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Modern therapeutic approaches in lower limb peripheral arterial disease

Thesis - Summary

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Doctoral thesis contains:
    159 pages (the overall 47-page personal side - 112 pages); 15 tables, 76 figures, 371
citations, 6 annexes, 3 scientific articles rated B +

Note: This summary is playing selective bibliography and iconography in the text, following the
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European Research Area"
Chapter I. Generalities (introduction, etiology, pathogenesis and natural development of peripheral arterial disease)

I. Introduction

Peripheral arterial disease (BAP) is a manifestation of systemic atherosclerosis and is defined by progressive stenosis or occlusion of the arteries lower limbs. PAD is the term currently accepted internationally for the symptoms (Group 2000), historically this disease was described and names were changed as well: peripheral arterial occlusive disease, arteriosclerosis obliterans, lower limb artery occlusive disease and peripheral vascular disease.

Normal artery morphology

Normal artery wall is made of three concentric layers: the internal one, called intima, the middle layer called the media, and adventitia external coating surface. The three layers are separated by concentric rings of elastin, known as the internal elastic lamina (separating vessel intima media) and external elastic lamina (separating media of adventitia). Internal surface of the artery (intima) consists of a single layer of endothelial cells lying on a basement membrane and extracellular matrix is bounded by the internal elastic lamina. The endothelial cells are attached to each other by series connection of intercellular junction complexes (Wang, Zeng et al. 2011).

II. Pathogenesis of atherosclerotic lesions

II. 1 The etiology of atherosclerosis

Atherosclerosis is the most common cause of arterial disease lower limbs. Major risk factors of nonreversible atherosclerosis are: age, male gender, and family history of early atherosclerosis onset. Reversible major risk factors in the development and progression of atherosclerotic disease are smoking, dyslipidemia, diabetes mellitus, hypertension and hyperhomocysteinemia.

Risk of developing intermittent claudication is at least two times higher for smokers than for smoking (Murabito, D'Agostino et al.1997). Diabetes is associated with a three times higher risk of intermittent claudication (Murabito, D'Agostino et al., 1997, van Pul, Kruidenier et al.2012 Qazi and Malik 2013). Dyslipidemia, particularly hypercholesterolemia, is present in 40% of patients with peripheral atherosclerosis. Patients with cholesterol levels above 270 mg/dl had a two-fold risk of developing claudication. Epidemiological studies have established an association between hypertension and a higher prevalence of atherosclerosis in vascular occlusive disease (Levy, Wilson et al.1990).

II. 2 Endothelium

Vascular endothelium constitutes about 1% of human body mass is five times harder than the heart and is a large endocrine organ, with a total area of approximately 5000 m² (Jaffe 1987). It is a single layer of endothelial cells covering the luminal surface of the vascular tree. Once thought to be a lifeless surface, nonthrombogenic, the endothelium is now recognized as an active organ that constantly reacts to metabolic factors, physical forces fluid flow and changes in concentration of vasoactive substances (Toborek and Kaiser 1999).
Endothelial functions include control of vascular tone, modulation of vascular structure regulation of angiogenesis and vascular proliferation, maintenance of a selective permeability barrier, regulating lipid oxidation and mediating immune response. Endothelium plays a major role in the interactions between circulating blood and the vessel wall in response to influences hemodynamic, neurohumoral and inflammatory (Shireman and Pearce 1996).

**Endothelial function**

Healthy endothelium normally acts as a barrier to maintain homeostasis role in the contraction of vascular smooth muscle cells, intimal proliferation, thrombosis, and the adhesion of monocytes (Toborek and Kaiser 1999). Although Rossi and his colleagues initially assumed that endothelial injury definite initial response to trauma is involved in atherogenesis has become clear that the endothelium can undergo subtle chronic dysfunction which can ultimately participate in the development of atherosclerotic lesion (Ross 1986). Dysfunctional endothelium can stimulate leukocyte infiltration, smooth muscle cell, muscle cell proliferation and the formation of foam cells (lipid inclusions) (Kozlov, Balachonova et al. 2012). A large number of evidence indicates that endothelial dysfunction is associated with most of the known risk factors for cardiovascular disease, thus providing a central mechanism of atherogenesis (Toborek and Kaiser 1999).

**III. The natural history of PAD**

PAD is a significant cause of morbidity and mortality because of its manifest systemic damage. Epidemiology PAD was evaluated in a number of international studies, some of which are presented in this paragraph. However, most clinicians believe that patients with intermittent claudication are patients in early stages of disease, the Rotterdam Study, which included 7715 patients, showed that most patients with PAD did not report symptoms of claudication (Meijer, Hoes et al.1998). In patients with severe PAD 5-year survival is 30%, and healthy subjects have a 80% survival.

![Figure 2. Survival of patients with PAD - reduced rate of survival in patients affected from healthy ones.](image)

Amended by Criqui, Langer et al.1992

In recent decades, the natural history of PAD was influenced by important medical advances in the treatment of arterial stenosis. Preconceptions about natural history, associated with fatalism, were based on the reality that atherosclerotic risk factors leading to cardiovascular events with other association, but this no longer applies in modern health care, and it is likely that
rates prevalence and cardiovascular ischemic be modified / upgraded successfully during long-term care, leading to a more benign natural progression and improve clinical outcomes.

Chapter II. Classic risk factors in the development of PAD

Specific risk factors have been associated with the development of BAP, coronary artery disease and cerebrovascular disease. These risk factors include smoking, diabetes, atherosclerosis positive family history, hypertension, hyperlipidemia (Criqui, Denenberg et al.1997 Murabito, D'Agostino et al.1997). The relative risk (RR) of developing PAD is closely associated with diabetes (RR, 4.05), smoking (RR 2.55), advanced age (RR 1.54 per 5 years), hypertension (RR, 1.51), hyperhomocysteinemia (RR, 1.44), elevated total cholesterol (RR 1.10 for an increase of 10 mg / dl) (Figure 4).

Figure 4. The relative risk of developing PAD associated risk of atherosclerosis.
Amended by Newman, Siscovick et al.1993

Chapter III. Prevalence of PAD

Epidemiological data show a high prevalence of PAD in people in the United States and Europe, the incidence of which increases with age and with increasing exposure to atherosclerotic risk factors.

Figure 5. PAD prevalence in relation to age in women and men.
Amended by Criqui, Fronek et al.1985

The relationship between the PAD and other atherosclerotic syndromes

Co prevalence of atherosclerotic syndromes investigated by Aronow and Ahn in a long term care facility was identified 25% of patients aged over 62 years who had at least two
manifestations of atherosclerosis (Aronow and Ahn 1994). Among patients with ischemic heart disease, 33% had also PAD and 32% had an ischemic stroke. Among patients with a history of ischemic stroke, 53% had ischemic heart disease and 33% had, PAD (Aronow and Ahn 1994).

People with PAD increased risk of developing angina, congestive heart failure, myocardial infarction, fatal and non-fatal MI, fatal stroke and non-fatal, and death (Cimminiello 2001 Zeymer, Parhofer et al.2009).

**Chapter IV. Diagnosis in PAD**

**IV. 3. Clinical diagnosis**

Signs and symptoms in PAD (Caralis and Bakris, 2005):

- Pain that occurs during exercise and resolves after discontinuation of the effort
- Cold Feet
- Nocturnal pain relieved by position latch
- Absent pulses
- Bleaching or paleness in lifting member
- Recolor delayed after positioning latch member position
- Redness standing member
- Atrophy of subcutaneous fatty tissue
- Glowing skin
- Hair loss on legs and toes
- Thickened nails often onychomycoses
- Ulcer and / or gangrene

Vascular examination includes auscultation for murmurs pressure which creates turbulence by a stenotic lesion. What is a noise systolic pressure common when there is obstruction, proximal to the site of auscultation. When a diastolic component is present, it means that collateral circulation is inadequate to allow diastolic blood pressure, distal to the affected area to rise to systemic diastolic pressure and thus flow during diastole is heard. Murmurs can be heard in the abdomen, in the femoral, carotid, subclavian, and right popliteal (Spittell 2004).

**IV. 4. Laboratory diagnosis**

**IV. 4. 1. Ankle-brachial index**

Clinical purposes, a normal ABI is considered to be above 0, 90. For any value smaller than this, the patient will be investigated in order to assess for the presence of occlusive diseases proximal to the site of recording. By default the index may switch to the severity of arterial disease (Sadeghi, Heidari et al. 2011).

- An ABI between 0.5 and 0.9 may be associated with damage to a single artery, such as the superficial femoral artery occlusion.
- An ABI of less than 0.5 is usually observed in diseases bunk stenosis (Signorelli, Anzaldi et al. 2012).

