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XXX CICLO

MONONUCLEAR PERIPHERAL CELLS AT HIGH CONTENT OF STEM CELLS CD34^{+} AFTER MOBILIZATION WITH G-CSF FOR THE TREATMENT OF CRITICAL LIMB ISCHEMIA (CLI).

CELLULE MONONUCLEATE PERIFERICHE AD ALTO CONTENUTO IN STAMINALI CD34^{+} POST MOBILIZZAZIONE CON G-CSF PER IL TRATTAMENTO DELL'ISCHEMIA CRITICA DEGLI ARTI

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SUMMARY

INTRODUCTION: The Peripheral Artery Disease (PAD) is a pathology characterized by different and progressive levels of damage generated by the cumulative reduction of blood flow to muscles, nerves and skin. Critical Limb Ischemia (CLI) is an evolution of occlusive arterial disease and it is one of the most severe clinical manifestations of PAD. Patients affected by CLI has a poor prognosis in term of limb salvage and survival too, despite they are submitted to the conventional surgical, endovascular and pharmacological treatments. A variable number from 25 to 40% of patients suffering from CLI is not eligible for either surgical nor endovascular treatment.

AIM OF THE STUDY: The aim is to evaluate the feasibility and the outcome of inoculation of mononuclear peripheral cells at high content of stem cells $CD34^+$ as treatment of critical limb ischemia in order to reduce the risk of major amputation of the inferior limbs.

MATERIALS AND METHODS: Patients affected by Critical Limb Ischemia and unresponsive to pharmacological therapy or with unsatisfactory clinical course after surgical or endovascular revascularization, are selected to be submitted to Mobilized Peripheral Blood MonoNuclear Cells implantation. To each patient is subcutaneously administered Granulocyte Colony-Stimulating Factor in order to mobilize $CD34^+$ haematopoietic bone marrow stem cells in the peripheral blood, then an apheresis procedure is performed. The amount of blood volume processed is implanted into the muscle of the ischemic lower limb or in area surrounding ulcers or gangrene lesions.

RESULTS: Between November 2014 and September 2017 sixty-two (62) patients have been treated. Amputation Free Survival (AFS) was 81% at 6 months and 77% at 1 and 2 years. The transplanted patients showed a reduction of pain after procedure, as well as a reduction of the severity

of the ulcers grade. Only 14 of all limbs undergo a major amputation with a median Time-To-Amputation (TTA) of 3 months. In some cases, new vessel formation with new capillary arteries has been observed in the follow-up angiography.

DISCUSSION: The gold standard treatment for severe PAD and CLI is direct revascularization. Despite the continuous progresses in surgical and endovascular treatments, still up to 30% of patients are not eligible for these therapies to date. The rate of Amputation Free Survival (AFS) of 77% obtained in this study permitted to achieve the primary end point and could be considered an encouraging result in limb salvage. A reason of this good result should be sought in the dose of cells administered that is about twice the amount reported in other studies.

This procedure seems to be safe. There were just few serious adverse events and no deaths related to the mobilization or transplantation procedures, even in elderly patients. Moreover, patients with PAD at stage IV of Fontaine Classification have not shown a significantly reduced capability of stem cells mobilization compared to patients with a lower grade.

CONCLUSIONS: Stem cells therapy confirms to be very promising for the effective treatment of “no-option” CLI patients with a favorable safety profile. $CD34^+$ cells could have a central role in this treatment as injection of higher doses of these cells seem to be associated with clinical benefit and better clinical results. On the other hand, the most advanced stages of arterial disease and limb ischemia are associated with worse clinical response to the stem cells’ transplantation treatment.

RIASSUNTO

INTRODUZIONE: l'Arteriopatia Ostruttiva Cronica Periferica (AOCP) è una patologia caratterizzata dalla progressiva riduzione del flusso sanguigno a muscoli, nervi e cute degli arti inferiori, che genera diversi e progressivi livelli di danno. L'Ischemia Critica degli arti (acronimo CLI in lingua inglese) è un'evoluzione ed una delle più severe manifestazioni dell'AOCP. Pazienti affetti da CLI hanno una cattiva prognosi sia per il salvataggio dell'arto che per la sopravvivenza nonostante siano sottoposti ai convenzionali trattamenti chirurgici, endovascolari e farmacologici. Una quota variabile dal 25 al 40% dei pazienti non può essere sottoposta al trattamento chirurgico o endovascolare.

SCOPO DELLO STUDIO: valutare la fattibilità ed i risultati dell'inoculo nei tessuti di cellule mononucleate periferiche ad alto contenuto di cellule staminali $CD34^+$ come trattamento dell'ischemia critica degli arti inferiori nel tentativo di ridurre il rischio d'amputazione d'arto.

MATERIALI E METODI: pazienti affetti da AOCP allo stadio di CLI, non rispondenti alla terapia farmacologica o con scarsi risultati clinici dopo rivascolarizzazione chirurgica o endovascolare, sono stati selezionati per essere sottoposti ad impianto di cellule mononucleate del sangue periferico mobilizzate. Ad ogni paziente viene somministrato Granulocyte Colony-Stimulating Factor per mobilizzare le cellule ematopoietiche $CD34^+$ dal midollo osseo al sangue periferico, poi prelevate mediante aferesi. Il sangue processato viene quindi iniettato nel muscolo dell'arto ischemico o nelle aree perilesionali di ulcere e gangrena.

RISULTATI: tra Novembre 2014 e Settembre 2017 sono stati trattati sessantadue (62) pazienti. La sopravvivenza con salvataggio dell'arto è dell'81% a 6 mesi e del 77% ad 1 e 2 anni. I pazienti trapiantati hanno mostrato una riduzione del dolore e della gravità delle ulcere dopo la procedura. Solo 14 arti sono stati sottoposti ad amputazione maggiore,

con una media di tempo di amputazione (TTA) di 3 mesi. In alcuni casi l'arteriografia al follow-up ha mostrato la formazione di nuova vascolarizzazione.

DISCUSSIONE: il trattamento gold standard dell'AOCP è la rivascolarizzazione diretta. Nonostante i progressi nella terapia chirurgica ed endovascolare ancora oggi fino al 30% dei pazienti non può essere sottoposto a tali terapie. Il valore ottenuto del 77% di sopravvivenza con salvataggio d'arto ha permesso di raggiungere l'end point primario dello studio e costituisce un incoraggiante risultato d'incremento delle possibilità di salvataggio dell'arto. Una ragione di questo buon risultato potrebbe essere individuata nell'elevata dose di cellule somministrate, che è circa il doppio del valore pubblicato in altri studi.

La procedura può essere considerata sicura e ci sono stati pochi seri eventi avversi e nessun decesso correlati alla mobilizzazione o al trapianto delle cellule anche nei pazienti anziani, inoltre i pazienti affetti da AOCP allo stadio IV di Fontaine non hanno mostrato una minore capacità di mobilizzazione delle cellule staminali rispetto a pazienti con grado di AOCP inferiore.

