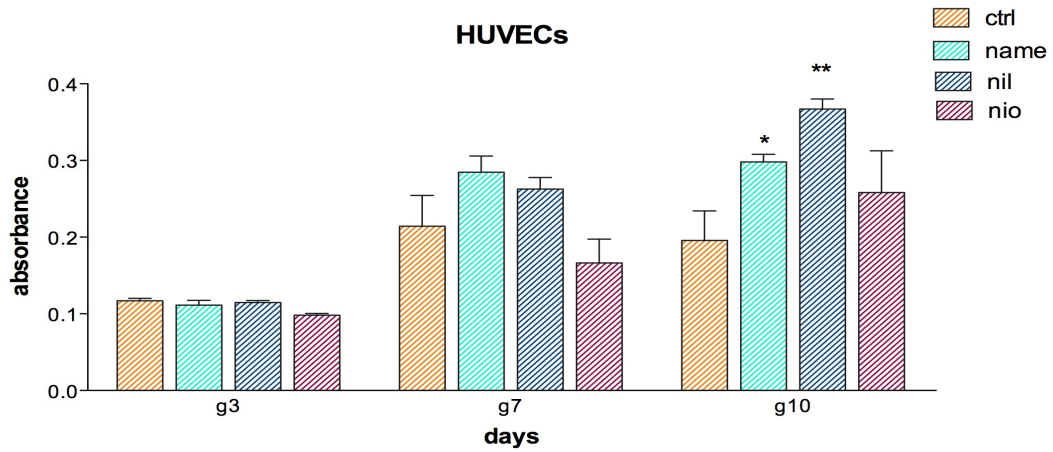


Fig. 24. Viability assay of HUVECs treated with NOS inhibitors.[L-NAME (1 mM), L-NIL (100μM) and L-NIO (10 μM)]. At day 10, the increase of proliferative rate for cells treated with L-NAME and L-NIL is significantly different. CTRL vs NAME, P<0.05, CTRL vs NIL, P<0.005.

Inhibition of iNOS resulted in the highest increase in proliferation rate in HUVECs. Subsequently, NOS inhibition was studied for EPCs too.

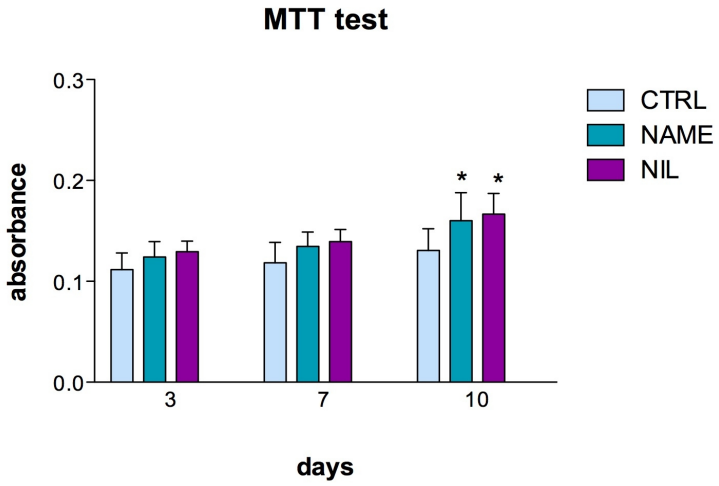


Fig. 25. Effects of NOS inhibition on EPC proliferation. .[L-NAME (1 mM), L-NIL (100μM) and L-NIO (10 μM)]. At day 10 increase of proliferation for L-NAME and L-NIL treated cells was significant. Ctrl s L-NAME, P<0.05, CTRL vs L-NIL, P<0.05.

Analogously to HUVECs, generic and iNOS specific inhibition resulted in higher proliferative capacity of treated cells. iNOS is the NOS isoform that produces the highest levels if NO. Thus, its inhibition may attenuate the apopotic function that elevated levels of NO have.

NO and differentiation

In order to investigate the role of NO on EPC differentiation, mononuclear cells were cultured at the presence of NOS inhibitors.

According to morphological characterization, no significant changes were observed in the number of spindle-shaped and attaching cells.

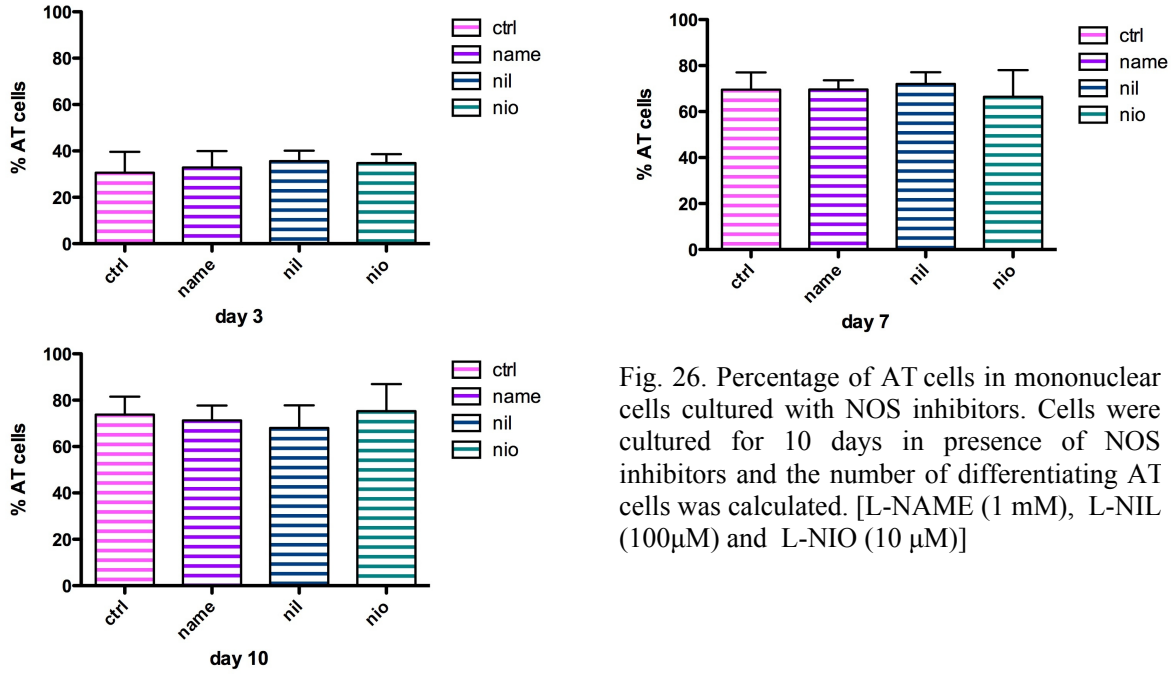


Fig. 26. Percentage of AT cells in mononuclear cells cultured with NOS inhibitors. Cells were cultured for 10 days in presence of NOS inhibitors and the number of differentiating AT cells was calculated. [L-NAME (1 mM), L-NIL (100μM) and L-NIO (10 μM)]

Cells cultured with NOS inhibitors were assessed for the double characterization with DilacLDL uptake and UEA-FITC binding. No significant differences emerged in terms of differentiation.

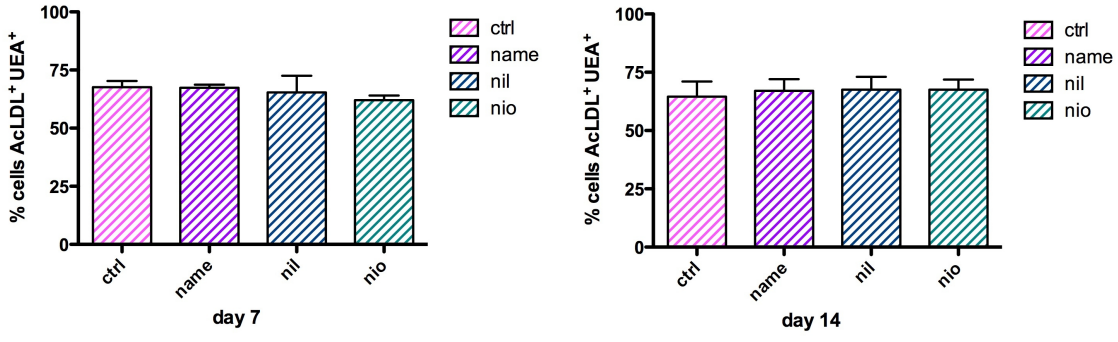


Fig. 27. Characterization of EPCs treated with NOS inhibitors .[L-NAME (1 mM), L-NIL (100μM) and L-NIO (10 μM)]

NO and angiogenesis

Cells were cultured with NOS inhibitors, and at day 14 th vasculogenesis ability was tested.

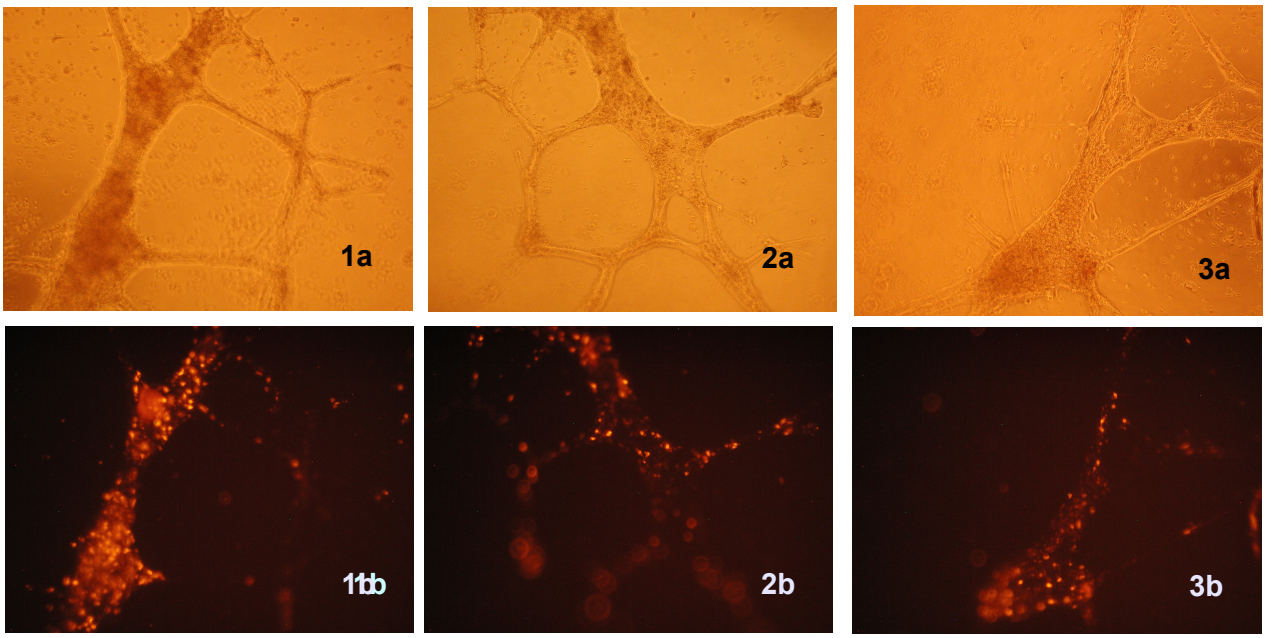


Fig. 28. Vasculogenesis assay of EPCs treated with NOS inhibitors. 1a,b: cells treated with L-NAME (1 mM); 2a,b: cells treated with L-NIL (100µM); 3a,b: cells treated with L-NIO (10 µM).