ABI may be used as an indicator of the severity of claudication. However, the estimates of ABI may be useful in critical limb ischemia. If ABI is below 0.40, it is important that these patients should be closely investigated (Kroger, Bock et al. 2010)
IV. 4. 4. **Vascular Doppler ultrasound**

This investigation may deny, justify, therefore confirming a medical or surgical treatment. Vascular Doppler ultrasound examination gives the doctor the image investigated structures and data about how blood flows through the vessel examined, including blood flow parameters and the effect of vessel pathological changes of blood flow (Abela 2004).

IV. 4. 5. **Angiography**

Angiographic evaluation of lower extremities includes the introduction of a contrast agent in the arterial system, usually through the femoral artery catheterization.

Angiographic detection of hemodynamically significant stenosis is considered to be extremely accurate. However, an interesting study carried out by Wikstrom and colleagues pressure measurements were obtained for all lesions iliac arteries, with a reduction of 50% luminal diameter (Abraham, Handler et al.2007).

IV. 4. 6. **Intravascular ultrasound**

Combines the features of angiography and intravascular ultrasound imaging with ultrasound. Use a probe mounted at the tip of a catheter (usually 3-9 G in diameter) is inserted into the vascular lumen with a guide wire (Nissen and Yock 2001). By this method, the artery is visualized from interior to outside. This technique can be an important adjunct to conventional angiography.

IV. 4. 8. **Computed tomography (Angio-CT)**

The development of computed tomography scans, made possible to use this technique to investigate the vascular tree.

Important information can be obtained concerning the vascular system through the use of intravenous contrast. All evaluations CT vascular pathology should start with a scan without contrast. Blood vessel size and the degree of vascular calcification can be easily evaluated on a native CT. The extravascular blood on a native CT appears dense, so the diagnosis of acute hemorrhage or rupture does not require intravenous contrast (Katz, Jorgensen et al.1999).

IV. 5. **The differential diagnosis**

IV. 5. 1. **Neurogenic claudication**

Neurogenic claudication is a form of lumbosacral radiculopathy due to narrowing of the spinal canal, with symptoms of claudication caused by motion (Simonetti and Pratesi 2006). Pathophysiology involves compression of neurovascular structures intramedullary spinal cord ischemia association, cauda equina or nerve roots during exercise. Compression of the spinal cord or cauda equina affects lower lumbosacral region, the venous drainage of spinal stenosis. In this case there is no damage to blood and the blood flow (Pearce 2005).

IV. 5. 2. **Arthritis**

Arthritis is characterized by inflammation and / or destruction of the joint articular space. The involvement of large joints of the lower limbs can lead to discomfort effort. This is described as a pain relieved by rest, which is located at the joints, or in a case in muscles. Physical examination may reveal evidence of inflammation in the joint effusion and joint destruction and deformity.
IV. 5.3. Aortoiliac Disease

Injuries involving the common iliac arteries, external iliac arteries can lead to signs of intermittent claudication. Because of the proximal location of these arteries, limbs are entirely devoid of normal blood flow during exercise.

IV. 5.4. Entrapment syndrome

Popliteal artery is particularly susceptible to catching and gastrocnemius muscle compression during exercise, leading to ischemia, which causes the typical symptoms of intermittent claudication.

This syndrome should be considered in young adults presenting with claudication. Usually before the age of 40, it was reported in the sixth decade of life. These symptoms can be vague and is slowly progressive but more than two thirds of patients are composed of claudication cramps after strenuous exercise, which is often one-sided (Roche-Nagle, Wong et al. 2009).

IV. 5.7. Venous claudication

Venous claudication is the most common result of chronic venous iliofemoral obstruction. Venous obstruction may be due to or may be iatrogenic iliofemoral thrombosis after surgical termination of the common femoral vein. Symptoms are described as severe pain in the thigh and tightness in effort. This discomfort lasts 15-20 minutes to subside after stopping effort and is facilitated by lifting the foot. Physical examination shows evidence of swelling of the limbs. Arterial examination including pulses at femoral, popliteal, and pedal pulses is usually normal.

IV. 5.8. Arterial embolism

Arterial embolism causing acute ischemia and intermittent claudication may result from an arterial embolus in a peripheral artery. The onset is usually sudden lameness. However, many patients develop even after claudication is an acute phase. These States are diminished or absent pulses as systolic blood pressure at the ankle.

IV. 5.9. Tromboangeitis obliterans

Tromboangeitis obliterans (Buerger disease) is characterized by segmental inflammation and occlusion of small and medium enterprises and neighboring the veins. Occlusive lesion foot vessels can lead to symptoms of intermittent claudication of the lower limbs. The diagnosis should be considered in young male smokers who develop claudication (Caralis and Bakris 2005).

Chapter V. Therapy in PAD

V. 7. Angiogenesis and gene therapy

V. 7.1. Introduction

Growth factors are a potential method of treatment for patients with BAP. It represents the possibility of using recombinant formulations of angiogenic growth factors to induce the development of collateral arteries by stimulating capillary growth in animal models. This strategy for the treatment of vascular insufficiency was called therapeutic angiogenesis. Studies have suggested that are three growth factors stimulating angiogenesis effectively, in particular, acidic
fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), are considered to be the most effective and deserve further analysis.

V. 7.2. Vascular development

Vascular development can be classified in vasculogenesis, angiogenesis, arteriogenesis. Vasculogenesis is restricted to embryonic stage and it is followed by the development and in situ differentiation of endothelial cell precursors - angioblasts and other subsequent organization of the primary capillary plexus formation.

The formation of new vessels, capillaries from existing capillaries or venules appears in a series of steps: nonproliferative endothelial cells are activated by cytokines, following the release of extracellular proteinases (plasminogen activators, metalloproteinases) that are required to degrade basal membrane and matrix components of the underlying endothelium.

Do not forget at least theoretically capable of stem cells to differentiate in endothelial cells and finally to be able to induce an effective angiogenesis.

V. 7.3. Therapeutic Angiogenesis

Nature has created mechanisms to adapt to regional ischemia partially offset by the development of functional collateral circulation, which is driven by angiogenic factors. Hypoxia is generally considered a fundamental stimulus for angiogenesis, although angiogenesis can occur in areas with normal oxygenation. Arteriogenesis is dependent on the gradient of the pressure increase rate and shear stress. Therefore, ischemia affects arteriogenesis.

The development of collateral vessels is quite slow and often cannot fully compensate for the effects of ischemia.

Using gene transfer as a potential therapeutic approach is an emerging field of fundamental and applied medical research that has recently matured in clinical studies, and acquired diseases. The purpose of the gene transfer door is increased protein expression through a process of introducing a gene that encodes a protein in the target cells that is required to induce angiogenesis. The use of viral vectors is based on the fact that viruses have evolved highly efficient mechanisms to transfer their DNA into target cells (Nicol 2007) both DNA and RNA viruses can be used.

V. 7.4. Physiology of arteriogenesis

Arteriogenesis is initiated by increasing the shear forces on the wall of the vascular narrowing and occlusion data. Therefore, the flow is redirected to branches of small collateral arteries (Pipp, Boehm et al.2004).

Shear stress are the primary and powerful arteriogenic stimulus (Schierling, Troidl et al., 2009), leading to a phase of inflammatory activation of several chemoattractants, inducing a chemotactic gradient for progenitor cells, monocytes (Heil, Eitenmuller et al.2006). Adhesion molecules are activated, followed by increased adhesion and invasion of circulating blood mononuclear cells (Chappell, Varner et al.1998). These cells migrate into the perivascular space and sometimes enters to the collateral vessel wall are derived from bone marrow cells, showing specific surface markers of monocytes and macrophages (Ziegelhoeffer, Fernandez et al.2004).

Therefore, the role of bone marrow-derived mononuclear cell is to act as perivascular cytokines, to provide the needed tool to start a process of angiogenesis (Heil, Ziegelhoeffer et al.2004). The concept of bone marrow-derived mononuclear cells is supported by the presence of circulating endothelial progenitor cells (Asahara, Murohara et al. 1997), which also originate from monocyte-macrophage lineage and are assumed to be identical with mononuclear cells
Two important aspects should be mentioned with regard to arteriogenesis 1) arteriogenesis is driven by shear stress, and does not by hypoxia, and therefore, it appears near to occluded artery proximal and parallel to it, and 2) the recruitment of monocyte cells, derived from bone marrow, which represent the central event of arteriogenesis.

**V. 8. Cellular therapy**

PAD is characterized by narrowing and occlusion of blood vessels, reduced distal perfusion and local compensatory mechanisms including capillary growth and development of collateral blood vessels (*arteriogenesis*). Burgeoning growth of capillary vessel appears as a small tube creates endothelial cells existing in capillary beds, in response to local hypoxia. This budding is mediated by hypoxia-induced release of cytokines (vascular endothelial growth factor-VEGF). The capillaries are made small, with a diameter of about 10-20 μm, and are unable to compensate for blood flow (Figure 7).