CONCLUSIONI: la terapia con cellule staminali conferma di essere molto promettente per la cura di pazienti con Ischemia Critica Cronica senza altre opzioni di trattamento e di essere una metodica sicura. Le cellule $CD34^+$ sembrano avere un ruolo centrale nel trattamento, infatti l'utilizzo di alte dosi di queste cellule si associa a beneficio e migliori risultati clinici. Lo stadio più avanzato della patologia, invece, è associato ad una peggiore risposta clinica al trattamento con trapianto di cellule staminali.

1 INTRODUCTION

1.1 ATHEROSCLEROSIS IN PERIPHERAL ARTERY DISEASE

The Peripheral Artery Disease (PAD) is a pathology involving principally the arteries of inferior limbs, but could affect also the superior limbs; it is the most common cause of walking disability and is characterized by presence of atherosclerotic or calcified plaques generating a progressive reduction of the lumen of the vessel until its complete occlusion.

Risks factors associated to atherosclerosis are several (Tab. 1), most of which are consequently associated to PAD.

RISK FACTORS FOR ATHEROSCLEROSIS
ADVANCED AGE
RACE (non-Hispanic blacks)
MALE GENDER
HYPERFIBRINOGENEMIA
DIABETES MELLITUS
HYPERHOMOCYSTEINEMIA
SMOKING
HYPERCOAGULABILITY
HYPERTENSION
ELEVATED C-REACTIVE PROTEIN
DYSLIPIDEMIA
CHRONIC RENAL INSUFFICIENCY

Table 1 – Risk factors for atherosclerosis. Modified from Norgren et al..[1]

The principal risk factors are advanced age, smoking, arterial hypertension, dyslipidemia, diabetes mellitus, obesity and

hyperhomocysteinemia. In particular: the risk for PAD increase 1.5- to 2-fold every 10-years rise in age;[2,3,4,5] hypertension is present in 55% of patients with PAD;[6] in diabetics patients PAD prevalence increases from 20% to 30% [7] and patients with diabetes are more likely to suffer of symptomatic peripheral arterial disease, risk increased especially in women;[8] excess abdominal adiposity plays a direct role in initiating atherosclerosis [9,10,11] and, according to the Cardiovascular Health Study, each 5-unit increase Body Mass Index (BMI: the weight in kilograms divided by the height in meters squared) in midlife and older age is associated with an increase of about 30% in PAD prevalence and incidence;[12] moreover the metabolic syndrome, present in 38% of the population with PAD, consists of having three or more of these characteristics: blood pressure elevation (≥ 130/85 mmHg), fasting blood glucose ≥ 110 mg/dL, triglyceride count ≥ 150 mg/dL, high-density lipoprotein count ≤ 50 mg/dL (for women) or 40 mg/dL (for men) and abdominal obesity (BMI > 30 kg/m^2 or waist circumference ≥ 88 cm in women and ≥ 102 cm in men).[13] Cigarette smoking is a recognized stimulus for atherosclerosis and is associated to an increased risk of developing PAD in men and women,[2,8] with higher risk for the latter. Moreover, smoking cessation is associated with a substantial risk reduction,[14] and the severity of PAD is directly proportional to the number of smoked cigarettes.[15] Hyperomocysteinemia is associated with a mildly increase risk for PAD, CAD, stroke and venous thromboembolism.[16,17,18] Normal plasma levels of homocysteine, differentiated by age are:[19]

- Age 0-30 years: 4.6-8.1 μmol/L.
- Age 30-59 years: 6.3-11.2 μmol/L (males); 4-5-7.9 μmol/L (females).
- Age > 59 years: 5.8-11.9 μmol/L.

Excessive plasma levels of homocysteine may promote medial smooth muscle cell proliferation and arterial wall inflammation.[20] A meta-analysis showed that patients with PAD had homocysteine levels higher

than those of control without PAD.[21] The potentially involved mechanisms are: enhanced thrombosis risk generating a less porous fibrin network, activation of platelet aggregation, procoagulant factors V and VII, thrombomodulin, tissue factor pathway inhibitor and Plasminogen Activator Inhibitor (PAI) and inhibition of activation of protein C.[22,23,24,25,26]

PAD is characterized by a progressive reduction of blood flow to muscles, nerves and skin generating different and progressive levels of manifestation. Symptoms could be absent in the initial stages of the disease, but often include pain that could occur with various patters, from Intermittent Claudication (IC), the typical exertional muscular pain, to ischemic rest pain, although not always the evolution of the IC progresses to rest pain. The more frequent site of inferior limb pain is the calf, but discomfort or pain could appear also in the thighs or the buttocks. Typically, the symptoms of IC disappear or are reduced after a brief period of rest, after which the patient can restart walking, to reappear after a certain distance is walked, and so on. As the pathology progresses, the symptoms occur more frequently and after distances of walk increasingly shorter. The worsening of the pathology may lead to rest pain.[20]

Other causes of Intermittent Claudication are non-atherosclerotic, among which can be encountered thromboangioitis obliterans, Takayasu's disease, fibromuscular dysplasia, pseudoxanthoma elasticum, remote trauma or radiation injury, thrombosis of persistent sciatic artery, arteritis, popliteal entrapment, adventitial cist of popliteal artery and primary vascular tumors.[20]

1.2 PAD CLASSIFICATIONS

In order to define different grades of progression of the Peripheral Artery Disease there are principally two classifications commonly used. Both are clinical staging systems with an objective scale to allow clinicians to describe and discuss about patients with peripheral artery disease using a common terminology. These classifications are clinical means of describing peripheral artery disease, as opposed to the angiographic morphological criteria proposed by the TASC II working group in 2007.[1]

The Rutherford Classification [27] has seven categories (Tab. 2); the Fontaine Classification [28] has four stages, divided in sub-stages (Tab. 3).

Rutherford Categories	
Category	Clinical description
0	Asymptomatic
1	Mild claudication
2	Moderate claudication
3	Severe claudication
4	Ischemic rest pain
5	Minor tissue loss
6	Major tissue loss

Table 2 – Rutherford's PAD Classification. Modified from Hardman et al..[29]

The Fontaine's Classification was introduced by its authors in 1952 during the first meeting of the European Society for Cardiovascular Surgery dedicated to aorto-iliac lesions.[28]

Fontaine Classification		
Stage I	Asymptomatic	
Stage II	Exercise-induced ischemia. ▪ Intermittent Claudication: pain during walking ▪ Relief of symptoms when standing	
	IIa	Compensated disease: walking distance ≥ 200 m
	IIb	Decompesated disease: walking distance < 200 m
Stage III	Rest pain	
Stage IV	Ulcers, necrosis and/or gangrene	

Table 3 – Fontaine's PAD Classification. Modified from Hardman et al..[29]

The Trans Atlantic Inter Society Consensus (TASC II), working group of representative of 14 surgical vascular, cardiovascular and radiologic scientific societies, proposed in 2007 a classification of PAD lesions providing treatment recommendations according to lesion type. The group classified anatomic patterns of disease of both the aortoiliac and femoropopliteal lesions (Fig. 1 and 2) in four types (A through D), based on recommended treatment (open surgery versus endovascular procedure).[1]