Apparently, incorporation of EPCs in newly formed structures was not reduced by NAME pretreatement. Otherwise, L-NIL seems to be more effective in reducing EPC incorporation new vessels created by HUVECs,. Analogously, L-NIO pretreatment reduced EPC incorporation, but in a smaller rate compared to L-NIL pretreatment.

The inhibition of NO production was not limited to the use of NOS inhibitors. A novel technique has been adopted in order to achieve a better NOS inhibition. siRNA techniques specifically interact with mRNA coding for the target product, allowing to obtain an efficient inhibition.

At day 7 of culture silenced EPCs were characterised by Dil-AcLDL uptake and UEA-FITC binding. Under inverted light fluorescence microscopy, it was observed a decreased rate of cells positive to AcLDL uptake.

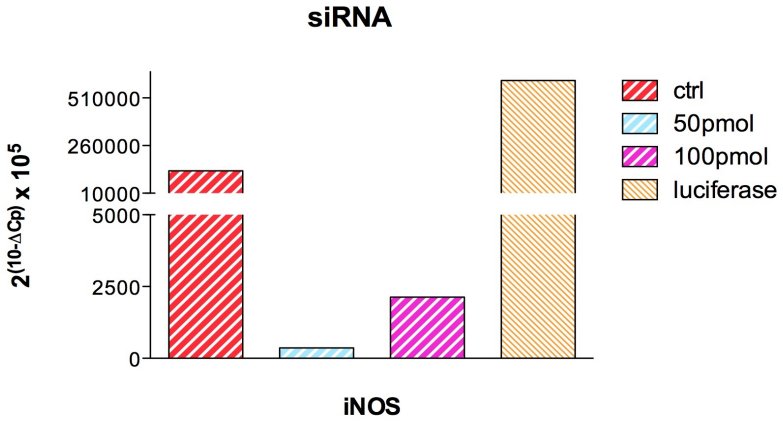


Fig. 29. Effectiveness of iNOS mRNA silencing. In this graph two concentrations of siRNA are compared. As negative control it was used luciferase siRNA. Increment of iNOS expression could be due to the treatment with the liposomial Transfection Reagent.

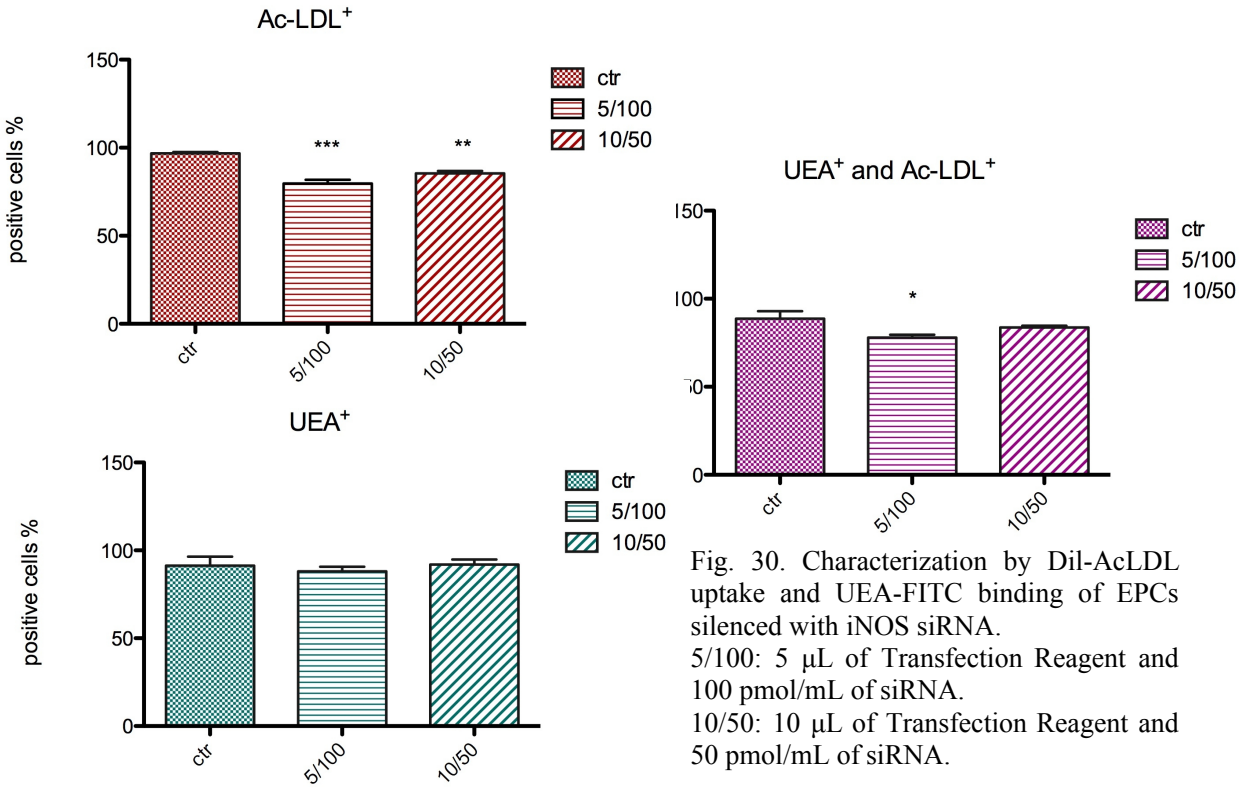


Fig. 30. Characterization by Dil-AcLDL uptake and UEA-FITC binding of EPCs silenced with iNOS siRNA.
5/100: 5 µL of Transfection Reagent and 100 pmol/mL of siRNA.
10/50: 10 µL of Transfection Reagent and 50 pmol/mL of siRNA.

Nadph oxidase

Given the inflfluence of ROS on NO metabolism, it was studied the expression of Nox isoforms by real time PCR techniques. Endothelial cells express all the phagocytic NADPH oxidase subunits, including Nox1, Nox2 and Nox4.

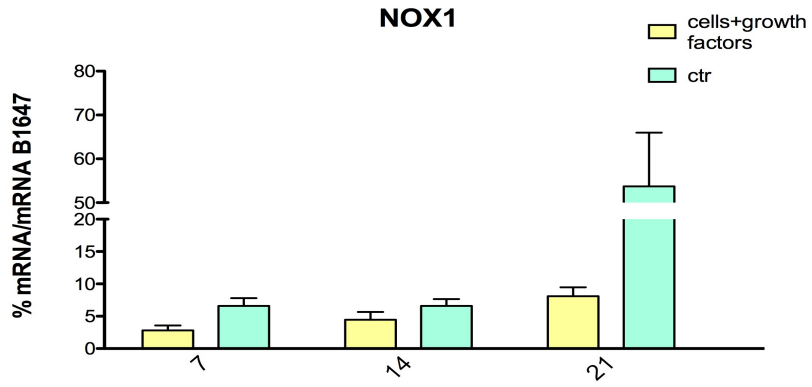


Fig. 31. Expression of Nox1 in EPCs under differentiating condition, control EPCs (ctr), and HUVECs. Values are expressed relative to human megakaryocyte cell line B1647.

EPCs showed to express both Nox1 and Nox2 at higher rate compared to mature endothelial cells, such as HUVECs. On the contrary, Nox4 mRNA levels, which is typically expressed in endothelial cells, were lower in the EPCs than in HUVECs.

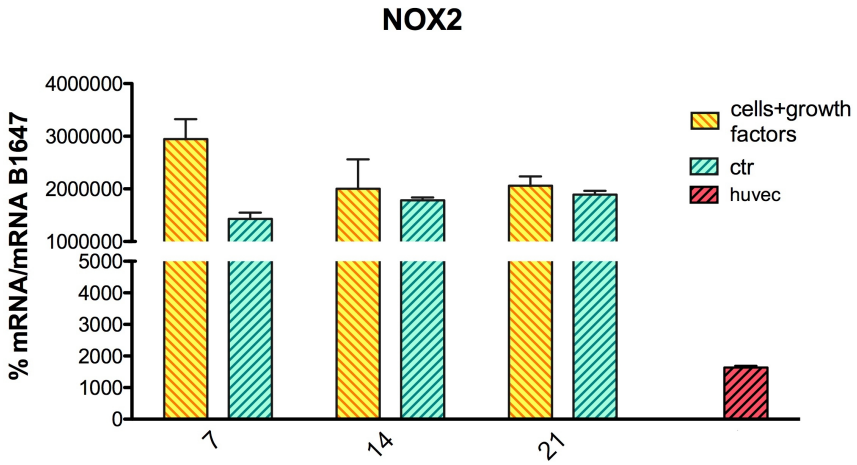


Fig. 32. Expression of Nox2 in EPCs under differentiating condition, control EPCs (ctr), and HUVECs. Values are expressed relative to human megakaryocyte cell line B1647.

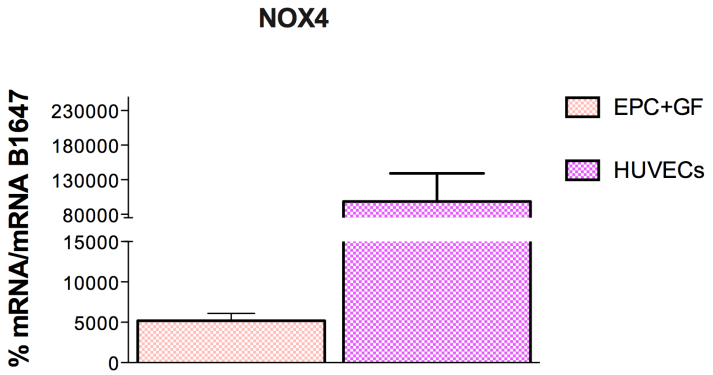


Fig. 33. Expression of Nox4 in EPCs under differentiating condition and HUVECs. Values are expressed relative to human megakaryocyte cell line B1647.

Aim II: EPCs and hyperglycaemia

Being hyperglycaemia a leading cause of endothelium dysfunction in diabetes, the effects of glucose on EPCs were studied. To this end, EPCs were cultured in normal glucose medium, and in medium with the addition of glucose, with a final concentration of glucose 22 mM, and of mannitol, 16,5 mM.