Arteriogenesis leads to the formation of collateral arterioles. The small diameter increases up to 20 times (Heilmann, Beyersdorf et al. 2002), during the arteriogenesis.

Endogenous arteriogenesis can restore almost completely normal vascular network induced in ischemia animal models of occlusion of large vessels. This correlates well with the clinical observation that many patients with PAD with femoral artery occlusion without ischemic symptoms because their network provides enough blood to meet the need of lower limb perfusion (Aboyans, Lacroix et al. 2004).

**V. 8. 1. Clinical results of autologous cell therapy**

TACT study (studying angiogenesis therapy and autologous cell transplantation) was the first major report on the use of bone marrow mononuclear cells in the treatment of limb ischemia (Tateishi-Yuyama, Matsubara et al. 2002). The protocol consisted of a pilot open randomized controlled.

In this pilot study recruited patients to receive bone marrow transplantation had chronic limb ischemia, including rest pain, ulcers without healing tendency, or both because of BAP, who have not responded effectively by a successful revascularization. Twenty-five patients with unilateral limb ischemia were treated with mononuclear cells injected into 40 points in the gastrocnemius muscle of the ischemic limb (ABI <0.6). As a control treatment, saline was injected into the contralateral leg, foot less ischemic (ABI> 0.6). The procedure was performed seemingly safe and improved ABI, transcutaneous oxygen pressure and led to pain relief at rest, increased walking distance of four and 24 weeks.

Another randomized controlled trial of 22 patients with bilateral leg ischemia who were recruited and randomly treated with either bone marrow mononuclear cells (active treatment) in one leg or the other unstimulated peripheral blood mononuclear cells taken as control leg. ABI has improved after four weeks of treatment with stimulated cells. ABI improved foot injected with bone marrow mononuclear cells of 13 out of 20 patients. Pain at rest was eliminated in 16 of 20 patients, the pain went also was improved (Sica, Williams-Ignarro et al. 2006 Jeon, Song et al. 2007 Zhang, Zhang et al. 2010).

Injection of bone marrow mononuclear cells had a beneficial effect on the parameters, the result proving that 1) unstimulated peripheral blood mononuclear cells did not induce angiogenesis, and 2) needle stings as well does not improve disease.
Endothelial progenitor cells, mesenchymal cells, stem cells, mononuclear cells, cells derived from adipose tissue, increased number of CD34+ hematopoietic stem cells and the large number of immature cells in the bone marrow are responsible for increasing the efficiency of angiogenesis (Awad, Dedkov et al. 2006).

After this study considerable interest has arisen in the use of stem cell / cell therapy in peripheral ischemia in a number of countries. Some studies had a small number of study subjects, the lack of control groups and the emergence of conflicting results.

Despite these limitations, the results of therapy with bone marrow derived stem cells have a remarkable consistency and positivity throughout various reports. The results show that combined treatment with autologous cells may induce an increase in the ABI values of 0.1 and 0.2, and an increase in oxygen saturation to 10 and 20 mmHg. Distance of walking, depending on the initial values can be increased from a few Steps to about 200 meters. In addition, no serious adverse effects were reported (Tateishi-Yuyama, Matsubara et al. 2002).

While the results of clinical trials of cell therapy in lower limb ischemia are promising, a number of methodological questions remain unresolved.

V. 8. 2. The role of the cell type

The implementation of cellular therapies of cardiovascular disease was first run of the theoretical concept that endothelial progenitor cells from bone marrow may incorporate into the flap damage to the endothelium, with the ability to form a new vessel. This process has been considered as a possible mechanism for cell therapy for ischemic diseases. Vasculogenesis is aimed at the formation of new blood vessels, but this process has not been convincingly demonstrated in adult humans. As a result, their role in human angiogenesis remains uncertain (Purhonen, Palm et al. 2010).

Arteriogenesis concept reinforces the importance of using several different types of cells. They migrate into perivascular space and inducing the growth and development of collateral arteries through the release of angiogenic growth factors. It appears that the cellular therapies PAD are the most successful methods using their isolated cell preparation, such as CD or CD 34 + 133 + purified from peripheral blood, mobilized with granulocyte colony-stimulating factor (G-CSF) (Kudo, Nishibe et al. 2003).

PERSONAL PART

Chapter VI. Mesenchymal stem cells: isolation, characterization, differentiation - for understanding the mechanism of neoangiogenesis

The role of integrins in mediating cellular response

Cells are activated by mediators released into the affected tissue. The stimuli activate cell migration; adhesion molecules mechanism involves the process of chemotaxis, which attracts cells to the site of injury. Cell receptor binds to the extracellular matrix and therefore appears cellular response. This is initiated by rolling on the surface of endothelial cells. During rolling the cells are forming some transitional bridges involving the presence of various adhesion molecules, at which integrins are activated by chemokines and soluble factors released by endothelial cells. This activation induces a strong adhesion to the endothelial cells, resulting in
immobilization (Duan, Cheng et al. 2006). Cytoskeleton recognizes signal molecules that induce changes at this level and consequently cell shape change. Cells extend pseudopodia some who pass the endothelial junctions, a process called transmigration. Once in the interstitial fluid leukocytes migrate through chemotactic gradient towards the site of injury or infection (Chavakis and Preissner 2005).

![Figure 10. Activation of integrins and transmigration mechanism](http://www.med.lu.se/english/expmed/about_the_department)

There are two potential mechanisms for homing the mesenchymal stem cells: a passive represented by slowing down and eventually blocking the mesenchymal stem cells in the microvasculature, or active, to cross the capillary wall by means of a mechanism similar to that described above (He, Ma et al. 2010).

The role of integrins is not fully known; a recent study has shown that the well-documented presence of beta1 integrin plays an important role in establishing cell polarity and lumen formation pressure (Zovein, Luque et al. 2010).

![Figure 12. Lumen formation, accumulation of vacuoles in the cell pole](Amended by: Zovein et al. 2010)
Mesenchymal stem cells can migrate to damaged tissue; the process is regulated by integrins expressed on their surface. It has been shown that in vitro cultured MSC to a confluence of 80%, express the following subunits: beta 1, beta 2, and alpha 3 at a rate of 20%-55%, alpha 1, alpha 2, alpha 4, alpha 5, alpha 6, and alpha V are expressed in about 10%, this was demonstrated by flow cytometry of Semon et al in 2010.

The specific role of integrins in the interaction stem cells - endothelium is a good tool to understand peripheral arterial disease, where stem cells may be used for therapeutic purposes and their adhesion to the vascular endothelium which is very important (Bradfield and Imhof 2004).

**VI. 2. Objectives**

This PhD thesis aims to identify opportunities to induce lower limb peripheral angiogenesis in atherosclerotic patients, who may be and such associated diseases: ischemic heart disease, myocardial infarction, arteritis.

In order to understand these aspects it is necessary to know:

- Marrow extraction procedure
- Spinal manipulation in the laboratory in order to retrieve / filter cells are directly involved in tissue regeneration
- Growth and differentiation of the cells
- The characterization of stem cells

**VI. 3. Material and Methods**

**VI. 3. 2. Isolation and culture of human mesenchymal stem cells**

Mesenchymal stem cells (MSC) were obtained from six healthy donors (2 women and 4 men) aged between 21 and 56 years. Donors have given specific consent and signed the forms as required by law. All of the bone marrow, will be used for transplantation in hematologic diseases, such as leukemia.

After 24 hours of culture, adherent cells are removed from the environment, with changes. Adherent cells will be grown up when she get a confluence of 60-80%, at which point they can be used for other experiments.

**VI. 3. 4. Differentiation ability of mesenchymal stem cells**

Mesoderm differentiation of mesenchymal stem cells is done using standardized mean difference. When cells are at a confluence of 80% is started differentiation, to take an average of three week period in which the culture will change 2 times per week.

**VI. 3. 5. Flow cytometric analysis**

To characterize MSC cell surface, we used flow cytometry. Cells were labeled to identify specific markers defined by (Dominici, Le Blanc et al.2006).

**VI. 3. 6. 3. Polymerase chain reaction - quantify gene (qRT-PCR)**

Polymerase chain reaction in real time quantitative (Q RT - PCR) PCR was performed on Applied Biosystem StepOne® On with primers TaqMan.

The result is derived from the calculation of the melting curve after PCR. Collected signal is given by the excitation light to the sample and its fluorescent waste collection.
VI. 4. Results

VI. 4. 1. Isolation and differentiation of human mesenchymal stem cells

This study focuses on the characterization of five donor mesenchymal stem cells.