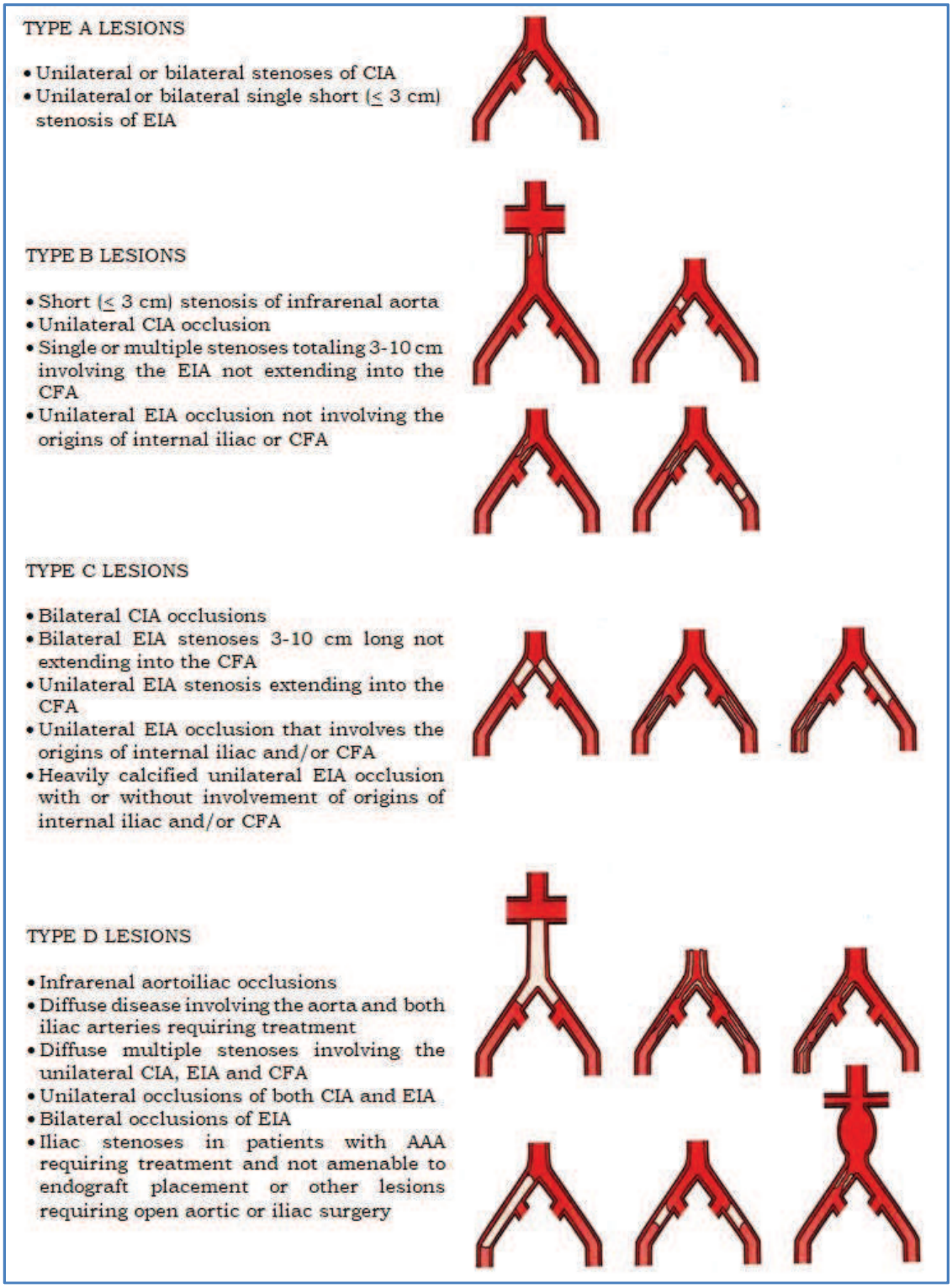


Figure 1 – Trans Atlantic Inter Society Consensus classification of aortoiliac lesions. AAA: abdominal aortic aneurism; CFA: common femoral artery; CIA: common iliac artery; EIA external iliac artery. Redrawn from Norgren et al..[1]