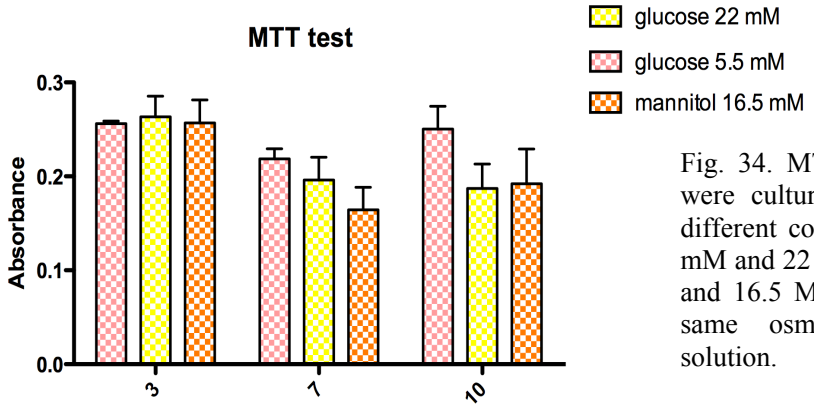


Fig. 34. MTT test for viability. Cells were cultured for 10 days under two different concentrations of glucose (5.5 mM and 22 mM) and at 5.5 mM glucose and 16.5 Mannitol, so that to have the same osmolarity of hyperglycamic solution.

Firstly, it was studied the proliferation of EPCs under the different culture conditions. High glucose levels effect cell proliferation, as well as hyperosmolarity. Secondly, it was

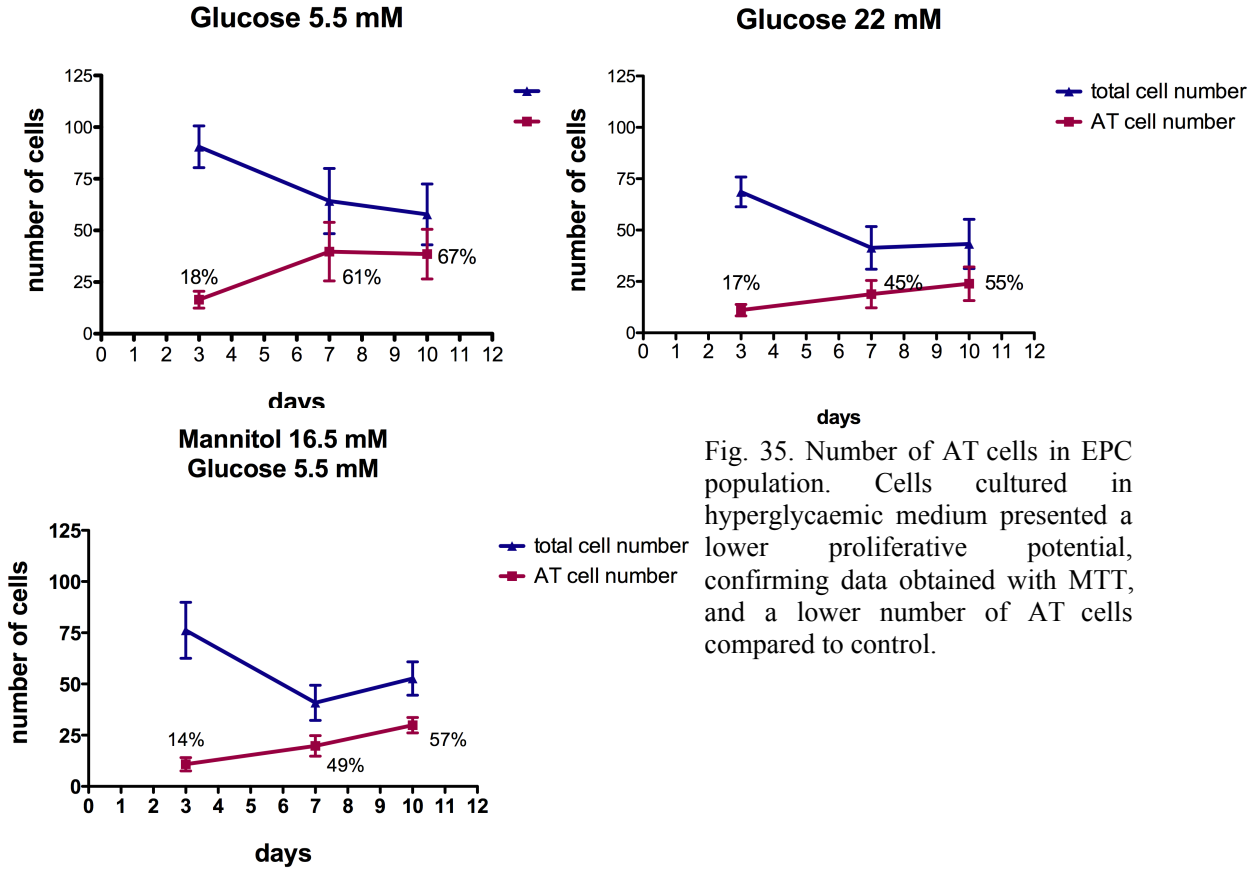


Fig. 35. Number of AT cells in EPC population. Cells cultured in hyperglycaemic medium presented a lower proliferative potential, confirming data obtained with MTT, and a lower number of AT cells compared to control.

investigated whether higher glucose concentration could influence EPC differentiation or not. Cells were cultured under previously described conditions and the number of AT cells was measured.

Effects of Glucose on NOS Expression

Since the NO is a key regulator of EPC function, it has been investigated if a lower proliferation was associated with a change in NOS expression.

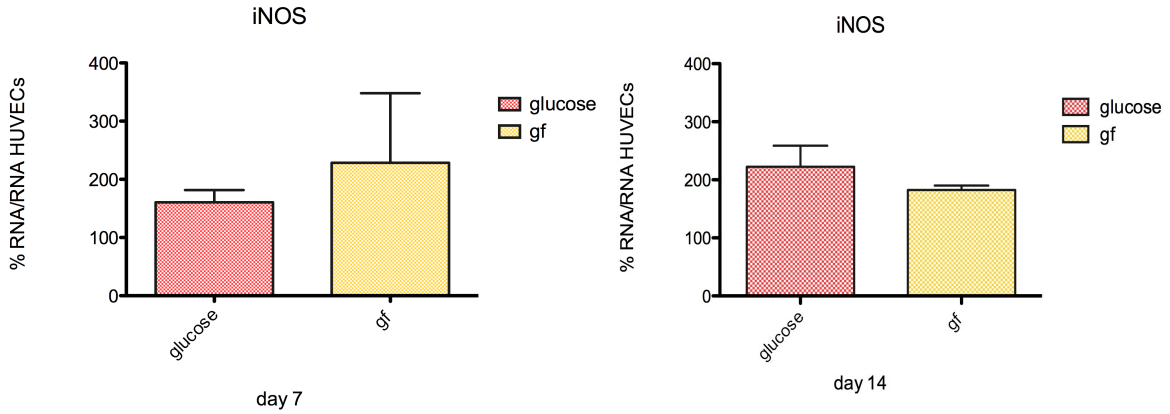


Fig. 36. Expression of iNOS mRNA after glucose conditioning. Values are expressed relative to HUVECs.

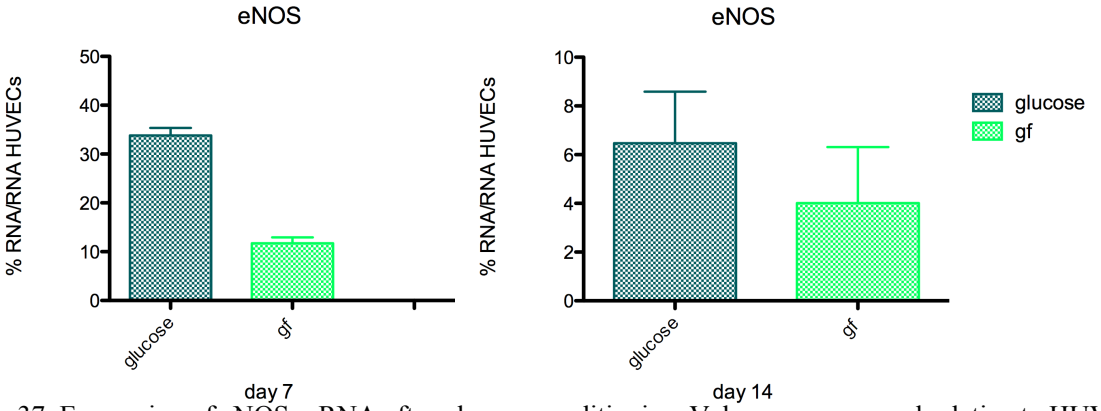


Fig. 37. Expression of eNOS mRNA after glucose conditioning. Values are expressed relative to HUVECs.

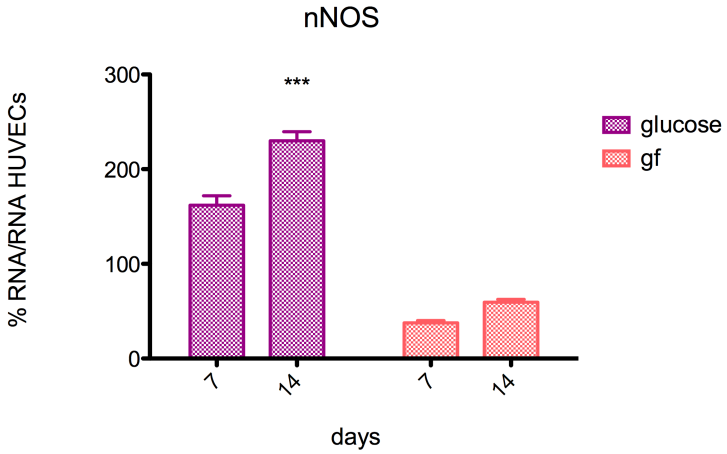


Fig. 38. Expression of nNOS. Values are expressed relative to HUVECs.

As resulted from data obtained by real time-PCR, high glucose concentration induced an enhanced expression of iNOS, eNOS and nNOS. Higher glucose concentration are known to enhance the expression if iNOS, causing an impaired response to insulin. Though eNOS expression seems to be higher compared to controls, experimental data suggest an impaired function of this enzyme.

Aim III: Study of EPCs in Patients with PAD and Diabetes

The third part of this study was dedicated to the characterization of the EPCs in patients with PAD and diabetes. A preliminar distinction of patient vascular situation was given by the number of circulating endothelial cells (CECs). In fact, an elevated number of CECs is an index of vascular damage.