Figure 17. Differentiation of mesenchymal stem cells (N = 5). A. appearance before cell differentiation B. Differentiated adipocytes - coloring with Oil Red O, C. Differentiate into osteocytes - color von Kossa D. Differentiated chondrocytes - with toluidine blue staining (100 x).

VI. 4. 2. Characterization of surface markers of mesenchymal stem cells

To fully characterize mesenchymal stem cells, identification of specific markers on their surface.

Figure 18. The expression of surface markers characteristics of mesenchymal stem cells (n = 5) (isotype control - black line, test - red line).
VI. 4. 3. 1. Membranar expression of integrins in mesenchymal stem cells

Figure 19. Integrin expression on the surface of mesenchymal stem cells (n = 5) (isotype control - black line, test - red line).

Figure 20. The expression of surface markers of mesenchymal stem cells nonspecific (n = 5) (isotype control - black line, test - red line).
The presence of CD133, CD44 and CD117 surface markers was analyzed and only CD44 was positive at the 5 donors, while CD133 and CD117 are negative (Figure 20).

**VI. 4. 3. 2. Relative expression of the integrins in the cell genome**

To confirm the results of the previous experiment it was decided to evaluate the expression of integrins in the RNA. The analysis confirmed the results obtained by flow cytometry, the absence of all integrin alpha subunits, alpha subunit less than X, which has a discreet expression of two donors.

Beta subunits have in principle a good expression represented by the beta subunit beta 3 and 5 and at the same time see a small variability between donors; in contrast integrin beta 1 subunit expression shows a high variability between donors (Figure 21).

![Figure 21. Relative expression of integrins to 5 different donors (between donors variability can be seen, in particular beta 1 subunit expressed in a standard high-pass)](image)

**VI. 5. Discussions**

The present study aimed to investigate the mesenchymal stem cells from different donors. Isolated mesenchymal stem cells were first subjected to experiments to show that indeed they are isolated from the bone marrow; they have undergone differentiation into the three specific lines described above. These things are consistent with the findings of researchers who declare that mesenchymal stem cells are able to differentiate into at least three cell lines of mesoderm: adipocytes, chondrocytes and osteocytes (Prockop 1997 Pittenger, Mackay et al.1999 Which was demonstrated in the case of isolated cells. They were able to differentiate into the three cell lines.

In addition to flow cytometry results confirmed the presence of markers CD105, CD90, CD73 and absence markers CD 11b C and CD34, CD45 (Dominici, Le Blanc et al.2006).

Integrin expression was identified by flow cytometry and quantitative gene expression. All alpha subunits analyzed were absent (alpha 1, alpha 4, alpha 5, alpha X, alpha M, alpha D, alpha L) as well as some of the beta subunits (beta 2, beta 7, beta 4, beta 6). A high expression was observed for beta 3 and beta 5 subunit. From only beta 3 was analyzed by flow cytometry and qRT-PCR, which demonstrated that it is not expressed at the cell surface, even so through PCR quantification integrin beta 3 have a very low value. This can be explained by possible cellular changes in the integrins, changes that can occur when extracellular preparation for
marking cells, cell culture from trypsinization. Or simply that the protein is not expressed on
the cell surface and was not possible to identify it by flow cytometry. Beta subunit gene
expression 6 shows a significant, but unfortunately the presence of this molecule on the cell
surface could not be highlighted, in the absence of specific antigens.

A special observation was observed in the analysis of beta1 integrin subunit, which
showed a high variability between donors characterized, it was observed by qRT-PCR and flow
cytometry, in a proportional manner. This observation raised several questions. One of them is
whether gene expression is correlated with a wide variability of surface markers, which is why it
was decided to compare the variability donors with high D 2 and D 4 (Figure 23). In this figure
one can see differences between these two donors in relation to the number of positive cells.
These data demonstrated that gene expression is associated with increased surface expression of
genes greater than if the donor 4.

![Figure 23. 1 integrin beta subunit expression in two different donors
(D 2 in the left and D. 4 on the right).](image)

Our results are related to the observations of Semon (Semon, Nagy et al. 2010) who
described that more than 80% Mesenchymal stem cells express the integrin beta 1 subunit, but
in contrast to his comments, we did not find any expression of beta 2 subunit and other integrin
alpha subunits.

Primers were checked by peripheral blood mononuclear cells, but these cells do not
express the integrin subunits following: ITGAM, ITGAD, ITGB3, ITGB4, ITGB5, ITGB6,
ITGB7, and ITGB8. For this confirmation it is necessary to carry out further tests on other cell
types that are known to be positive for specific primers and thus to verify the operation of
primers q RT - PCR.

**VI. 6. Conclusions**

Experiments were performed on mesenchymal stem cells presenting all three
characteristics: adherence to plastic, tripotentă cell surface markers present features.

Integrin expression in mesenchymal stem cells is donor dependent for integrin beta 1
subunit. These results are not entirely in agreement with previous studies, and discrepancies in
the literature are not fully understood.
Chapter VII. Role of Proprotein convertase subtilisin / kexin type 5 (PCSK5) as predictor of atherosclerotic risk in peripheral arterial disease

PCSK5 gene product is a protein called proprotein convertase subtilisin / kexin type 5 is an enzyme that is part of the family of subtilisin-like proprotein convertazelor. Members of this family are proprotein convertase, which are proteins produced biologically active precursor (van de Loo, Creemers et al. 1996). This protein encodes postranslationala processing endoproteolitică for integrin alpha subunits, particularly alpha 6 (Kappert, Meyborg et al. 2009).

Figure 30. The mechanism of action of PCSK5 the LE and its influence on serum HDL.
Adapted Iatan, Dastan et al. 2009

A recent study published in 2009 by Julia Iatan et al showed that: PCSK5 gene polymorphism is important in the metabolism of HDL-cholesterol (Iatan, Dastan et al. 2009). Fertile field of investigation in molecular genetics and pathophysiological is the research of HDL deficiency. Despite the fact that there is much information about the protective role of HDL's, the fundamental mechanisms are not fully known. The study reports show that the variability of PCSK5 gene modulates serum levels of HDL-cholesterol. Seven non-coding variants were found in patients with deficiency of HDL - cholesterol, but nowhere among them was a mutation missens (wrong-way) of the gene (Iatan, Dastan et al. 2009).

Other researchers in the field have identified PCSK5 involvement and presence in atherosclerotic plaque. Product gene suggests an important involvement in the mechanisms of ateriosclerosis (Turpeinen, Raitoharju et al. 2011).

VII. 2. Materials and methods

We analyzed the presence of mutations in gene exons PCKS5. Genomic DNA was extracted from the blood of 48 patients.

VII. 2.3. MLPA technique

MLPA technique can be divided into five major steps:

1. DNA denaturation and hybridization with probes specific MLPA
2. ligation
3. PCR amplification
4. separation the amplified products by electrophoresis
5. data analysis

Following this procedure, the samples will be migrated - by capillary electrophoresis. These data were applied to the filter according to the instructions provided by the kit and standardized data.

The primary analysis gives us information about how particular evidence that they have been working properly migrated.

The next step in the analysis gives us information about the number of copies produced in amplification, but to know whether it is or is not clinically significant.

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**Figure 38. An example of a successful experiment**

**VII. 3. Results**

The study included 48 patients hospitalized in the period 1 January 2012-21 October 2012, with the diagnosis of peripheral arterial disease, confirmed angiographically. These patients were evaluated clinically and laboratory completely.
Patients included in the study had the following distribution by sex, age and area of origin Figure 39, 40, 41.

From the data collected we mention that smoking patients (81%), the average age of onset of smoking is 20.76 years, median 20 years and mode of 18 years. Standard error of 1.12 years. Minimum age of smoking onset is 12 years. All this is related to a period of 48 years (median, and mean age 49.6) by going to the doctor, most of whom were patients presenting for Pima date, which is the time they are diagnosed.

Of particular interest cunt we HDL-cholesterol values shown in Figure 44, the cholesterol ratio also, Figure 45. This ratio is very important to assess the risk for heart disease. For example, the optimum ratio in the risk for cardiovascular disease is at least 3.5:1. Values allowed up to a ratio of 5:1, after which cardiovascular risk increases exponentially.
Total cholesterol, suggests that the study population has a nursery in circulating blood lipid levels (average - 213 mg / dl, normal value exceeds) module default HDL-cholesterol is a risk factor with a value of 32 mg / dl, values indicating increased risk for cardiovascular disease. Module LDL - cholesterol (169 mg / dl) is also well above normal, optimal, suggesting that this indicator is a risk factor for cardiovascular disease. Mean triglycerides (165.35 mg / dl) also exceeds the optimal Table IX, when they are considered to have an increased cardiovascular risk.

According to the *National Cholesterol Education Program* of the U.S. plasma levels of cholesterol, triglycerides, LDL and HDL should be as it is shown in Table X.