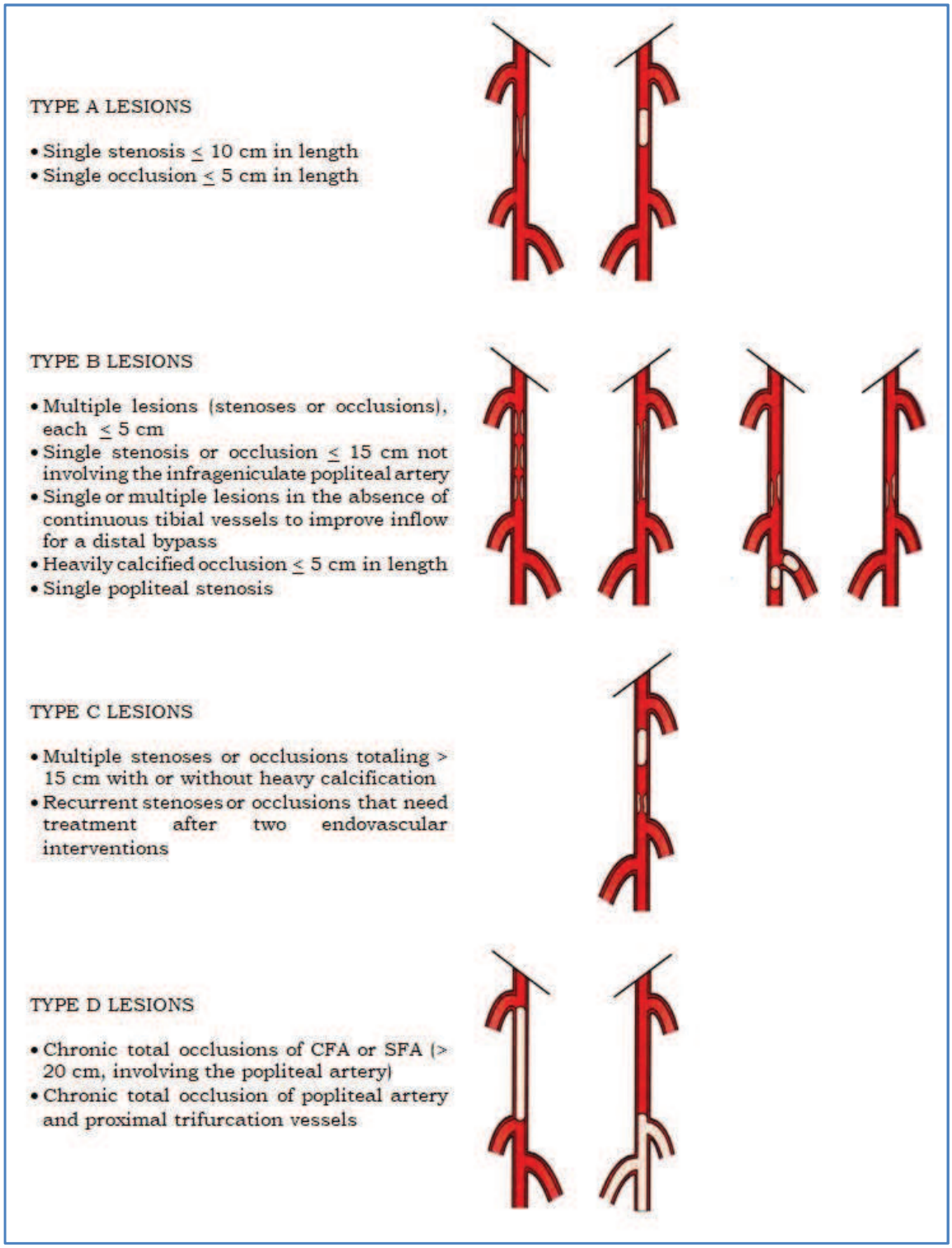


Figure 2 – Trans Atlantic Inter Society Consensus classification of femoropopliteal lesions. CFA: common femoral artery; SFA: superficial femoral artery. Redrawn from Norgren et al..[1]

1.3 ULCERS CLASSIFICATION

Foot ulcers occur mainly at an advanced stage of artery disease or in diabetic patients. The evaluation of ulcers is fundamental for the treatment of PAD; classifications permit to compare patients' level of damage and have a standard method to confront the evolution of the ulcers during the follow-up.

In the last years several ulcers classifications have been proposed. The two more diffused and widely accepted are the Wagner Classification and the University of Texas System Classification.

The **Wagner classification** [30] consists of 6 grades and is based mainly on wound depth:

- GRADE 0:– intact skin;
- GRADE 1:– superficial ulcer;
- GRADE 2:– deep ulcer extension to tendon, joint or bone without osteomyelitis;
- GRADE 3:– deep ulcer extension with abscess or osteomyelitis;
- GRADE 4:– forefoot gangrene;
- GRADE 5:– gangrene involving more than two/thirds of the foot.

The **University of Texas System Classification** divides the ulcers in grades (0-3) by depth, and in stages (0-3) depending on the presence or absence of infection and ischemia:[31]

GRADES:

- 0 pre- or post-ulcerative site;
- 1 superficial wounds involve either the epidermis or the epidermis and dermis, but do not penetrate to tendon, capsule or bone;
- 2 wounds penetrate to tendon or capsule, but do not involve bone and joints;

- 3 wounds penetrate to bone or into a joint.

STAGES:

- A: clean wounds;
- B: non-ischemic wounds;
- C: ischemic wounds;
- D: infected ischemic wounds.

Other used classifications are:[32]

- The S(AD) **SAD classification** divides ulcers evaluating five features (size, depth, arteriopathy, sepsis and denervation) on a four-point scale (0-3).
- The **PEDIS Classification**, proposed by the International Working Group on the Diabetic Foot, grades ulcers evaluating five features (extension/area, perfusion/arterial supply, depth, infection, sensation).
- The classification proposed in the **guidelines of Infectious Diseases Society of America (IDSA)** considers only infected diabetic foot lesions which are divided in three categories:
  - MILD: the involvement is restricted to skin and subcutaneous tissues;
  - MODERATE: the involvement is more extensive affecting deeper tissues;
  - SEVERE: the local infection is accompanied by metabolic instability or systemic signs of infection.

Another classification is the **WIfI classification system**, intended for any patient with a diabetic foot ulcer, non-healing foot ulcer present for two or more weeks, foot or lower extremity gangrene, or ischemic rest pain.[33] This classification, proposed for threatened lower limbs, has been published in 2014 by The Society for Vascular Surgery Lower Extremity

Guidelines Committee and is structured categorizing and grading the three major risk factors leading to amputation: Wound, Ischemia and foot Infection (WIfI).[34] The WIfI system merges existing classification systems, including the Infectious Diseases Society of America (IDSA) classification for diabetic foot infections, into a single concise system.[33] After grading each category, can then be clinically staged the affected limb to estimate risk of amputation at one year. WIfI system, in the intention of the authors, can predict amputation risk and is able to standardize outcome comparisons for accurate analysis of the increasing number of available therapies.[35]

The WIfI classification grades on a four-point scale (0-3) the three mentioned limb amputation risk factors, obtaining a score with the sum of the values:

WOUND – divided by the degree of tissue loss and anticipated level of intervention/amputation required for healing:

- GRADE 0: No ulcer, no gangrene. Ischemic rest pain.
- GRADE 1: Minimal tissue loss. No exposed bone (unless limited to distal phalanx). Intervention requires no more than a toe amputation or soft tissue covering. No gangrene.
- GRADE 2: Moderate tissue loss. Ulcer extends to tendon, joint, or bone. Localized gangrene to digits only. Intervention requires transmetatarsal amputation (TMA) or less.
- GRADE 3: Extensive tissue loss. Gangrene to forefoot, midfoot and hindfoot. Intervention requires more than a transmetatarsal amputation and/or complex soft tissue rearrangement.

ISCHEMIA – evaluates perfusion status to the foot using objective hemodynamic indices such as Ankle Brachial Index (ABI), Transcuteneous partial Pressure of Oxygen ($TcPO_2$), Pulse Volume

Recording, Skin Perfusion Pressure (SPP) or toe pressure (see below – Paragraph 1.5).[34]

- GRADE 0: No ischemia. ABI ≥ 0.80; toe pressure ≥ 60 mmHg.
- GRADE 1: Mild ischemia. ABI 0.60-0.79; toe pressure 40-59 mmHg.
- GRADE 2: Moderate ischemia. ABI 0.40-0.59; toe pressure 30-39 mmHg.
- GRADE 3: Severe ischemia. ABI ≤ 0.39; toe pressure < 30 mmHg.

FOOT INFECTION – this part derives from the IDSA and PEDIS clinical staging systems.[34]

- GRADE 0: No infection.
- GRADE 1: Superficial infection. Localized cellulitis ≤ 2 cm.
- GRADE 2: Moderate (deep) infection. Erythema > 2 cm. Abscess present or infection extends to joint or bone.
- GRADE 3: Severe infection. Local infection with Systemic Inflammatory Response Syndrome (SIRS).

After calculating the WIfI score, the classification system can assess the clinical stage (1–5) and is able to estimate the risk of major amputation at one year. The stages derive from a grid of 64 (4^3) theoretically possible outcomes assigned by a 12-member expert group using a Delphi consensus method.[34]

- STAGE 1: Amputation risk very low;
- STAGE 2: Amputation risk low;
- STAGE 3: Amputation risk moderate;
- STAGE 4: Amputation risk high;
- STAGE 5: Unsalvageable foot.