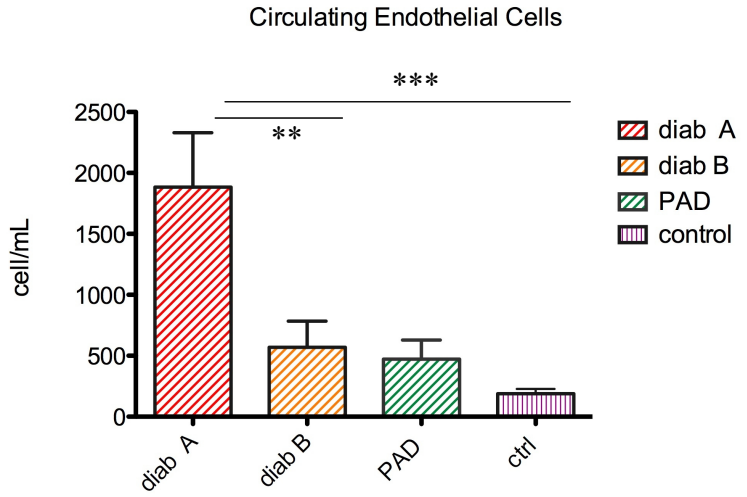


Fig. 39. Number of CECs in peripheral blood samples in patient with peripheral arterial disease and diabetes. Measure was effected by cytofluorimetric analysis. Diab A: group of diabetic cohort with elevated CEC number. Diab B: group of patient with CEC number comparable to that of PAD patients. Diab A vs Ctrl, $P<0.0005$; Diab A vs Diab B, $P<0.005$.

It was possible to distinguish to subpopulation in diabetic cohort. One group had an elevated number of CECs, compared to the other group of diabetics and to PAD patients. In the end, all patients showed a at least 2-fold higher number of CECs compared to heathy subject.

Another parameter was the number of mononuclear cells isolated from peripheral blood samples.

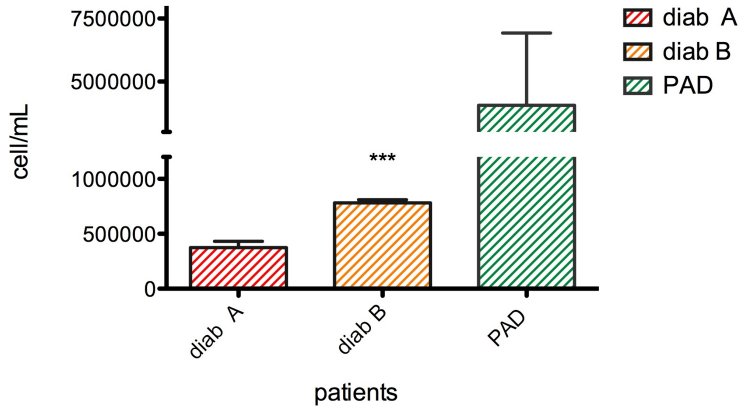


Fig. 40. Number of isolated MNCs per mL of blood. PAD vs Ctrl, $P<0.0005$.

Those patients belonging to group A presented a lower number of MNCs, indicating that, even if the number of CECs indicated a vascular damage, vascular repair is impaired.

Characterization of EPCs

Cells were cultured under differentiating conditions and observed under an inverted light microscope.

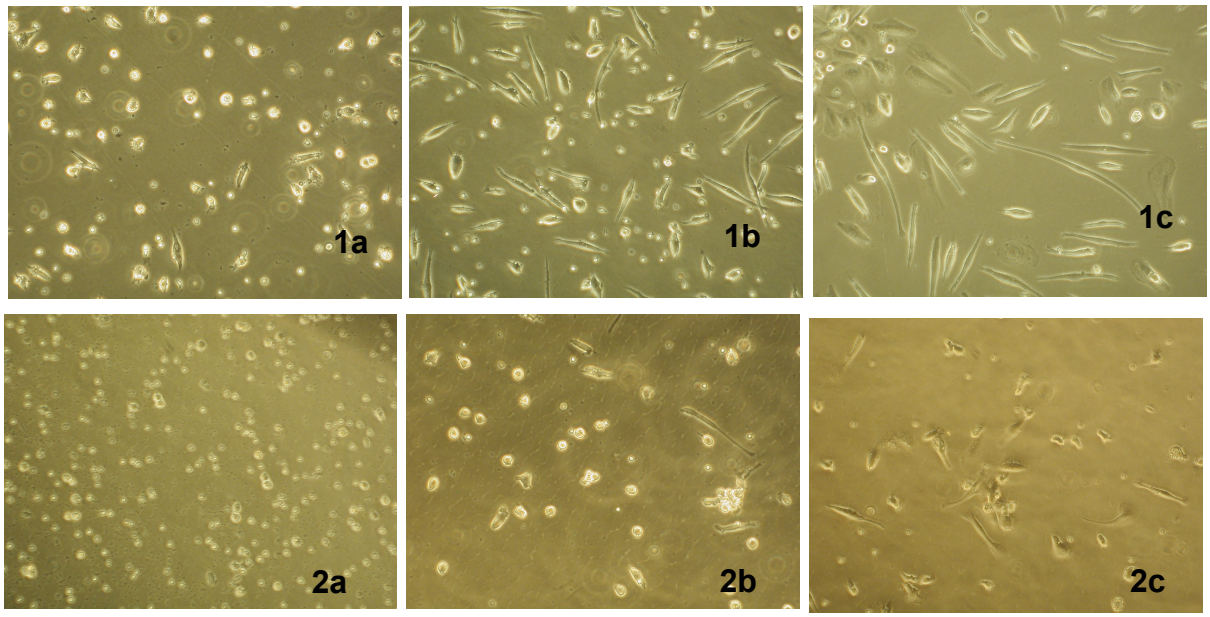


Fig. 41. Endothelial progenitor cells at different time of culture. a) day 3; b) day 7; c) day 10. Cells of 1a, b, and c present good proliferation, while Cells of 2a, b, and c are less proliferative

It was possible to distinguish two groups of patients, one which had good proliferative ability and another one who poorly proliferated. The less proliferative group, since now on called group A, corresponded to those diabetic patients with high levels of CECs and low levels of MNCs and to PAD patients. The other one, since now on called group B, corresponded to diabetic patients with low levels of CECs.

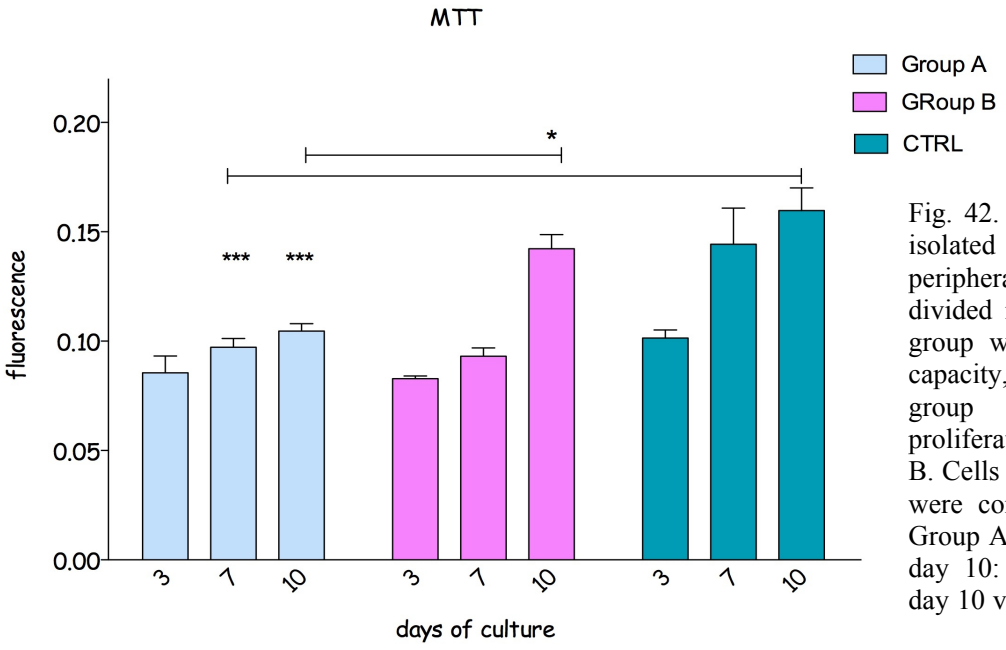


Fig. 42. Proliferation of cells isolated from patient peripheral blood. Cells can be divided into two groups: one group with low proliferative capacity, group A, and a group with normal proliferative capacity, group B. Cells from healthy subjects were considered as controls. Group A day 7 and 10 vs Ctrl day 10: $P<0.0005$. Group B day 10 vs Ctrl day 10: $P<0.05$

Also the test of viability, the MTT assay, confirmed this preliminary distinction of patients into two different group.

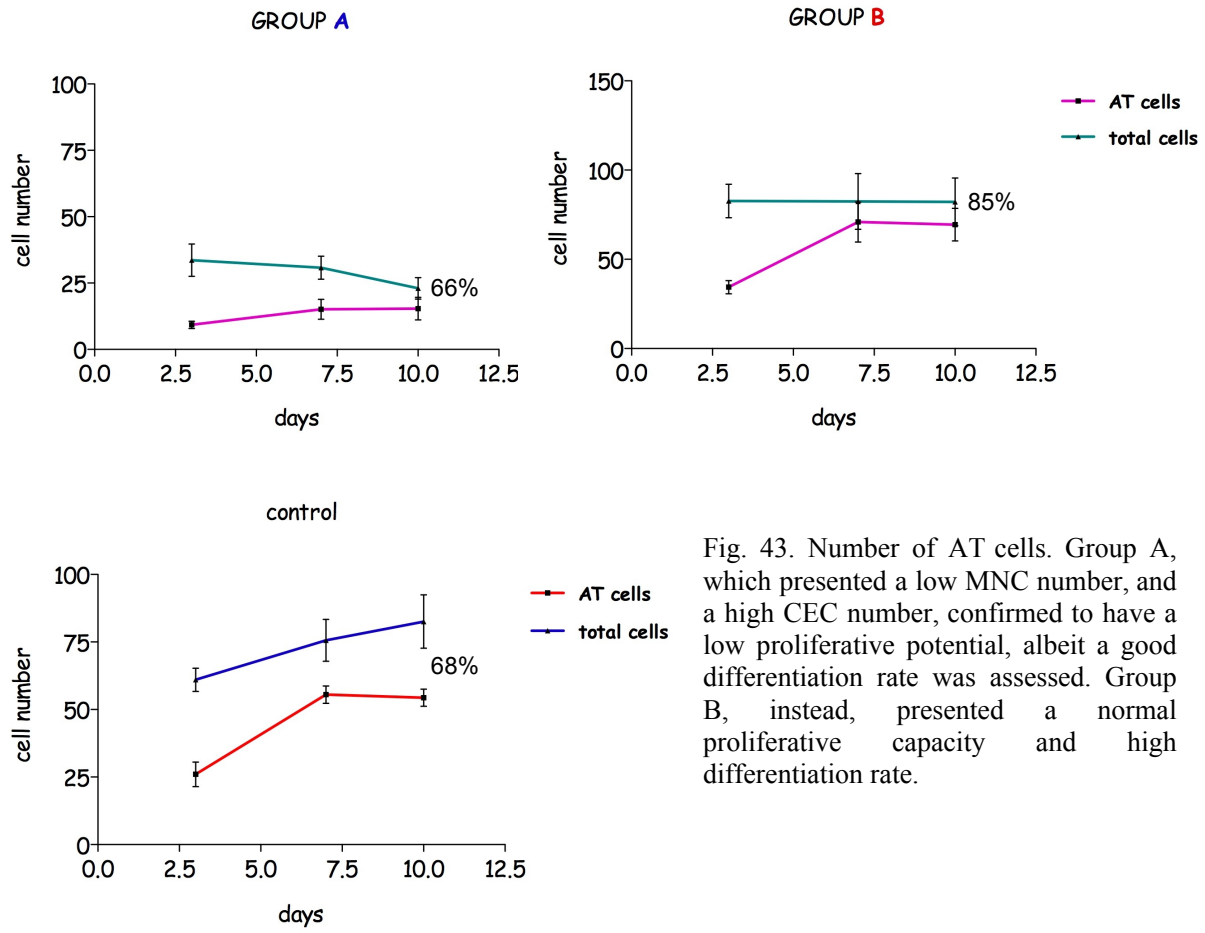


Fig. 43. Number of AT cells. Group A, which presented a low MNC number, and a high CEC number, confirmed to have a low proliferative potential, albeit a good differentiation rate was assessed. Group B, instead, presented a normal proliferative capacity and high differentiation rate.