As noted in previous data inflammatory syndrome is present, but is not very significant.

<table>
<thead>
<tr>
<th></th>
<th>Optimal</th>
<th>Level limit</th>
<th>High Risk</th>
<th>Very high risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>&lt;200mg/dl</td>
<td>200-239 mg / dl</td>
<td>&gt; 240 mg / dl</td>
<td></td>
</tr>
<tr>
<td>HDL - Cholesterol</td>
<td>&gt; 60 mg / dl</td>
<td>40-60 mg / dl</td>
<td>&lt;40 mg / dl</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>&lt;150 mg / dl</td>
<td>151-199 mg / dl</td>
<td>200 - 499mg/dl</td>
<td>&gt; 500 mg / dl</td>
</tr>
<tr>
<td>LDL - cholesterol</td>
<td>&lt;100 mg / dl</td>
<td>100-129 mg / dl</td>
<td>130-159 mg / dl</td>
<td>&gt; 160 mg / dl</td>
</tr>
</tbody>
</table>

The extraction procedure carried out genomic DNA according to the protocol described above, then the technique of amplification of the gene PCSK5 exons.

The results have identified the

- 45 patients in the normal range, the results of the analysis showed amplification of between 0.8 and 1.2, values considered normal for this gene on both alleles. Figure 49.
- 3 patients had deletion in exon 9 of the gene PCSK5. Figure 50.

![Figure 50. Graphics in the 2 cases of MLPA results for PCSK5, identification of a deletion in exon 9.](p273-24_H04_12071907X0CEQsCSV.csv)
test comparing the average values and independent. We used 95% confidence level. The results are shown in Table XIV.

**VII. 4. Discussions**

Evaluation of dislipidemia status confirmed previous research that the level of LDL - cholesterol, triglycerides and total cholesterol are a risk factor for cardiovascular disease (Kroger, Buss et al., 2000), thus low values of HDL - cholesterol levels are at increased risk for these (Papademetriou, Narayan et al.1998). To confirm that these data are accurate to assess the total cholesterol/ HDL – cholesterol ratio.

<table>
<thead>
<tr>
<th>Table XIV. Comparative analysis between the two independent study groups.</th>
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</thead>
<tbody>
<tr>
<td><strong>Levene test (assessing variability)</strong></td>
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<tr>
<td><strong>f.</strong></td>
</tr>
<tr>
<td>LDL</td>
</tr>
<tr>
<td>HDL</td>
</tr>
<tr>
<td>Triglycerides</td>
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<tr>
<td>Total Cholesterol</td>
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</table>

Total cholesterol, suggests that the study population has increased lipid levels in the bloodstream (average - 213 mg / dl, normal value exceeds) module default HDL - cholesterol is a risk factor having a value of 32 mg / dl, values indicating increased risk for cardiovascular disease. Module LDL - cholesterol (169 mg / dl) is also well above normal, optimal, suggesting that this indicator is a risk factor for cardiovascular disease. Mean triglycerides (165.35 mg / dl) also exceeds the optimum, in which are considered to have an increased cardiovascular risk.

PCSK5 gene is part of ongoing research genes to identify their role in lipid metabolism, therefore to emphasize that mutations in this gene are a risk to patients of developing atherosclerotic lesions. Being a gene encoding a protein, it can be very important in lipid metabolism, which motivated us and more choice.
This technique is a screening method is quite fast, but that any screening method requires confirmation by other methods, such as PCR, qPCR, FISH, Southern blot, etc.

This confirmation is necessary for us to exclude including this normal variants that are considered normal (over 50 known normal variants of the gene). Or identification of possible new mutations risk for developing atherosclerotic disease (Carter, Church et al. 2012).

For example, a complete duplication of a gene is not necessarily to be pathological; instead a partial duplication of the gene can cause disease. in other situations, a variability of a specific gene variants can lead to disease expression, disease light, medium and severe (Forsberg, the Faire et al.2001).

We have identified by MLPA technique, deletion of exon 9, namely its lack of amplification on one gene alleles.

This lack of amplification can be caused by several conditions:
- Complete deletion of exon
- Partial deletion of the exon
- Exon mutations (point mutations, deletions, insertions, duplications, translocations, etc.).

That said, we conclude that mutations in the gene PCSK5 of leukocyte genome are statistically insignificant in patients in the study group.

**VII. 5. Conclusions**

Characteristic of patients with peripheral arterial disease:
- 15% of the study populations are women
- It shows a high prevalence of rural patients 58%, with the average age around 66.5 years
- 81% of the study populations are smokers,
- Patients assigned to the dyslipidemia of the following: 213 mg / dL for total cholesterol 165 mg / dL triglyceride and 137 mg / dL LDL - cholesterol, 41 mg / dL HDL - cholesterol.

It is necessary to study the relationship between the type of smoked cigarettes, the amount and period to assess how real smoking is important in the development of peripheral arterial disease.

It is also important to identify whether there are differences between patients coming from rural areas, compared to those coming from urban areas to identify other potential risk factors related to socioeconomic status of patients.

At 6.25% of patients in the study group was identified a mutation in exon 9 of the gene PCSK5 without statistical significance p<0.05, but this statistical significance for a confidence interval of 90%, where p = 0.000489. To increase the statistical significance test can increase the number of patients participating in the study. The presence of this mutation should be confirmed by other molecular biology techniques.

Survey of gene expression in the plaque is also important because endothelial lipase concentration is high in the vascular endothelium and its inhibition will be held at the same level, therefore this proprotein convertase would act directly on endothelial lipase.

These data should be investigated in our study to identify whether similar abnormalities are present in the group of patients investigated and have clinical significance.
PCSK5 gene is involved in many biological processes, yet unknown, any modification can lead to various diseases: cancer, neurodegenerative diseases, cardiovascular diseases, immunological diseases, kidney disease, etc. This confirms the need for further studies.

Chapter VIII. Of autologous bone marrow aspirate unprocessed - viable therapeutic alternative lower limb peripheral arterial disease?

VIII. 1. Introduction

Gene Therapy
Gene therapy is the use of DNA as a pharmaceutical agent to treat the disease. In gene therapy, DNA encoding a therapeutic protein is packaged in a "vector" is used to carry the DNA into the cells of the body. Once in the body, the DNA is expressed in cells, leading to production of therapeutically active proteins, which, in turn, theoretically designed to treat the patient's condition.

In peripheral arterial disease it can be used the therapy with vascular endothelial growth factor (VEGF), to stimulate endothelial cell growth and to induce angiogenesis respectively.

Recent studies performed in Romania had promising results (Taranu Ionac et al. 2010).

Cellular Therapy
Cell therapy is the process of introducing new cells into a tissue in order to treat a disease. Cell therapies often focus on treating hereditary diseases, in combination or not with gene therapy. Cell therapy is a type of regenerative medicine (Gerlach and Zeilinger 2002).

Cell therapy and other regenerative medicines, such as tissue engineering, represents a separate technology and have diverse therapeutic platform.

This initiative has its roots in blood transfusion, bone marrow and organ transplantation, tissue and in vitro fertilization. This trip lasted 200 years and it was based initially on trial and errors and, more recently, research laboratories have to face to the unique challenges that justifies a new separate industry (Sanchez, Schimmang et al. 2012).

There are many forms of cell therapy (Ramos and Hare 2007)

- Transplantation of stem cells or progenitor autologous cells (from the patient) or allogeneic (from another donor).
- Mature cell transplantation, functional (cell replacement therapy).
- Application of modified human cells that are used to produce a required substance (cell-based gene therapy).

These forms of cell therapy are not widely used today, but it is possible that in the future, depending on the results of research and ethical concerns to be a therapeutic method widely used in many kinds of diseases.

Non-human xenotransplantation of cells are used to produce a required substance. For example, treatment of diabetic patients by entering their insulin-producing pig cells directly into the muscle.

Transplantation of transdifferentiated cell derived from the patient's own cells. For example, the use of insulin-producing beta cells in isolated hepatocytes transdifferentiation as a treatment for diabetes.
For arterial disease were conducted many trials involving the use of stem cells isolated from bone marrow, they were injected intra-arterial and intravenous. These results are quite promising.

**VIII. 1. 2. Composition of bone marrow aspirate**

Marrow aspirate contains numerous progenitors because this tissue is known to be the main source of hematopoiesis even intrauterine period.

Bone marrow cellularity can be investigated by analyzing bone marrow smear of bone marrow aspirate or biopsy. What light is influenced by the processing technique Figure 52.