1.4 CRITICAL LIMB ISCHEMIA

For the first time, the expression “critical ischemia” appeared in the literature in 1982, used by Jamieson to describe lower limb ischemia of such severity that major amputation became necessary in the absence of successful revascularization.[36]

Critical Limb Ischemia (CLI) can be defined as the presence of ischemic rest pain symptoms for more than 2 weeks, ischemic ulceration or gangrene objectively attributable to the evolution of arterial occlusive disease associated with an ankle pressure of less than 50 mmHg or a toe pressure of less than 30 mmHg or ankle-brachial index less than 0.40 (see below - Paragraph 1.5).[20,37] CLI is one of the most severe clinical manifestations of PAD, is associated with a very poor prognosis and quality of life [38] and occurs in about 5-10% of patients with PAD. Arterial occlusions reduce the blood flow to muscles and tissues, the arterioles become maximally vasodilated and insensitive to vasodilator stimuli due to chronic exposure to vasorelaxing factors.[20] Under these conditions, the arterioles decrease wall thickness and cross-sectional area, leading to edema. Edema is worsened by the analgesic attitude of keeping the limb dependent and changes in function and structure of endothelial cells, platelet activation and leukocyte adhesion, which could bring to micro-thrombi formation in capillaries and generate an impaired oxygen exchange in the tissues.[39,40]

The most common symptoms are rest pain and ischemic ulcerations or gangrene in the toes or the forefoot (Categories 4, 5 and 6 of Rutherford Classification – Tab. 2; Stages III and IV of Fontaine Classification – Tab. 3). Cranley was one of the first authors to describe the ischemic rest pain, whose definition was a “pain that occurs in the toes or in the area of the metatarsal heads. Occasionally, it occurs in the foot proximal to the metatarsal heads. Elevation of the limb above or at the horizontal position aggravates the pain and pendency, to some degree at least,

brings relief".[41] Rest pain is often described as a burning sensation or coldness or paresthesia of sufficient intensity to interfere with sleep and in more than 90% of cases the toes are involved.

Becker et al. describe three degrees of ischemic rest pain:[42]

I. the pain starts at primo-decubitus and declines quickly — the patient can thus stay supine. Patients can experience numbness or tingling instead of pain;
II. the patient needs to angle his leg to relieve the pain;
III. the patient has to remain seated to relieve the pain.

It is important to note that rest pain depends on pain perception, which can be reduced or abolished in the case of sensory neuropathy (e.g. secondary to diabetes, ageing, or to ischemia itself). Since many of these patients are affected by diabetes and suffer of numbness, it could be difficult to define if these neuropathic symptoms are due to the ischemia alone.[43] Rest pain is enhanced by leg elevation because of reduction of gravitational pull of blood to the foot and the patient try to reduce the pain placing the limb dependent, usually dangling the foot of the side of the bed, increasing the tissue edema, worsening the ischemia because the increased tissue pressure exceeds capillary pressure.[20,42]

Ischemic ulcer and toe gangrene are more difficult to identify as lesions clearly attributable to end-stage PAD.[37,44,45]

Schematically, according to Becker et al., three situations can be encountered:[42]

- Skin lesion occurs spontaneously or after a minor trauma in presence of severe arterial insufficiency.
- Arterial insufficiency is moderate but severe enough to impair the healing process of any skin lesion (skin flow needed for wound healing is higher than skin flow necessary for baseline nutritional requirements of an intact skin).

- PAD is only an associated condition without evident causal relationship with skin lesions.

Even in the presence of toe gangrene, if perfusion pressure remains well above threshold values for critical ischemia, the potential morphological lesions of lower limb arteries identified by imaging techniques may be innocuous. In diabetic patients, foot lesions present numerous potential confounding factors, so in these subjects the relation between PAD and trophic changes should be documented with particular attention.

As previously mentioned, the clinical diagnosis of CLI should be confirmed by diagnostic tests (see below – Paragraph 1.5) in case of values of ankle blood pressure inferior to 50 mmHg, toe blood pressure inferior to 30 mmHg and ABI calculation inferior to 0.40.[20,37] Diabetic patients with ulcers could have values that exceed these criteria and still have distal blood flow inadequate for healing. According to Jamieson, the term CLI should not be applied to this kind of patients.[36]

Every year 500-1000 new cases of CLI per million of population are diagnosed in United Kingdom, with an estimated cost for the National Health System (NHS) of about 200 million pounds.[46] Patients affected by CLI, despite being submitted to the conventional surgical, endovascular and pharmacological treatments, have a poor prognosis in term of limb salvage and survival. According to TASC II guidelines,[1] after one year, about 30% of patients with CLI lose an inferior limb and 25% die. Inferior limb major amputations are related to 40% of mortality rate after 2 years.[47] It is estimated that in Italy, every year, 10000 limbs major amputations are performed, with high social and economic costs. A number between 25% and 40% of patients suffering from CLI is not eligible for either surgical nor endovascular treatment because of coexistent morbidities, high surgical risk or technical unfeasibility.

1.5 PAD AND CLI DIAGNOSIS

1.5.1 DIAGNOSTIC METHODS AND TESTS

The more common tests used to confirm and define the grade of PAD are:

- Ankle and toe blood pressure measurement.
- Ankle/Toe Brachial Index.
- Transcutaneous partial Oxygen Saturation.
- Skin Perfusion Pressure.
- The six minutes walking test.
- The treadmill test.

The **systolic ankle blood pressure measurement** evaluates the blood pressure at the level of the ankle when the patient is horizontal with the leg at the same height of the heart. Values of ankle pressure lower than 50 mmHg typically confirm the clinical diagnosis of CLI. **Systolic toe blood pressure measurement** evaluates the blood pressure at the level of the forefoot when the patient is horizontal with the leg at the same height of the heart. It is useful in case of distal arterial occlusions and in case of feet ulcers, also used to confirm the clinical diagnosis of CLI. According to TASC II, "ischemic rest pain most commonly occurs below an ankle pressure of 50 mmHg or a toe pressure of 30 mmHg".[1] Moreover, according to Wickström at al., toe pressure values lower than 30 mmHg increase significantly the risk of cardiovascular mortality and limb amputation.[48] For patients with ulcers or gangrene (Categories 5 and 6 of Rutherford Classification – Tab. 2; Stage IV of Fontaine Classification – Tab. 3), ankle or toe pressure values inferior to 70 mmHg or 50 mmHg, respectively, are suggestive of CLI presence.[1] Diabetic patients could have elevated values of ankle or toe pressure even in case of a low real perfusion flow. These false high-pressure readings may be generated by the extensive calcification of artery walls in the case of an

advanced diabetic pathology, a condition that prevents the sphygmomanometer to completely compress the artery.[20,36] Despite critical limb ischemia has been studied for decades, it is necessary to underline that, according to TASC II, "there is not complete consensus regarding the vascular hemodynamic parameters required to make diagnosis of CLI".[1,42]

The **Ankle Brachial Index (ABI)** is one of the first parameters to evaluate in patients suffering of peripheral vascular disease. It has been proposed in 1969 by Yao et al. [49] and it is the ratio between the higher of the dorsalis pedis or posterior tibial systolic pressure and the higher of the brachial arteries pressure measurements.[50] Bilateral arm pressure measurement is necessary to obtain accurate ABI values in case of coexistent subclavian or innominate artery stenosis, condition for which patients with PAD are at increased risk;[51,52,53] differences in blood pressure higher than 15/20 mmHg are suggestive for subclavian or innominate artery stenoses.