By observing the number of AT cells, it emerged that, although cells belonging to group A were less proliferative, their differentiation ability was not altered. Characterization by Dil-AcLDL uptake and UEA-FITC binding further confirmed this data.

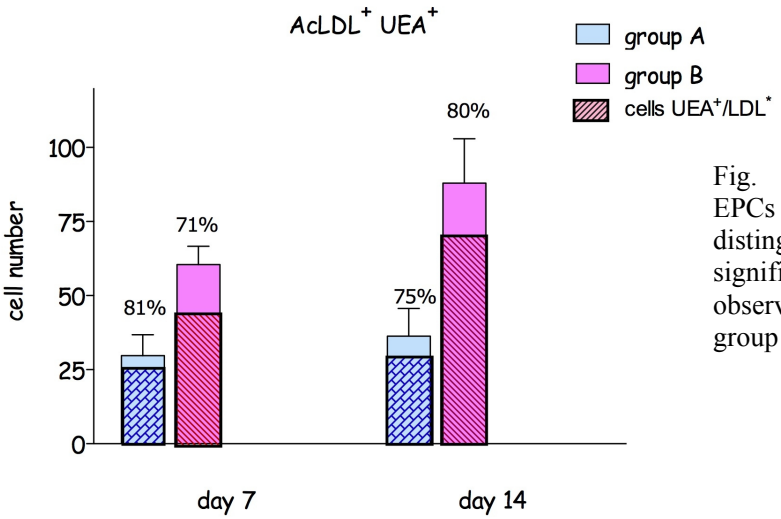


Fig. 44. Characterization of EPCs belonging to the distinguished groups. No significant differences were observed between group A and group B.

Vasculogenec potential

As resulted from the in vitro angiogenesis model, the two populations showed different ability to participate to tube formation. The first group presented a low incorporation rate in the new capillary-like structures formed by HUVECs, while the second group showed a higher vasculogenic potential.

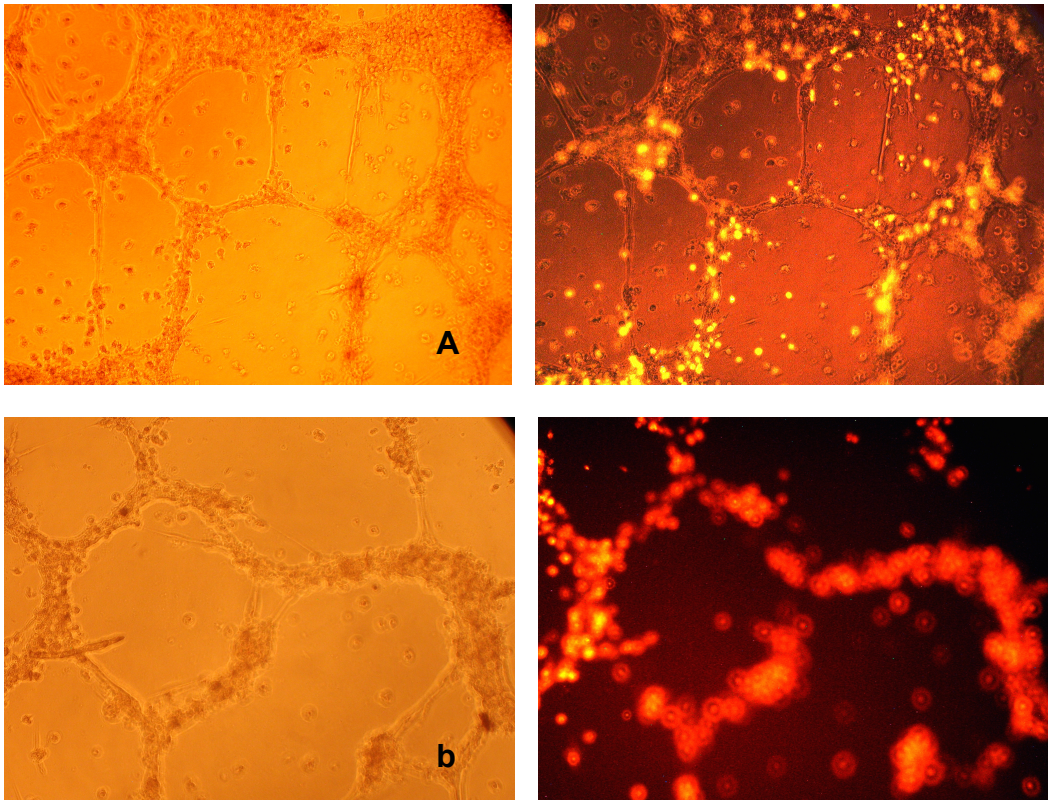


Fig. 45. Participation of EPCs to new vessel formation. EPCs from group A (a) and from group B (b) were plated together with HUVECs on Matrigel matrix. Group A showed a limited capacity to incorporate to newly formed structures.

Since EPCs belonging to group B had shown no significant impairment in participation to vasculogenesis, it was tested whether they could be able to autonomously form capillary-like structures. If seeded at 3-fold higher concentration, they gave origin to tube formation.

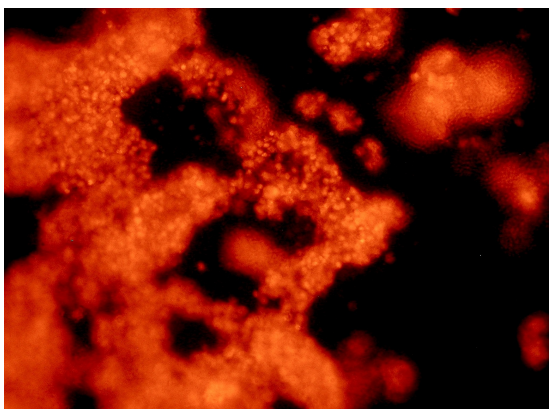


Fig. 46. Angiogenic capacity of diabetic EPCs. Cells were seeded at 30000 cell/well on matrigel coated plates and their vasculogenic ability was assessed. As hypothesised, the cells of group B were able to form approssimative capillary like structures as well as normal EPCs do.

NOS Expression

The following step was to understand if there was a correlation between the reduced proliferative and angiogenic potential and the expression of NOS isoforms.

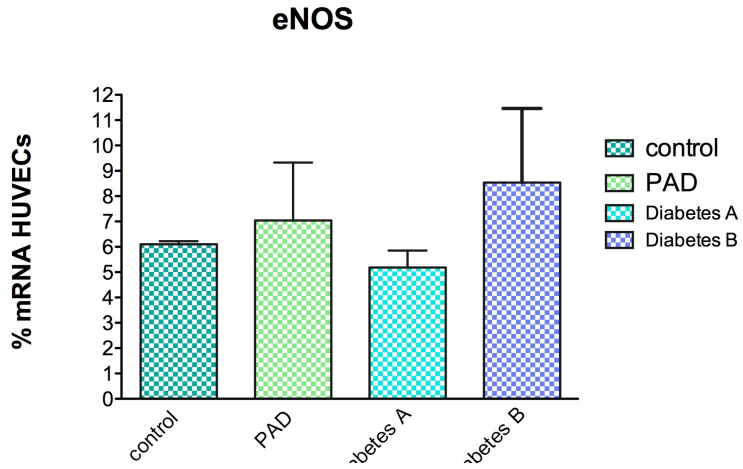


Fig. 47. Expression of eNOS mRNA in patients with PAD and in diabetic patients with low (A) and normal (B) proliferative capacity.

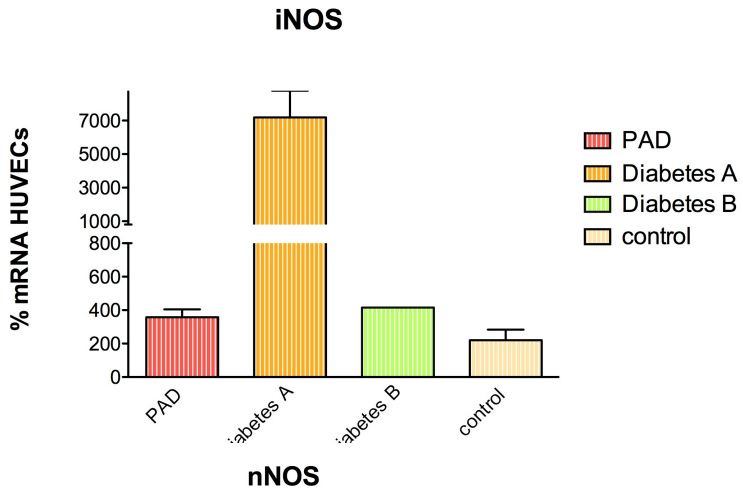


Fig. 48. Expression of iNOS mRNA in patients with PAD and in diabetic patients with low (A) and normal (B) proliferative capacity.