**VIII. 2. The purpose of the study**

- Induction of angiogenesis in patients with peripheral arterial disease
- Forming a network of vessels that provide collateral perfusion in the periphery
- Improving the quality of life in patients with peripheral arterial disease
  - a. Reducing pain
  - b. Increasing distance away
  - c. Reduction of trophic changes
- Identifying patients who will most benefit from this procedure
- Identifying the stage of the disease where the procedure will have maximum effect

**VIII. 3. Materials and methods**

**VIII. 3. 1. Patients inclusion in the study**

This study is an experimental study, it was decided to include a small number of patients, and was therefore included 8 patients with lower limb peripheral arterial disease, stage III and IV after Leriche classification - Fontaine have read, understood, and agreed information sheet and
informed consent (Annex 1 and 2). Internal Medical Clinic, Emergency County Hospital of St. "Spiridon", who had inoperable stage of the disease, with the ankle arm index below 0.6. The study was conducted in accordance with the regulation of research ethics committee and was approved by the Ethics Committee of the University of Medicine and Pharmacy "Grigore T. Popa" Iasi (Annex 3).

**Inclusion criteria**
- Patients hospitalized in the Clinic cardiologist Emergency Hospital "St. Spiridon"
- PAD - inoperable stage
- Understanding and signing informed consent and information sheets
- Trophic changes
- IGB under 0.06
- Effect of minimum one member
- Smokers and non-smokers

**Exclusion criteria**
- Patients who refused participation in the study
- Patients who have cancer and leukemia
- Mentally ill
- Chronic users of alcohol withdrawal
- The presence of cutaneous staphylococci in the hip and calf,
- Obese with a body mass index over 35 kg / m$^2$
- Decompensate diabetes patients
- Patients who have chronic hepatitis B or C,
- HIV / AIDS.

**VIII. 3. 2. Patients clinical status evaluation**

Patients included in the study were evaluated clinical, laboratory, in order to collect objective data about their health, they have applied some questionnaires (quality of life and pain evaluation), which aimed a subjective evaluation of their health status.

The **objective** evaluation of the health of the set: performing routine blood counts and chemistries which are typically in these patients when they are confined in the clinic:

- **General laboratory tests:** Hemoleucogam, glucose, urea, creatinine, SGPT, SGOT, GGT, alkaline phosphatase, bilirubin.
- **Local and general clinical examination:** We evaluated locally specific trophic changes: lesion area (both diameter) lesion depth by damaged tissues (pilosity, thin skin, ulcerated lesion with loss of substance, etc..)
- **Ankle-brachial index** (sphygmomanometer consists in applying the leg cuff patient tracking with pulse Doppler vascular pumping air into the cuff until the disappearance of the Doppler signal, then gradually release air to recurrence beep, record the time when the gauge procedure to be carried out on both hands and feet, and then is the ratio between the values of ankle and arm on the same side).
- **Angiography:** determining the level of occlusion / arterial stenosis, evaluation indication operators. The procedure consists of inserting a catheter into the arterial system, arterial puncture (femoral), at its injection of contrast,
examination of the arterial system on a fluoroscopic screen to identify the location and severity of arterial stenosis.

- Vascular Doppler

In the study procedures were performed: harvesting bone marrow aspirate, bone marrow aspirate intramuscular injection.

VIII. 3.3. Bone marrow aspirate harvest

Harvesting bone marrow aspirate was performed under local anesthesia from the iliac crest using trocars provided by Gallini Medical Devices which have a diameter of 15 G and 11 cm length. Getting marrow aspirate and its transplantation was performed by the classical procedure from the iliac crest (Figure 53). It were harvested around 50-60 ml, relatively small amount, which poses significant challenges to the quality of "donor" post the transplant procedure may feel heavy, or the patient may be fatigued for a few days. Within a few weeks the bone regenerates in principle patients can return to normal routine in one to two days.

Puncture aspiration of the bone marrow (MO) seated astride his back in his chair-stands. It was performed under local anesthesia with Xilina at periosteum level Figure 54.

It was revealed puncture site and trocar was introduced through the skin into the iliac crest Figure 55, subsequently the bone marrow was aspirated in the syringe attached to the trocar Figure 56 and 57. Bone marrow is rich in stem cells, precursors of hematopoiesis, adipocytes, bone fragments and growth factors.

There have been drawn from 20 to 60 ml of bone marrow amount which varied from patient to patient.

The obtained marrow aspirate was used wholly within autotransplantation procedure, which reduces the risk of contamination by minimizing the time interval from the aspiration to injection puncture; this avoids numerous maneuvers of cell separation and processing.

![Figure 54. Prepare the puncture site - local anesthesia (at periosteum)](image1)

![Figure 55. Iliac crest puncture](image2)
VIII. 3. 4. Procedure of intramuscular injection of marrow aspirate

Autotransplantation was made intramuscularly into the calf muscles and / or thigh intramuscular injection of integral bone marrow.

This procedure was performed in 12 to 22 points, at a distance of 2 cm between them, it was used collection a needle with a diameter of 18 G. Verification of compliance was checked by palpation and intramuscular injection was required. For this injection it was followed several steps:

1. Determination of the number and location of injection points
2. Checking intramuscular location of the needle
3. Aspirate injection

The establishment of injection site was done by correlating clinical and laboratory data of patients with identification of affected aria, and the amount of harvested bone marrow.

These decisions have varied from case to case situations described below.

Intramuscular needle localization was checked by attaching the needle to a syringe with saline isotonic water, it was placed intramuscularly, and the aspiration confirmed intramuscular position Figure 58.

Injection needle aspiration was performed by leaving the needle in the same position and changes the injection syringe at the other end to Figure 59, for each injection site the needle was changed.
Case 1. Patient aged 60 years, smoker, has stage IV PAD with severe skin changes Figure 60, IBG = 0, the affected lower limb angiography revealed stenosis of the femoral artery was originally thrombosis distal to the spacing of the knee joint. Collateral circulation in the calf and visible in the proximal third interosseous artery at which there are multiple serial stenosis. In this patient it was extracted 30 ml of bone marrow aspirate that has been injected in the 17 to the leg.

Case 2. Patient aged 42 years, smoker, has stage IV PAD with severe skin changes (Figure 62 and 63), ABI = 0.1; angiographic stenosis was revealed superficial femoral artery at the origin vascularity in the thigh by only collateral circulation in the calf extremely low. In this patient were collected 60 ml of bone marrow aspirate, which was injected into 28 points distributed as follows: 10 points antero-external face of the thigh, and 17 points in box gastrocnemian muscle of the calf.
Case 3. Patient aged 70 years, smokers with stage IV PAD, shows skin changes (Figure 64), the ABI = 0, angiography has distinguished superficial femoral artery thrombosis in the lower third of the thigh. In this patient we could harvest 20 ml bone marrow aspirate, which was injected into 6 points, 2 in the lower third of the thigh and 4 in the upper third of the calf.

Case 4. Patient vast 69 years, former smoker, diagnosed with stage IV PAD, shows the left lower limb, finger amputation 2 and 4 with the third finger gangrene limited laterally by the lack of tissue to the finger and partly on foot, with a visible tendon. Plantar it was observed a hole without inflammatory signs and no expression on the dorsal foot (channel blind), Figure 65, IBG affected limb is 0.15; angiographic stenosis is distinguished floor superficial femoral artery, stenosis of the joint spacing knee. Collateral circulation in the calf is present only in the proximal third of the leg. In this patient were extracted 40 ml of bone marrow aspirate which was injected into 18 points in the gastrocnemius muscle lodge.
Figura 65. The appearance of lower limb Case 3.
A. Anterior aspect B. Plantar aspect.

Case 5. Patient aged 72 years, former smoker with stage IV PAD, leg appearance is shown in Figure 66. Angiography is distinguished: sub occlusion of the left common femoral artery, superficial femoral artery occlusion from home with proximal popliteal artery refilling without circulation in the calf. In this patient were extracted from two punctures only 15 ml of bone marrow aspirate (possibly due to medullar atrophy observed in a new hematologic evaluation by reducing the number of erythrocytes and platelets slightly below the lower limit). Aspirate extract was injected in 5 points in the upper third of the leg affected.

Figure 66. The appearance of lower limb Case 5.
**Case 6.** Patients aged 62 years old, smoker with stage IV PAD. The left lower limb, these trophic changes Figure 67, ABI is 0.2. The patient was assessed by CT angiography, Figure 68 A and B, are observed: multiple stenosis in the common femoral artery, deep and superficial, poor circulation in the calf with multiple stenosis. In this patient were extracted 45 ml of bone marrow aspirate that has been injected at 18 locations in the left leg.

![Figure 67. Appearance limb Case 6](image)

![Figure 68. Angio CT appearance of Case 6, A. Thigh level, B. Lower leg.](image)

**Case 7.** Patient 63 years old, smoker with stage IV PAD. In the right leg, this trophic changes Figure 69. IGB is 0.1 patient was assessed by CT angiography, Figure 70 A and B, are observed: multiple common femoral artery stenosis, thrombosis in the deep and superficial femoral artery in the lower third of the right thigh, the absence of contrast in the artery right popliteal. Inefficient circulation in the calf, this patient was extracted 55 ml of bone marrow aspirate, which was injected into 22 points in the calf straight.