Calculation of the ABI is indicated as a first-line non-invasive test for screening and diagnosis of PAD (Recommendation Class I Level B) in the guidelines of European Society of Cardiology.[54]

ABI results are differentiated as follow:[55]

- non-compressible vessel for values > 1.40;
- normal for values between 1.00 and 1.40;
- borderline for values between 0.91 and 0.99;
- abnormal for values of 0.90 or less.

Values inferior to 0.40 are suggestive for CLI.[20,37]

These ranges of values are indicative and the ABI calculation is a useful tool, but has some limitations, since about 30% of patients with CLI have borderline or normal ABI, in case of isolated arterial disease below the knee. Moreover, ABI evaluates perfusion to the ankle and not

distal foot excluding from the evaluation the microvascular vessels in foot and plantar arch and the capillaries. For these reasons some investigators proposed to use the **Toe Brachial Index (TBI)** calculation as an additional perfusion tool to establish the diagnosis of PAD in the setting of non-compressible arteries or in suspected CLI. TBI is the ratio between the higher blood systolic pressure at the level of the forefoot and the higher of the brachial arteries pressure measurements. In contrast to the well-defined and evidence-based limits of the ABI, the diagnostic criteria for a pathologic TBI remain ambiguous. Although several guidelines and reviews of PAD diagnostics suggest a TBI < 0.70 as cut-off value, this recommendation is not strictly based on evidence.[56] Values of TBI inferior to 0.25 could increase significantly the risk of cardiovascular mortality and limb amputation.[48]

As previously mentioned, the possible presence of non-compressible or partially compressible arteries in diabetic patients may result in falsely elevated ABI and TBI calculations that are not significant.

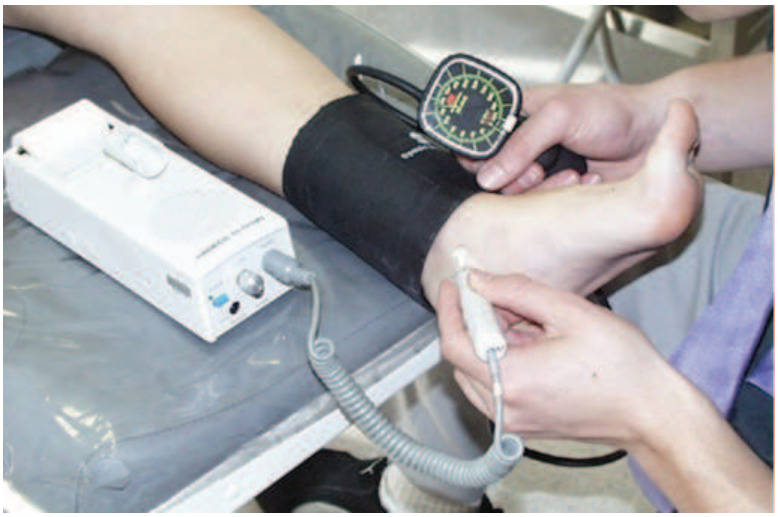


Photo 1 – ABI measurement.

The **Transcuteneous partial Pressure of Oxygen ($TcPO_2$)** measures the oxygen tension 1-2 mm deep in the skin evaluating local capillary perfusion.[57] The cut-off value considered indicative of ischemia is oxygen

saturation inferior to 40 mmHg. This measurement could be a good predictor of ischemic ulcers healing for cut-off values of less than 20 mmHg or more than 40 mmHg.[58] According to Nishio et al., $TcPO_2$ of 30 mmHg would be an appropriate value for wound healing after amputation and 20 mmHg could be a valid cut-off value to bring a decision for limb amputation.[59] $TcPO_2$ could also be used to assess hyperbaric oxygen response in patients with lower extremity wounds. Unfortunately, this tool has some limitations of efficacy: its measurements are affected by lower extremity edema and skin thickness and furthermore its sensibility is reduced by pulmonary disease, heart failure and high altitude. In addition, the duration of the procedure is quite long, usually 20 minutes for each measurement.

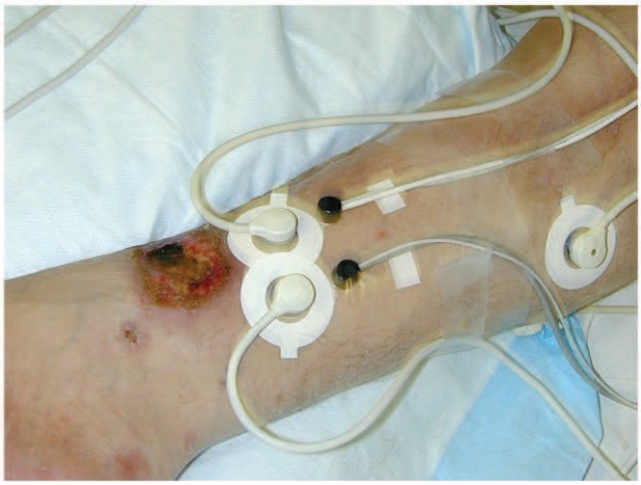


Photo 2 – $TcPO_2$ measurement.

The **Skin Perfusion Pressure (SPP)** is another method used to measure perfusion.[60] A cuff, inflated until no Doppler sounds are heard (using a LASER Doppler), is placed at the level of metatarsal bone, then it is slowly deflated and when any movement in the capillaries is detected, the pressure value is captured. According to Castronuovo et al., SPP measurement could be considered an objective, noninvasive method to use to diagnose CLI with approximately 80% accuracy in case of values lower than 30 mmHg.[58] A pressure value inferior to 40 mmHg in diabetic

patients is considered significant for a reduction of the chances of wound healing.[61]

Photo 3 – SPP measurement.

The **Six Minutes Walking Test (6MWT)** is a measure of the capability of patients to walk. It is a practical test that requires only a hallway long at least 30-40 meters, without any specific instrumental equipment or need of advanced training for technicians. This test measures the distance that a patient can quickly walk on a flat, hard surface in a period of 6 minutes (the 6 Minute Walking Distance - 6MWD).[63] During this simple test is possible to evaluate cardiac and pulmonary function, peripheral circulation, neuromuscular efficiency and muscle metabolism together with other systems involved during exercise. However, exercise is also one of the limitations of this test, because arterial disease is often associated with other pathologies and some patients cannot complete the walk, due to severe pulmonary or cardiac distress. The 6MWT gives a general view on the clinical conditions, but unfortunately is not able to provide specific information on the function of each individual organ and system involved in exercise and not even the mechanism of exercise limitation.