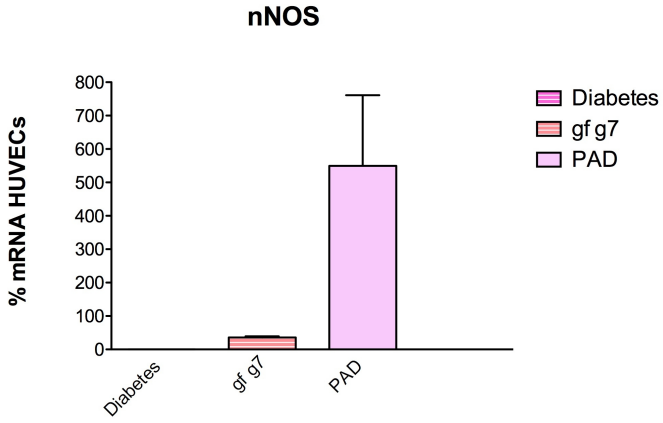


Fig. 49. Expression of nNOS mRNA in patients with PAD and in diabetic patients with low and normal proliferative capacity.

As regards eNOS mRNA expression in the studied cohorts, it did not emerged a significant variation in its levels in EPCs. However a slight increase was observed in diabetic patients with normal proliferation, thus probably allowing cells to be more functional. On the contrary,

diabetic patients with low proliferative capacity presented a significant increase in iNOS expression, much more higher than the other populations considered in this study.

Moreover, both groups of diabetic patients did not express the neuronal isoform. Likewise other cell populations in diabetic patients, EPCs showed an impairment in nNOS expression.

Modulation of the NADPH Oxidase Expression

Finally, it was investigated whether the endothelium dysfunction could be not only due to an impaired NOS expression, but also to oxidative stress.

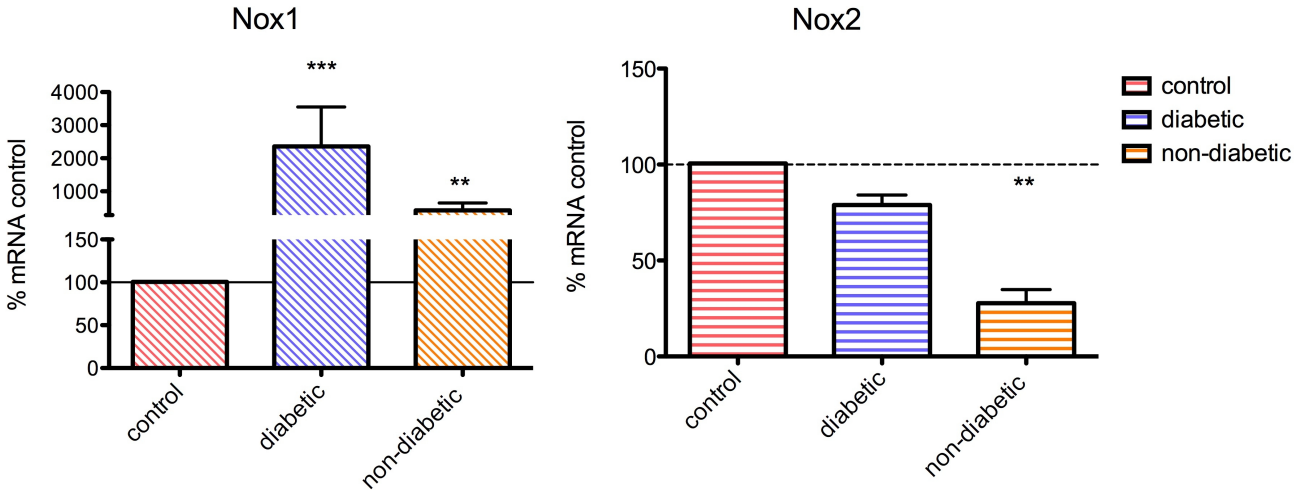


Fig. 50. Expression of Nox isoforms, Nox1 and Nox2 in diabetic and non diabetic patients affected by PAD (n=2).Nox1 graph: Diabetic vs control P<0.0005, Non-diabetic P<0.005. Nox2 graph: Non-diabetic vs control P<0.005.

Data indicated that Nox1 levels were extremely high in patients affected by PAD, and in particular, in diabetic ones. As a result, EPC dysfunction could be caused by an increased oxidative stress and consequent impairment of NOS production, because of NOS uncoupling. Moreover, Nox2 is less expressed in non-diabetic patients. Nox2 is known to be involved in VEGF-signalling, and subsequent VEGF-induced neovascularization. Hence low levels of Nox2 may be associated to a reduced vascularization.

Chapter 10

Discussion

Changes in human lifestyle and healthcare have resulted in a dramatic increase in the prevalence of cardiovascular disease. Atherosclerosis-related vascular disease is responsible for half the deaths in Europe and the growing population of diabetic patients is particularly prone to occlusion of arteries and suffers a more complicated clinical outcome after myocardial infarction or stroke compared with non-diabetics (129).

Initially pharmacological treatement was considered the only instrument to alleviate symptoms. Afterward, the advent of mechanical revascularization by angioplasty improved the survival rate and life quality of patients. However, not all patients can undergo this kind of therapy. Thereby, it was essential to find an alternative method.

In the last decade a novel concept arose, that improvement in perfusion can be achieved by new vessel formation. This new approach postulate a lack of or an insensitivity to angiogenic factors, thus, supplementation with cytokines would counteract the deficiency of endogenous growth factors typical of vascular disease. However, the increasing evidence that neovascularization in adults is not uniquely dependent on resident ECs but also involves bone marrow derived EPCs, suggested the use of these cells for therapeutic use. In fact, therapeutic vasculogenesis, which is a cell-based version of biological revascularization, postulates that the transplantation of endothelial progenitor cells can enhance native regenerative mechanisms and thereby produce clinically valuable improvement in blood flow. EPCs can be isolated from different sources. However, isolation from peripheral blood is less invasive, so that .hematopoietic cells can be harvested and readministrated for autologous transplantation

This study was focused on better understanding the vasculogenic potential of EPCs isolated from blood, their differentiative and proliferative capacity with respect to NO signalling and glycaemia, and to a pathological condition, such as peripheral arterial disease.

The hematopoietic population was isolated and cultured under differentiating conditions. After few days it was possible to observe spindle-shaped and attaching cells, also referred to as AT cells. This is already considered an endothelial-like morphology. Moreover, these cells tended to form clusters and subsequently, to assume a cobblestone aspect, that is very similar to that of mature endothelial cells, such as HUVECs.

Being the morphological characterization not sufficient to define this population, the adherent cells were characterised by a double staining, the Dil-acetylated LDL uptake and UEA-I

binding. After one week, about 70% of cells was positive to both markers, and this value increased throughout time.

Once differentiation toward an endothelial phenotype was assessed, the next step was to determine whether these cells were able to form capillary-like structures. If seeded on Matrigel membrane, EPCs were not able to do tube formation ex novo. They were only able to give origin to rough vascular structures. On the contrary, when plated together with mature endothelial cells, EPCs showed ability to partecipate to vasculogenesis. In fact, labelled EPCs were visibly incorporated into the newly formed structures. In the end, this would be more similar to the *in vivo* situation. Actually EPCs are known to home to sites of neovascularization sites and work in concert with existing endothelial cells so that to form vessels.

Increasing evidence demonstrated the key role of NO in angiogenesis and vasculogenesis (95). On the basis of these data, it was examined the metabolism of NO in EPCs. By use of fluorescent probe DAF-2DA, it emerged that these cells produced NO. In mature endothelial cells, NO effects angiogenic process via transduction pathway VEGF-eNOS-NO and eNOS is considered the principal NOS isoform involved in vasculogenesis (73). Hence, the expression of this isoform in EPCs was investigated. Both eNOS protein, assessed by Western Blot analysis, and mRNA, assessed by qPCR, resulted to be much lower compared to the expression of this enzyme in HUVECs. Also the other constitutive isoform, nNOS, was less expressed than in HUVECs. On the contrary, the trend of iNOS was completely different. iNOS protein was detectable by Western Blot, and its mRNA values were 2-3fold higher than those in HUVECs.

In order to understand which was, actually, the principal isoform responsible for NO production in EPCs, two weeks cultured cells were pretreated with NOS inhibitors, the generic inhibitor L-NAME, the iNOS specific one L-NIL, and the eNOS specific one L-NIO and the NO production was measured by using DAF-2DA. Results seemed to confirm the previous data. In fact, L-NIL caused the highest inhibition of NO production. Although L-NAME was a generic inhibitor and was supposed to strongly inhibit NO production, it was less effective than L-NIL. It has to be considered that, finally, L-NAME presents a higher affinity for constitutive isoforms. The lesser inhibitory power registered for L-NAME would support the previous findings of a lower expression of constitutive NOS isoforms in EPCs, in comparison to inducible NOS.

Then, the following step was to assess the role of the three isoforms in EPC homeostasis, as regards proliferation, differentiation and vasculogenesis. By treating cells with NOS inhibitors

for two weeks, it was observed an increase in the proliferative rate for cells cultured with L-NAME, and, in particular, for those cultured with L-NIL. The positive effect of NO depletion on proliferation could be explained by the dual role of NO, either apoptotic or not. Highest levels of NO are, in fact, associated with an enhanced cell apoptosis.

iNOS inhibition in EPCs also exhibited a reduction in vasculogenic ability of these cells. Matrigel assay was performed on EPCs pretreated with NOS inhibitors and cells inhibited with L-NIL showed to be less incorporated in newly capillary-like structures. In their work, Mayr and coworkers had actually reported a central role of inducible NOS in vein graft reendothelialization (101). Moreover, it was investigated if iNOS silencing affected EPC differentiation. Cells treated with iNOS siRNA exhibited a lower Dil-acetylated LDL uptake, thus reducing the rate of cells positive to double staining.

It is known that NO inhibition can also be caused by enhanced ROS expression. Hence, it was studied the expression of NADPH oxidase complexes in physiological conditions. It was evaluated the expression of Nox1, Nox2 and Nox4. The last two ones represent the major source of ROS in endothelial cells, while Nox1 is principally expressed in phagocytic cells. EPCs presented a higher expression of Nox1 and Nox2, compared to mature endothelial cells. This can be considered as a consequence of the macrophagic origin of EPCs. On the contrary, Nox4 was less expressed in EPCs compared to HUVECs. Being Nox4 highly expressed in endothelium, its lower levels in EPCs were a further evidence of progressing but not concluded EPC differentiation toward an endothelial phenotype.

Since the final aim of this research was the study of EPCs isolated from patients affected by PAD and diabetes, it was investigated the effect of the most damaging factor in diabetes: hyperglycaemia. Control cells, isolated from healthy volunteers, were cultured under hyperglycaemic conditions (22 mM glucose) and under hyperosmotic conditions (5.5 mM glucose and 16.5 mM mannitol). Cells under hyperglycaemic conditions exhibited a reduced proliferation, compared to controls. Hyperosmosis also induced a decrease in EPC proliferation, but less than high glucose concentration did. Analogously, also differentiation was altered. Hyperglycaemic EPCs showed a lower number of adherent cells, and most importantly, a lower percentage of AT cells. As regards NOS expression, cells exposed to high glucose concentrations had an increase in iNOS expression, and also of the other constitutive isoforms.