![Figure 69 Appearance limb Case 7](image)

![Figure 70 Appearance Angio CT Case 7 A. Thigh level, B. Lower leg.](image)
Case 8. Patient aged 59 years, smoker with stage IV PAD. In the right leg, this trophic changes Figure 71. ABI is 0. The patient was evaluated by angiography are observed common femoral artery occlusion, thrombosis, absence of movement in the middle third of the thigh, are present fine collateral arteries and the lower third of the thigh and lower leg circulation is absent Figure 72 A, B and C. At this patient were extracted 50 ml of bone marrow aspirate that has been injected into the anterior view 10 points in the thigh and 15 points in the posterior face of the shank.

![Figure 71. Leg appearance. Case 8.](image)

![Figure 72. Angiography. Case 8.](image)

A femoral artery occlusion, B. Circulation in the middle third of the thigh, C. Absence of circulation

Patients were included in the study for a period of 6 months or until amputation because angiogenesis can be investigated in their case, if the research team had been included an anatomic pathologist to identify the product then it would be amputated been identified, but the absence of pathology lab, led to exclusion from the study patients after amputation. Also death is another criterion out of the study.

Patients were assessed at one month by phone, during the stem cells determine their direction of differentiation, then 3 months clinical evaluation and ultrasound and angiographic assessment 6 months or angio CT.
In the study the patients used their normal medication for other associated cardiovascular disease.

**VIII. 8. Results**

Telephone assessment of patients a month consisted of: questioning how evolved their pain; the discussion was directed specifically to identify changes between period before intervention and after intervention and possible local reactions.

**Results in one month**

Case 1. It was amputated after 15 days of intervention in hospital from Tg.Neamt - removed from the study.

**Results in three months**

Case 2. Appearance leg is shown in Figure 73, where we can compare with the previous aspect before intervention Figure 62 and 63, we see: amputation of the distal phalanx of the finger 2, discrete limb edema, and changes in skin color from light pink cyanotic. ABI in the revaluation was 0.2. At 2 weeks of evaluation the patient suffer a set of tree strokes with seizures, paraparesis, aphasia, and the last of them is fatal.

![Figure 73. The appearance of leg 3 months after autotransplantation Case 2.](image)

Case 3. Appearance leg Figure 74, where Figure 64 compared to the previous point, there is discoloration, and the petechial lesions disappeared. ABI in the revaluation was 0.25 vascular ultrasound highlights the repermiaillisation of the normal vascular tree, average popliteal artery plaque, flux phase, multiple stenosis, distal popliteal artery flow enabled, posterior tibial artery, current flow, phase, stenosis storiied interosseous artery and anterior tibial without flow. Noted that patients during the 3 months following therapy with vasodilating prostaglandins.

Case 5. The patient declares a slight improvement in pain, but instead he made a stroke with hemiparesis on the right. Because of the difficulty of going to reevaluations he requested the exclusion from the study.

Case 6. Appearance of the leg Figure 75, where the previous point of intervention the appearance was like in Figure 67, were we see: skin color changes from light cyanotic to pink,
the walking distance have grown, with a improvement of ABI at the revaluation it is 0.3, vascular ultrasound was done and showed: present flux in the popliteal artery, below the knee is observed numerous small vessels, suggesting collateralised circulation. Current flow in the posterior tibial artery and the previous phase flow with low speed, and the absence of interosseous artery flow.

**Figure 75. The aspect of the inferior leg, after 3 months after intervention. Case 6.**

Case 7. Without significant changes, the same IGB, without changes in walking distance. Had the same appearance in outer side of the lower leg.

Case 8. Presents of the pain in the region of gangrene, especially in the located areas of pressure, the pain intensity does not improve with the administration painkillers, leads to lower limb amputation at the level below the knee. Amputation was performed at almost 5 months after the procedure. Deficiency of amputation stump, required the strump reamputation, with repetition of the procedure was performed above the knee.

**Results at six months**

Case 1. The patient was excluded from the study after amputation at one month.

Case 2. The patient died after 2 months after the intervention following a stroke.

Case 3. The patient made spontaneous thrombolysis and it was observed in ultrasound that in the vascular tree he had better blood flow, this was in direct relation with the improving appearance of the limb, visible from 3 months, ultrasound flow was observed in the femoral artery, popliteal, tibial anterior and posterior, these data are consistent with significant improvement IGB, from 0.1 to 0.3.

Case 4. The patient was excluded from the study after amputation in a month and a half.

Case 5. In a four and a half months after the procedure, during which pain relief was observed in transplanted lower limb, the patient suffers a stroke, followed by hemiparesis, aphasia, and increased contralateral leg pain. The patients and his family asked for exclusion from the study because of the difficulties of mobilization (the patient being in the province and special transport needs to present the following assessment).

Case 6. There was a slight improvement in clinical symptoms with increasing ABI 0.15, and increased walking distance by 10 meters, pending claudication. The patient is happy with such a development. Ultrasound are emphasized: dish network side, the thigh, the popliteal vascular refilling distal axis, but in the present wave is specific for an arterial bunk stenosis persistence and movement of a collateral blood flow still lead sufficient to compensate for the local aspect.
Case 7. The patient returns to the revaluation, which shows a discrete clinical improvement of symptoms with increasing of walking distance by 7 m, and the same ABI 0.1 (although values below 0.15 are considered to have no clinical relevance in measurement because of the particular index, and sensitivity of the method). Ultrasound shows no change in the local blood supply; however we can mention the symptoms getting worse during the six-month assessment.

Case 8. The patient is amputated at an interval of three months after the procedure, initially below the knee then above the knee. Reamputation was decided after persistent stump pain and scarring deficit.

**Final results.**

The overall results are shown in Table XV, in which we see no major efficiency of the therapeutic standpoint, which most likely is due to the fact that the patients included in the study were in advanced stages, some of them at the time they present indications of amputation.

Major complications in other vascular territories such as death, stroke brings into question severity and seriousness of the problem, and raises the need for an effective treatment of atherosclerotic lesions and the timing of treatment initiation.

**Table XV. Results of the bone marrow autotransplantation in patients with PAD, and their evaluation.**

<table>
<thead>
<tr>
<th>The initiation</th>
<th>One month</th>
<th>Three months</th>
<th>Six months</th>
<th>Ultrasound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IGB DCI</td>
<td>IGB DCI</td>
<td>IGB DCI</td>
<td>IGB DCI</td>
</tr>
<tr>
<td>Case 1</td>
<td>0 0</td>
<td>Amputation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Case 2</td>
<td>0.13 9</td>
<td>0.22 17</td>
<td>Died</td>
<td>Repermiabilisation of vascular tree</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>from stroke (complications in other vascular territories)</td>
<td></td>
</tr>
<tr>
<td>Case 3</td>
<td>0 10</td>
<td>0.17 20</td>
<td>(0/2) 24</td>
<td>0.27 47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stroke (hemiparesis and aphasia) - requires exclusion from the study because of difficult mobilization</td>
<td></td>
</tr>
<tr>
<td>Case 4</td>
<td>0 0</td>
<td>Amputation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Case 5</td>
<td>(0/2) 6</td>
<td>0.28 19</td>
<td>Stroke (hemiparesis and aphasia) - requires exclusion from the study because of difficult mobilization</td>
<td></td>
</tr>
<tr>
<td>Case 6</td>
<td>0.1 11</td>
<td>0.12 18</td>
<td>0.18 23</td>
<td>0.27 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>The presence of collateral circulation</td>
<td></td>
</tr>
<tr>
<td>Case 7</td>
<td>0.15 18</td>
<td>0.15 22</td>
<td>0.1 29</td>
<td>(0/2) 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absence of collateral circulation</td>
<td></td>
</tr>
<tr>
<td>Case 8</td>
<td>0.1 0 0</td>
<td>Amputation</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**VIII. 5. Discussions**

Treatment for peripheral arterial disease is dependent on the severity of symptoms and stage. We can use from conservative therapy and drugs, in association with treatment of the modifiable risk factors influencing the endovascular or open surgery. The study included patients in severe, end-stage disease without other therapeutic options, to assess first of all the procedure safety. At that time the amputation was the only treating option.

The goal of treatment is to promote angiogenesis. Bone marrow cells include different types of primitive cells that are able to differentiate into hematopoietic and mesenchymal cells and growth factors that promote angiogenesis and endothelial formation (Esato, Hamano et al.2002 Higashi, Kimura et al.2004 Li, Su et al. 2011).