This test is useful in patients suffering of PAD because it is simple and can be performed in a hospital ward. Moreover, it gives information on

the clinical results of the therapy or the treatment with precise distance values, when repeated during the follow-up, even in elderly patients.[64]

The **Treadmill Test** is widely used to determine walking distance in case of Intermittent Claudication, but could also evaluate the effects of exercise on the heart and systemic circulation. This test consists on walking in place on a treadmill while monitoring the electrical activity of the heart. During the test the speed and incline of the treadmill increase every two minutes, according to a preprogrammed protocol, with precise speed and slope. The speed usually starts at 2 mph (miles per hour) corresponding to 3.2 km/h (kilometers per hour) and the inclination varies by 2% increments, from 0% to a maximum of 12%. The results show the response of the heart and systemic circulation to the stress of different levels of muscular exercise and point out the possible presence of muscular pain during increasingly higher exercise, defining at which level of muscular work Intermittent Claudication appears.[65,66]

The use of graded exercise testing instead of a constant load treadmill stress test is well established and accepted in clinical medicine in patients with PAD.[67] Graded testing has been used in clinical cardiology for decades to help evaluate cardiac ischemia and functional limitations in patients with coronary artery disease.[68,69]

Photo 4 – Treadmill test execution (L) and treadmill machine (R).

The importance of some of these tests is confirmed by the flow chart for the diagnosis of CLI (Fig. 3) presented in 2016 AHA/ACC (American Heart Association/American College of Cardiology) guidelines on the management of patients with lower extremity peripheral artery disease.[55]

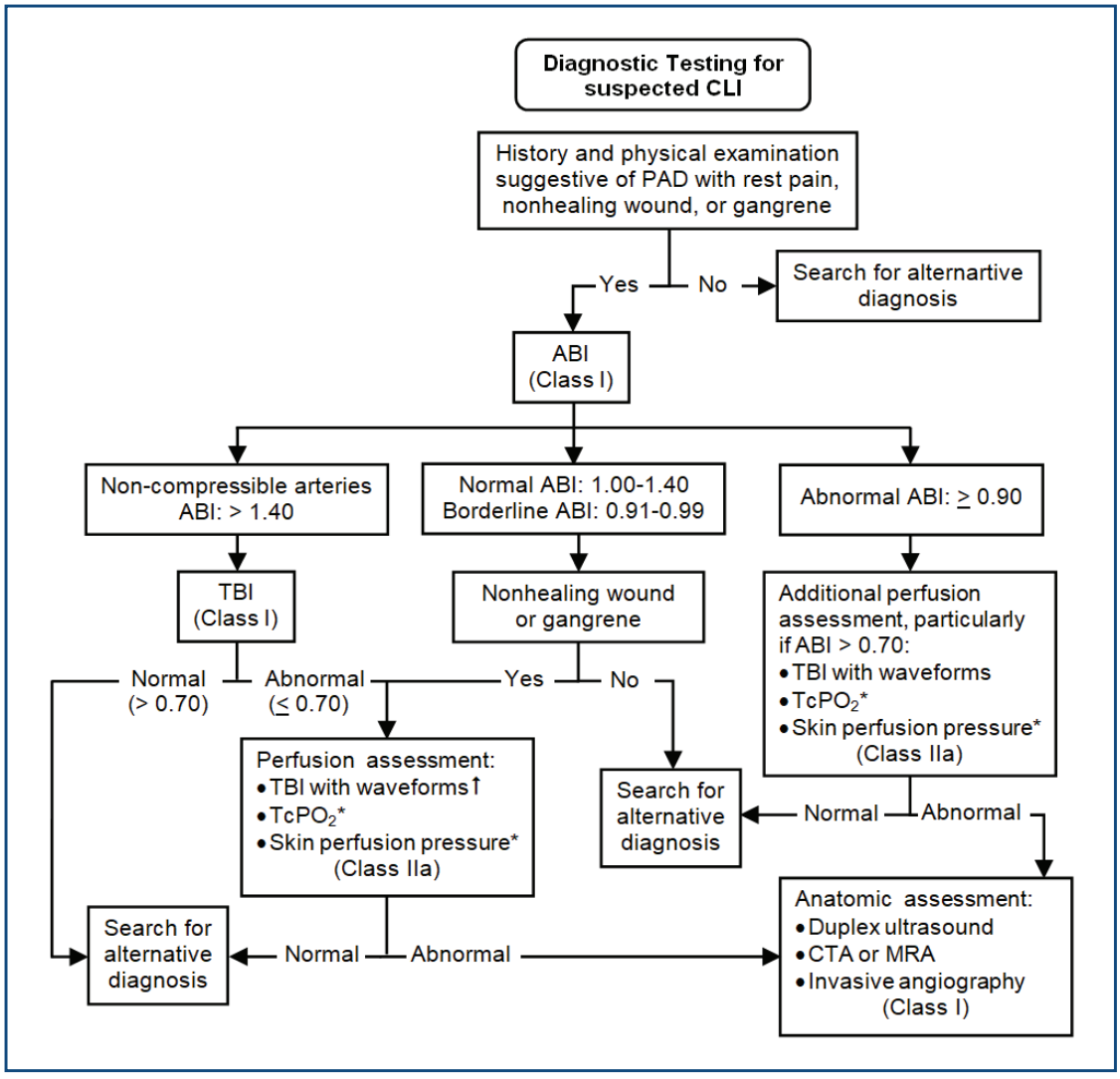


Figure 3 – Flow chart for diagnostic testing for suspected CLI, modified from Gerhard-Herman et al..[55]

1.5.2 PAIN EVALUATION

Pain is a central symptom in this pathology and is used to classify the level of progression of the disease and evaluate the efficacy of the treatment. Hence, it is necessary to have values as much as possible

precise and repeatable. One of the most utilized methods for classifying the pain is the Visual Analog Scale.

The pain **Visual Analog Scale (VAS)** [70] is a method to measure the intensity of pain and is a unidimensional, single-item scale. The scale usually starts from “no pain” (score of 0) and ends with “pain as bad as it could be” or “worst imaginable pain” (score of 10). The pain VAS is administered to the patient as a paper and pen and is self-completed placing a sign perpendicular to the VAS line at the level that represents the pain intensity subjectively perceived by the patient. No training is required and cannot be administered verbally or by phone, it is very easy and fast, usually takes less than 1 minute for the completion.

1.6 ATHEROSCLEROSIS AND PAD THERAPY

Peripheral Artery Disease is a pathology associated to elevated cardiovascular mortality rates due to the important role that atherosclerosis plays. For this reason, all patients with PAD require medical management of their cardiovascular disease. Atherosclerosis is a systemic pathology and the first step for treatment of lower extremity PAD is the modification and correction of atherosclerosis risk factors (see above – Paragraph 1.1, Tab. 1) to try to limit the progression of atherosclerotic process. The primary treatment goal in ameliorating symptoms of PAD, independently of the type of intervention (e.g. pharmacological therapy exercise training or direct revascularization), is to improve the patient’s ambulatory function and quality of life. The effectiveness of an intervention can be understood on the basis of improvements in peak and sub-maximal exercise capacity because these in turn are determinants of ambulatory function.[69]

The initial treatment should consist on smoking cessation, reducing sedentary living habits, increasing muscular exercise and cardiovascular activity, optimize the diet reducing fats assumption. To these behavioral modifications should be associated the specific medical treatment in case of hypertension, dyslipidemia, diabetes mellitus and an adequate antiplatelet therapy. Another important point is the exercise therapy that is one of the most beneficial interventions to preserve exercise tolerance and improve symptoms in PAD patients.[71]

1.6.1 PHARMACOLOGICAL THERAPY

Therapy for atherosclerosis is very important in patients suffering of PAD.