EPCs isolated from patients affected by PAD and diabetes also showed an impairment in cell proliferation, although it has to be done a distinction among the different patients.

A preliminar characterization of vascular situation of these patients was given by the number of circulating endothelial cells. It is widely recognized that an increased CEC number is associated with a more serious vascular damage. Non-diabetic cohort affected by PAD presented high CEC number in peripheral blood compared to control cells, and showed an impaired proliferation. On the contrary, diabetic cohort could be classified into two groups. One group had a very high number of CECs and a poor proliferative ability. The other group had a CEC number similar to that of non-diabetic PAD patients, but presented quite good proliferative ability. The dual functionality of diabetic patients could be referable to the number of mononuclear cells, and thus of EPCs, isolated from patient peripheral blood. In fact, patients belonging to the first group had a lower number of MNCs compared to patients belonging to the second group. However, PAD patients showed a very high number of MNCs, but that resulted unable to adequately proliferate.

With respect to EPC differentiation, it was not detected any significant difference between patients with good and those with bad proliferative capacity. Although, when it was tested their vasculogenic potential, the first ones showed a relative good rate of incorporation into new structures formed by HUVECs, and even demonstrated to be able to form approssimative vessels ex novo, as well as EPCs from healthy subjects do. Patients with low proliferative capacity exhibited low incorporation rate, instead.

Concerning NOS mRNA expression, eNOS was not significantly different among examined patients. Only a slight increase was observed in diabetic patients with good proliferation rate. Also iNOS mRNA expression was similar to that of controls, except for the diabetic patients with less functional EPCs. In this group iNOS expression was extremely high. Probably in these cells with increased iNOS levels could result in too high NO release, causing its apoptotic effects rather than positive for reendothelialization. Finally, nNOS was not or too poorly expressed in diabetic patients. Studies in other cell types have already evidenced an impairment in nNOS in diabetic patients, being this enzyme important in insulin-response to arginin. In animal model, eNOS and nNOS deficiency causes insulin resistance in skeletal muscle cells, while induction of iNOS is associated with impaired insulin-stimulated muscle glucose uptake (130).

Finally, being atherosclerosis associated to inflammation and increased oxidative stress, it was investigated the expression of NADPH oxidases, Nox1 and Nox2, in the two groups. Both diabetic and nondiabetic patients showed a high enhancement of Nox1 levels compared to controls. Nox1 is not constitutively activated in endothelial cells, thus, in atherosclerosis there are stimuli which cause the translocation of cytosolic subunits and subsequent Nox1 activation.

On the contrary, Nox2 resulted to be less expressed compared to controls. Hence, a lower response to the angiogenic factor VEGF could occur as a consequence of the reduction of Nox2 mRNA expression.

In conclusion, NO is confirmed to play a crucial role in EPC ability to differentiate and to take part in vasculogenesis. Moreover, the NOS activity seems to be involved in cellular response to glucose and insulin metabolism, as registered for diabetic patient, and especially nNOS. Albeit non-diabetic patients did not present a significant difference in eNOS and iNOS expression, it has not be excluded an impairment of enzymatic function, due to increased Nox1 expression and subsequent enhanced ROS production.

Since a group of patients presented a quite normal EPC function in vitro, it has to be hypothesised the presence of negatively conditioning factors in vivo. Hence, concerning the use of autologous transplantation of EPCs for reendothelization of vein graft, it becomes essential to find preconditioning substances which would counteract the endogenous negative factors. NO could considered an elegible candidate for this.

References

1. **Tomson J et al.** Peripheral arterial disease: A high risk-but neglected- disease population. BMC Cardiovasc Disord, 2005, 5:15.
2. **Watson K et al.** Peripheral Arterial Disease: a review of disease awareness and management. Am J Geriatr Pharmacother,
3. **Criqui MH et al.** The epidemiology of peripheral arterial disease: importance of identifying the population at risk. Vasc Med 1997; 2:221-226.
4. **Dormandly JA**, The fate of patients with critical leg ischemia. Seminars in Vascular Surgery 1999; 12:142-147.
5. **Caro JJ et al.** The morbility and mortality following a diagnosis of peripheral arterial disease: Long-term follow up of a large database. BMC Cardiovasc Disord, 2005; 5:14.
6. **Stoffers HE (file-hooi)**, et al. The prevalence of asymptomatic and unrecognized peripheral arterial occlusive disease. Int J Epidemiol 1996;25:282–90.
7. **Weitz et al**., Diagnosis and treatment of chronic arterial insufficiency of the lower extremities: a critical review. Circulation 2000;102(9):1074.
8. **Greenland P**, Clinical significance, detection and medical treatment for peripheral arterial disease. J Cardiopulm Rehabil. 2002; 22:73-79.
9. **Allaqband S**. et al. Endovascular Treatment of Peripheral Vascular Disease, Curr Prob Cardiol 2006; 31:711-760.
10. **Allison MA et al.** Association between the ankle-branchial index and future coronary calcium (the Rancho Bernardo study). Am J Cardiol, 2006; 97:181-186.
11. **Wang JC** et al. Exertional leg pain in patients with and without peripheral arterial disease. Circulation 2005, 2005; 112:3501-3508.
12. **Newman AB** et al. Ankle-arm index as a marker of atherosclerosis in the Cardiovascular Health Study. Circulation 1993; 88:837-845.
13. **Hiatt WR et al.** Medical treatment of peripheral arterial disease and claudication. N Engl J Med. 2001; 344:1608-1621.
14. **Meijer WT et al.** Determinants of peripheral arterial disease in the elderly: the Rotterdam Study. Arterioscler Thromb Vasc Biol. 1998; 18:185-192.
15. **Kennedy M et al.** Risk factors for declining ankle-branchial index in men and women 65 years or older: the Cardiovascular Health Study. Archives of Internal Medicine. 2005; 165:1896-1902.

16. **Hooi JD et al.** Incidence of and Risk Factors for Asymptomatic Peripheral Arterial Occlusive Disease: A Longitudinal Study. Am J Epidemiol 2001;153:666–72.
17. **White CJ et al.** Endovascular Therapies for Peripheral Arterial Disease: An Evidence-Based Review. Circulation, 2007; 116:2203-2215.
18. **Sottiurai V et al.** Extensive Revascularization or Primary Amputation: Which Patients with Critical Limb Ischemia Should Not Be Revascularized? Semin Vasc Surg, 2007; 20:68-72.
19. **Endemann DH et al.** Endothelial Dysfunction. J Am Soc Nephrol 2004; 15:1983-1192.
20. **Fan J at al**, Inflammatory reactions in the Pathogenesis of Atherosclerosis. Journal of Atherosclerosis and Thrombosis, 2003; 10:63-71.
21. **Hansonn GK et al.** Inflammation and atherosclerosis. Annual Review of Pathology. 2006;1:297-329.
22. **Krieger M**, Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. Journal of Clinical Investigations. 2001; 108:793–797.
23. **Glass CK et al.** Atherosclerosis, the road ahead. Cell, 2001; 104:503-516.
24. **Haroaka S et al**. Role of T lymphocytes in the pathogenesis of atherosclerosis: animal studis using athymic nude rats. Annals of the New York Academy of Sciences. 1997; 811:515-8.
25. **Watanabe et al**. Role of macrophages in atherosclerosis. Sequential observations of cholesterol-induced rabbit aortic lesion by immunoperoxidase technique using monoclonal antimacrophage antibody. Lab Invest, 1985; 53:80-90.
26. **Cybulsky MI et al.** Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991; 251, 788–791.
27. **Blakenberg S et al.** Adhesion molecules and atherosclerosis, Atherosclerosis 2003; 170 (2):191–203.
28. **Ross R**, Atherosclerosis an inflammatory disease. N Engl J Med 1999; 340:115-126.
29. **Bruschke AV et al**. The dynamics of progression of coronary atherosclerosis studied in 168 medically treated patients who underwent coronary arteriography three times. Am. Heart J. 1989; 117: 296–305.
30. **Rajavashisth TB et al.** Inflammatory cytokines and oxidized low density lipoproteins increase endothelial cell expression of membrane type 1-matrix metalloproteinase. J Biol Chem 1999; 274:11924–11929.

31. **de Boer OJ et al.** Leucocyte recruitment in rupture prone regions of lipid-rich plaques: a prominent role for neovascularization? Cardiovasc. Res. 1999; **41:**443–449.
32. **Panza JA et al.** Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N Engl J Med, 1990; 323:22-27.
33. **Szmitko PE et al.** New Markers of Inflammation and Endothelial Cell Activation. Circulation, 2003; 108:1917-1923.
34. **Verma S et al.** C-reactive protein attenuates endothelial progenitor cell survival and differentiation. Can J Cardiol, 2002; 18(suppl B): 325.
35. **Robin J et al.** Protease-activated receptor 2-mediated vasodilation in humans in vivo: role of nitric oxide and prostanoids. Circulation, 2003; 107:271-276.
36. **Iwaguro H et al.** Endothelial progenitor cell culture and gene transfer. Meth Mol Med; 2002, 117:239-247.
37. **Rahman S et al.** Diabetes-associated macrovasculopathy: pathophysiology and pathogenesis. Diabetes Obes Metab, 2007; 9:767–780.
38. **Varughese GI et al**. Type 2 diabetes mellitus: a cardiovascular perspective. Int J Clin Pract, 2005; 59 (7):798-816.
39. **Nguyen LI**. Percutaneous treatment of peripheral vascular disease: The role of diabetes and inflammation. J Vasc Surg, 2007; 45(6):A149-A157.
40. **Jansonn PA**. Endothelial dysfunction in insulin resistance and type 2 diabetes. J Intern Med 2007; 262: 173–183.
41. **Zeng G et al**. Roles for insulin receptor, PI 3-kinase, and Akt in insulin signaling pathway related to production of nitric oxide in human vascular endothelial cells. Circulation 2000; 101: 1539–1545.
42. **LeRoith D et al.** Recent advances in our understanding of insulin action and insulin resistance. Diab Care 2001; 24: 588–597.
43. **Montagnini M et al**. Inhibition of phosphatidylinositol 3-kinase enhances mitogenic action of insulin in endothelial cells. J Biol Chem 2002; 277: 1794–1799.
44. **Reusch JEB et al**. Atherosclerosis in diabetes and insulin resistance. Diabetes Obes Metab, 2007; 9:455–463.
45. **Varma S et al**. Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction. Am J Physiol Heart Circ Physiol 2005; 289: H1744–H1751.