The results lead to the idea of establishing the therapy much earlier in the disease evolution, that have to be addressed to the patients with no indication for amputation, to let cells from bone
marrow aspirate sufficient time for differentiation. They are capable of differentiation and typical features of a particular tissue obtained only after at least 2 weeks in vitro experiments.

For each patient included in the study there was a particular and specific adaptability. Some of them were already needed to be amputated but patient and their caregivers in despair insisted to introduce them in the study group to see first and foremost the safety of the procedure.

Phase I clinical trials are designed to establish the safety primary procedure (Manoonkitiwongsa 2011), which we performed and our patient without other therapeutic solution.

The procedure proved to be safe, at least in the short term. And in the timp of intervention one patient had discrete vagal reaction, manifested by lowering systolic blood pressure of 20 mmHg, when we made a break between injections, and we resumed shortly injection.

Local reactions at the puncture was not met, any small interval of injection or injection every great post.

In three patients, apparently favorable evolution is most likely on injection therapy with prostaglandins, as it was observed in ultrasound - normal blood flow increase in the vascular tree of the patient. These can be attributed to the fact that the patient is older than 72 years, and when he could not get a puncture amount of 20 ml of aspirate, which was injected into only 6 points.

Death confirm that peripheral arterial disease is a severe, multisystemic and potentially fatal and disabity (Khandanpour, Jennings et al. 2011).

**VIII. 6. Conclusions**

The number of stem cells administered is an important criterion for carrying out therapeutic angiogenesis, but there are standard guidelines for this.

Angiogenesis has been described as the development of new capillaries from pre-existing vessels, which lead to the formation of new capillary networks (Kawamura, Horie et al. 2006). Currently it is known that angiogenesis can be induced by many types of cells and cytokines in both ischemic animal models and humans. (Kim, Kim et al. 2006). Circulating stem cells such as endothelial cells, progenitor cells are an attractive source for the regeneration of tissues, and are now widely accepted that the source of cells may promote angiogenesis (Aoki, Yasutaka et al. 2004).

In this study, we tried to identify effective autograph of integral bone marrow aspirate to severe peripheral arterial disease patients without other therapeutic option.

As research results we mention that this therapy may be effective in patients who have a free period of amputations at least for three months, during which mesenchymal stem cells and endothelial progenitor cells have the ability to differentiate into endothelial cells (Fukunaga, Uchida et al. 1999 Goerke, Plaha et al. 2012), then their polarization and the formation of new capillaries (Zovein, Luque et al. 2010). Do not forget that post fracture hematoma is angiogenic (Glowacki 1998 Kolar, Schmidt-Bleek et al. 2010), which I induced medically, in these transplantations, local hematoma formation, which aimed angiogenesis.

Marrow cellularity, as is known, to decreases after the age of 60 years exponentially after reaching the age of 70 years it is below 20% (Tuzuner, Cox et al. 1994), making it necessary to include in the study the patients with younger age (up to 65 years).

Our results are uncertain, possibly because they were already included patients who had indication for amputation, or very old patients with severe reduced bone marrow cellularity, having a permanently reserved enough stem cells. They require a more serious depth in subsequent studies. And containing at least one pathologist or histologist, to analyze the amputation products to be investigated, to reveal whether or not we had formed blood vessels,
and if these vessels are efficient in terms of hemodynamic here highlights the need for a multidisciplinary team that includes: a cardiologist or internist, hematologist, vascular surgeon, general surgeon, or orthopedic surgeon, a specialized laboratory service for marrow aspirate analysis, including a service technician, biologist, medical lab.

The need for such a team is important to prevent data loss that refers to amputation products and especially in the regional hospital services, the loss to follow-up.

This technique has a large apparent therapeutic potential that has to be investigated, and the inclusion of patients to make well established criteria: to provide a rest period of at least three months amputations, be relatively young patients who have a good marrow cellularity more than 50%.

**Chapter X. Conclusions**

Exploring new treatment strategies in patients with PAD is most important because of the high risk of major amputations of feet and subsequent mortality if surgical or endovascular revascularization procedures have failed or are not possible. Autologous cell therapy is a new promising treatment option for these patients, and clinical trials are consistent in reporting clinical benefits, including improvements in ABI, peripheral perfusion, reduce pain and reduce the need for amputation. However, there is still a need for large randomized trials, placebo-controlled, double-blind trial to provide a definitive role for this treatment option.

A growing number of clinical evidence supporting the routine use of stem cell therapy, several issues will have important practical applicability. In this context, a single closed containment system quickly without the need for special equipment and without legal obstacles.

**Elements of originality of the thesis**

The thesis brings up a completely new approach to stem cell therapy of bone marrow origin, separated, unprocessed, which has not been addressed so far to minimize the handling time of the cells outside the body, reducing the risk of contamination, feature very useful for developing country within limited financial resources, the possibilities for Good Clinical Practice are difficult if not impossible.

In addition a part of the thesis attempts to identify a method of screening increased risk of atherosclerosis; to identify individuals with increased risk of atherosclerosis is (by identifying a gene expression PCSK5). In order to use this procedure as a screening were not used similar methods.

Another element of originality is the attempt to characterize stem cells by the view of integrin expression, which identified the conflicting with other authors with the presence of beta1 integrin subunit, which is very important in the development and subsequent tubular capillary blood vessels.

**Perspective that opens the thesis**

The thesis opens horizons in the management of arterial occlusive diseases, minimizing surgical approach to this disease, and as a result opened the possibility of starting large studies in this regard, including a large number of patients, which may benefit from autologous cell therapy and bring significant benefits at relatively low cost.

We hope that the following studies will be done with less reticent from authorities and patients, not only in eastern Romania, but also the possibility of collaboration with vascular surgery clinic in Timisoara, led by Professor Dr. Michael Ionac, which is open to possible future
collaborations. The thesis also established that medullary autotransplantation procedure is a safe procedure, however it must be established whether there is a relationship transplanted patients suffered strokes and autologous away.

In addition to collaborating with Opened for Timisoara County Hospital for further studies, particularly comparative studies.

This thesis also initiated collaboration with the Department of Medical Genetics, by genetic study when attempting to establish a method of screening for patients with increased risk of atherosclerosis. These collaborations have not lost since an interdisciplinary approach to modern medicine open important doors to the future.

**Bibliography**


Aponte, J. The prevalence of peripheral arterial disease (PAD) and PAD risk factors among different ethnic groups in the US population. *J Vasc Nurs* 2012; 30(2): 37-43.


Beksac, M. *Bone marrow and stem cell transplantation*, Humana Press. 2007.


Appendixes

Annex 4. List of published works

Articles as first author published / accepted by publishing in journals BDI


Items sent / being assessed as first author in ISI
- Popa, S., Baroi, G., Ivanov, A., Datcu, DM, V. Aursulesei "Ethical issues in the use of therapeutical angiogenesis in Cardiovascular Diseases "Rev Ro Bioethics

Published Abstracts


Annex 5. List participation in courses, conferences, internships abroad and awards received

National Congress and Conference Participation:
1. Rare Disease Day Symposium 2011 held at University of Medicine and Pharmacy "Grigore T. Popa" Iasi, on 5 March 2011.
2. Symposium News for diagnosis and treatment in pediatric neurology, conducted Iasi, on 24 November 2011.
3. Medical Genetics Symposium - turntable between specialties - Rare Disease Day 2012, held at the University of Medicine and Pharmacy "Grigore T. Popa" Iasi, on 3 March 2011.
4. Symposium doctoral within HRD project, titled "Doctors for PhD scholarships competitive POSDRU/88/1.5/S/63117 the European Research Area", held in Timisoara in September 8 2011.
5. The 5th Conference of PhD Students in Medicine and Pharmacy and 2th Conference of Postdoctoral Researches, held in Targu Mures, Romania, between 4 to 6 July 2012.
6. 63117 HRD Conference International Conference, 28-29 September 2012, Timisoara, Romania, Quality Management in Medical Academic Research
7. The VI National Conference of Medical Genetics with international participation organized by SRGM, held in Iasi, 5-8 October 2012.
8. National Symposium, Section doctoral students, the HRD project, entitled "Contributions to the Development of Young Researchers in the medical and pharmaceutical research", held in Iasi, 7-9 November 2012.

International Congress and Conference Participation:
2. European Human Genetics Conference, held in Nuremberg, Germany, from 23 to 26 June 2012.

Awards:
1. Second prize in National Conference on Genetic Iasi (Romania) 2012, for the poster "Role of proprotein convertase subtilisin / kexin type 5 (PCSK5) as predictor of atherosclerosis risk in peripheral arterial disease."
2. Second prize of 14th in the Conference Euroregional DKMT Szeget (Hungary) 2012, for the poster "Natural Regeneration Stem Cells and Human Capacities In Inducing Angiogenesis In Lower Limbs."