46. **Basta G et al.** At least 2 distinct pathways generating reactive oxygen species mediate vascular cell adhesion molecule-1 induction by advanced glycation end products. Arterioscler Thromb Vasc Biol 2005; 25:1401–1407.
47. **Hotamisligil GS et al**. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993; 259:87-91.
48. **Asahara T et al.** Isolation of putative progenitor endothelial cells for angiogenesis. Science, 1997; 275:964-967.
49. **Semenza GL**, Vasculogenesis, Angiogenesis, and Arteriogenesis: Mechanisms of Blood Vessel Formation and Remodelling. J Cell Biochem, 2007; 102:840-47.
50. **Lin Y et al.** Origins of the circulating endothelial cells and endothelial outgrowth from blood. J Cli Invest, 2000; 105-71-77.
51. **Ingram DA et al.** Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. Blood, 2005; 105(7):2783-6.
52. **Gill M et al.** Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res, 2001; 88(2):167-74.
53. **Edelberg JM et al.** Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. Circ Res, 2002; 90(10):E89-93.
54. **Asahara T et al**. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological conditions. Circ Res, 1999; 85:221-228.
55. **Crosby JR et al**. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res, 2000; 87:728-730.
56. **Murayama T et al.** Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. Exp Hematol, 2002; 30(8):967-72.
57. **Reyes M et al.** Origin of endothelial progenitor cells in human postnatal bone marrow. J Clin Invest, 2002; 109:337-346.
58. **Tamaki T et al.** Identification of myogenic endothelial progenitor cells in the interstitial space of skeletal muscle.J Cell Biol 2002; 157:571-577.
59. **Beltrami AP et al.** Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114:763–776.
60. **Planat-Bernard V et al.** Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives, Circulation 2004; 109:656–663.

61. **Wurmer AE er al.** Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. Nature 2004; 430: 350–356.
62. **Yin AH et al**. AC133, a novel marker for human hematopoietic stem and progenitor cells, Blood 1998; 90: 5002-12.
63. **Rehman J et al.** Peripheral blood endothelial progenitor cells are derived from monocyte/macrophages and secrete angiogenic factors. Circulation 2003; 107:1164-169.
64. **Kim SY et al.** Differentiation of endothelial cells from human umbilical cord blood AC133-CD14+ cells. Ann Hematol 2005; 84(7):417-2.
65. **Urbich C et al.** Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 2003; 108:2511-2516.
66. **Weiss MJ et al.** In vitro differentiation of murine embryonic stem cells. New approaches to old problems. J Clin Invest 1996; 97:591-595.
67. **Peichev M et al.** Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood. 2000; 95: 952–958.
68. **Gulati R et al.** Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ Res, 2003; 93(11):1023-5.
69. **Ingram DA et al.** Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. Blood. 2005;106:1525-1531.
70. **Choi K et al**. A common precursor for hematopoietic and endothelial cells. Development 1998; 125: 725-32.
71. **Schmeisser A et al.** Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res 2001; 16, 49(3):671-80.
72. **Hristov M et al.** Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. J Cell Mol Med 2004, 4(8): 498-508.
73. **Aicher A et al.** Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells, Nat Med 2003; 9:1370-1376.
74. **Urbich C et al.** Endothelial Progenitor Cells: Characterization and Role in Vascular Biology. Circ Res. 2004; 95:343-353.
75. **Hristov M et al.** Regulation of endothelial progenitor cell homing after arterial injury. Thromb Haemost 2007; 98: 274–277.

76. **Chavakis E et al**. Role of β2-integrins for homing and neovascularization capacity of endothelial progenitor cells. J Exp Med 2005; 201: 63–72.
77. **Duan H et al**. LFA-1 and VLA-4 involved in human high proliferative potential-endothelial progenitor cells homing to ischemic tissue. Thromb Haemost 2006; 96: 807–815.
78. **Tepper OM et al.** Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures, Circulation, 2002; 106:2781-2786.
79. **Valgimigli M et al**. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation, 2004; 110:1209-121.
80. **Rauscher FM et al.** Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation* 2003; 108: 457–63.
81. **Werner N et al**. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med. 2005; 353: 999–1007.
82. **Hu Y et al.** Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. Circulation 2003; 108: 3122–7.
83. **Seeger FH et al.** p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. Circulation 2005; 111:1184-91.
84. **Liew A et al**. Endothelial progenitor cell for treatment of diabic vasculopathy: panacea or Pandora's box? Diabetes Obes Metab, 007 Sep 6 [Epub ahead of print]
85. **Fadini GP et al.** Endothelial Progenitor Cells and the Diabetic Paradox. Diabetes Care, 2006; 29(3): 714-716.
86. **Iwaguro H et al.** Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation. 2002; 105: 732–738.
87. **Hiasa K et al**. Gene transfer of stromal cell-derived factor- 1alpha enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization, Circulation, **109:**2454-2461, 2004.
88. **Kocher AA et al**. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function, Nat Med, 2001; 7:430-436.

89. **Tateishi-Yuyama E et al.** Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial, Lancet, 2002; 360:427-435.
90. **Kong D et al.** Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells, Circulation, 2004; 109:1769-1775.
91. **Losordo DW et al**. Therapeutic Angiogenesis and Vasculogenesis for Ischemic Disease. Part II: Cell-Based Therapies. Circulation, 2004; 109:2692-2697.
92. **Hur J et al.** Characterization of two types of human endothelial progenitor cells and their different contributions to neovasculogenesis. Thromb Vasc Biol 2004; 24: 288-93.
93. **Brown GC et al.** Nitric oxide, citochrome c and mithocondria. Biochem Soc Symp 1999; 66; 17-25.
94. **Stamler JS et a**l. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci U S A 1992; 89:444-448.
95. **Duda DG et al**. Role of eNOS i neovascularization: NO for endothelial progenitor cells. Trends Mol Med 2004; 10(4):143-5.
96. **Buchwalow IB et al.** Inducible nitric oxide synthase in the myocard. Mol Cell Biochem 2001; 217 (1-2):73-82.
97. **Förstermann U et al.** Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation, 2006; 113:1708-1714.
98. **Dimmeler S et al**. Nitric oxide-an endothelial cell survival factor. Cell Death Diff, 1999; 6:964-968.
99. **Knowles RG et al**. Nitric oxide synthases in mammals. Biochem J, 1994; 298:249-258.
100. **Fukumara D et al**. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Nat Acad Sci U S A, 2001; 98: 2604-2609.
101. **Mayr U et al**. Accelerated arteriosclerosis of vein grafts in inducible NO synthase / mice is related to decreased endothelial progenitor cell repair. Circ Res 2006; 98:412–420.
102. **Gallagher KA et al**. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1α. J Clin Invest, 2007; 117: 1249-1259.
103. **Thum T et al**. Endothelial Nitric Oxide Synthase Uncoupling Impaires Endothelial Progenitor Cell Mobilization and Function in Diabetes. Diabetes, 2007; 56: 663-674.

104.**Lajoix AD et al**. A neuronal isoform of nitric oxide synthase expressed in pancreatic β-cells controls insulin secretion. Diabetes, 2001; 50:1311-1323.

105.**Novelli M et al.** Alteration of β-cell constitutive NO synthase activity is involved in the abnormal insulin response to arginine in a new rat model of type 2 diabetes. Mol Cell Endocrin. 2004; 219:77-82.

106.**Bradley SJ et al**. Skeletal muscle neuronal nitric oxide synthase protein is reduced in people with impaired glucose homeostasis and is not normalized by exercise training. Met Clin Exp 2007; 56:1405-1411.

107.**Leal EC et al**. Inducible Nitric Oxide Synthase Isoform Is a Key Mediator of Leukostasis and Blood-Retinal Barrier Breakdown in Diabetic Retinophaty. IOVS, 2007: 48(11):5257-5265.

108.**Heitzer T et al.** Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation, 2001; 104: 2673–2678.

109.**Ushio-Fukai M**. Redox signaling in angiogenesis: role of NADPH oxidase. Cardiovasc Res, 2006; 71: 226–235.

110.**Lambeth JD**. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol, 2004; 4: 181–189.

111.**Griendling KK et al**. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol, 2000, 20, 2175–2183.

112.**Bayraktutan U et al.** Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. Arterioscler Thromb Vasc Biol, 2000, 20, 1903–1911.

113.**Bendall JK et al.** Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. Circulation, 2002, 105, 293–296.

114.**Guzik TJ et al.** Systemic regulation of vascular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. Arterioscler Thromb Vasc Biol, 2004, 24, 1614–1620.

115.**Dworakowski R et al**. NADPH oxidase-derived reactive oxygen species in the regulation of endothelial phenotype Pharmacol Rep, 2008; 60(1):21-8.

116.**Weidig P et al**. High glucose mediates prooxidant and antioxidant enzyme activities in coronary endothelial cells. Diabetes Obes Metab, 2004; 6:432–441.

117.**Wendt M et al.** Differential effects of diabetes on the expression of the gp91phox homologues nox1 and nox4. Free Radic Biol. Med, 2005; 39:381–391.

118.**Uchizono Y et al**. Expression of isoforms of NADPH oxidase components in rat pancreatic islets. Life Sci. 2006; 80:133–139.

119.**Hoeldtke RD et al.** Oxidative stress and insulin requirements in patients with recent-onset type 1 diabetes. J Clin Endocrinol. Metab, 2003; 88:1624–1628.

120.**Nakayama M et al.** Increased expression of NAD(P)H oxidase in islets of animal models of Type 2 diabetes and its improvement by an AT1 receptor antagonist. Biochem Biophys Res Commun, 2005; 332:927–933.

121.**Busik JV et al**. Glucose-Induced Activation of Glucose Uptake in Cells from the Inner and Outer Blood–Retinal Barrier. Invest Ophthalmol Vis Sci, 2002; 43:2356-2363.

122.**Frum R et al**. HDM2-binding partners: interaction with translation elongation factor EF1alpha. J Proteome Res, 2007; 6(4):1410-1417.

123.**Imamura A et al**. The effects of endothelial nitric oxide synthase gene polymorphisms on endothelial function and metabolic risk factors in healthy subjects: the significance of plasma adiponectin levels. Eur J Endocrinol 2008; 158(2):189-195.

124.**Tsui JC et al**. Alterations in nitric oxide synthase isoforms in acute lower limb ischemia and reperfusion. Angiology, 2007; 58 (5):586-592.

125.**Brenman JE et al**. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. Cell, 1995; 82 (5):743-752.

126.**Hashimoto K et al.** Hypoxia-induced hyaluronan synthesis by articular chondrocytes: the role of nitric oxide. Inflamm Res 2006; 55:72-77.

127.**Strausberg RL et al.** Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A, 2002; 99 (26):16899-16903.

128.**Bendall JK et al**. Endothelial Nox2 overexpression potentiates vascular oxidative stress and hemodynamic response to angiotensin II: studies in endothelial-targeted Nox2 transgenic mice. Circ Res, 2007; 100(7):1016-1025.

129.**Emanueli C et al**. Changing the logic of therapeutic angiogenesis for ischemic disease. Trends Mol Med, 2005;11(5):207-16.

130.**Kashyap SR et al**. Insulin resistance is associated with impaired nitric oxide synthase activity in skeletal muscle of type 2 diabetic subjects. J Clin Endocrinol Metab, 2005; 90:1100–5.