Statins are one of the first drug to consider in the treatment of PAD patients, they could reduce cardiovascular events and have been shown to improve walking distance and reduce the progression of symptomatic claudication.[72]

Antiplatelet therapy is a key component of secondary prevention measures in patients with diabetes and diagnosed atherosclerotic vascular disease and is shown that platelet antagonist reduce the risk of myocardial infarction (MI), stroke and vascular death in patients with high risk of vascular disease [73] and is now widely accepted among physicians for the treatment of cardiovascular diseases. The more diffused antiplatelet drugs are:

- **AcetilSalycilic Acid (ASA)** is one of the oldest drugs, widely diffused for cardiovascular diseases secondary prevention. Its antitrombotic effect is produced by irreversibly acetylating and inhibiting platelet cyclooxygenase (COX-1), a critical enzyme in the biosynthesis of TXA_2, which reducted release attenuates platelet activation and recruitment on the site of vascular injury.[74] At high

doses, ASA also inhibits COX-2, an isoform COX found in endothelial and inflammatory cells.[75] Its action helps prevent the formation of blood clots reducing the platelet aggregation, thus lowering the probability that a blood clot will form and block a narrowed artery. It is used also after peripheral arterial angioplasty, usually in combination with other antiplatelet drugs, to reduce the risk of restenosis.[76] ASA, as side effect, increases risk of extracranial (mainly major gastrointestinal events) and intracranial bleeding events,[77] so its use in primary prevention is controversial.[78,79,80]

- **Clopidogrel** (Plavix; Bristol-Meyers Squibb, New York, NY – USA) is a thienopyridine (a family including ticlopidine and prasugrel) whose action is an irreversible block of the $P2Y_{12}$, a key ADP receptor on the platelet surface, resulting in a selective inhibition of ADP-induced platelet aggregation. Clopidogrel is a prodrug (as prasugrel) that must be metabolized by the epatic cytochrome P450 (CYP) enzyme system to acquire activity, but its activation is inefficient because only 15% of absorbed drug undergoes activation through two oxidative steps.[74] Clopidogrel use is preferred over ticlopidine because of enhanced safety and tolerability. The major side effect of the thienopyridines is bleeding, even if in gastrointestinal region is rare.[81] Clopidogrel is approved by the FDA (United States Food and Drug Administration) for the secondary prevention of atherosclerotic vascular disease, including PAD.[82]

- **Ticagrelor** (Brilinta, Brilique, and Possia; Astra Zeneca, Cambridge, England - Great Britain) is a reversible inhibitor of $P2Y_{12}$. In contrast to thienopyridines, it does not require metabolic activation and has a more rapid onset and offset of action than clopidogrel.[74] Ticagrelor is approved by the FDA in the treatment of acute coronary syndromes.

The EUCLID study is a trial performed from 2014 to 2016 on 13,885 patients to study the effect of antiplatelet treatment in patients

with peripheral artery disease comparing Clopidogrel and Ticagrelor.[83,84] EUCLID trial investigates whether Ticagrelor monotherapy versus clopidogrel monotheraphy reduces major cardiovascular and limb-specific events in a representative population of PAD patients.[85] According to the results of this large international, multicenter, randomized, double-blind, parallel-group, end point driven trial is evident that these two drugs "are equally effective and tolerated in symptomatic PAD; however they are not optimal in the prevention of major adverse cardiac events and major adverse limb events, specifically in CLI patients"; about this the authors underline how an optimal antithombotic treatment has not yet been found and further studies are required in a carefully defined CLI population.[86]

ACE inhibitors also play a role in PAD therapy. According to Ahimastos et al., in a randomized study on 212 claudicants, ramipril treated patients enjoyed significant increase in pain-free walking time, total walking time and ankle-brachial index compared with placebo.[87]

1.6.1.1 PAD specific therapies

Specific therapies for PAD and in particular for intermittent claudication have been studied for decades and in the years have been proposed several drugs and supplements with different mechanisms of action including enhanced nitric oxide production (e.g. L-arginine), vasodilatatory effects (e.g. statins, buflomedil, prostaglandins, ACE inhibitors) and variations in tissue metabolism (e.g. levocarnitina, naftidrofuryl).

Nowadays, the most used and widely diffused drugs are pentoxifylline, cilostazol and naftidrofuryl.

- **Pentoxifylline** (Trental; Sanofi-Aventis, Paris – France) is a methylxanthine derivative and its main effect is thought to be the

improvement of oxygen delivery to tissues thanks to a rheolytic effect on flexibility and deformability of the wall of the red blood cells, reducing blood viscosity. Another supposed action is to increase fibrinogen levels and inhibit platelet aggregation. It is a safe, well tolerated and widely used drug despite the improvement in walking distance demonstrated during clinical trials have been modest. Pentoxifylline is FDA approved for use.[82]

- **Cilostazol** (Pletal; Otsuka Pharmaceutical Ltd., Tokyo – Japan) is a phosphodiesterase III inhibitor and increase the cyclic Adenosine MonoPhosphate (cAMP). The main effects are inhibitions of platelet aggregation, reduction of contraction and proliferation of smooth muscle cells, increase in HDL and reduction of triglycerides serum concentration. Its proved positive effect on intermittent claudication should probably be generated by a combination of previously mentioned effects, since the exact mechanism is still unknown. Although the cilostazol is generally well tolerated by patients, it has some adverse effects that reduce its use as headache, diarrhea and gastrointestinal discomfort; besides it is contraindicated in patients with congestive heart failure, a pathology unfortunately quite diffused between patients suffering of CLI. The American Association of Cardiovascular (AAC) and American Heart Association (AHA) guidelines recommend, in addition to traditional antiplatelet therapy, the use of cilostazol to increase the overall ambulation distance.[88] Cilostazol is FDA approved for use.[82]

- **Naftidrofuryl** (Naftidrofuryl oxalate) is a serotonin antagonist thought to improve aerobic metabolism. It acts as a selective antagonist of 5-HT2 receptors. In the United Kingdom National Institute for Health and Care Excellence (NICE) final guidance published in may 2011,[89] Naftidrofuryl oxalate is recommended as

an option for the treatment of patients who have Intermittent Claudication caused by PAD and for whom vasodilator therapy is considered appropriate after taking into account other treatment options. Naftidrofuryl is not yet approved by the FDA.[82]

1.6.2 OPEN SURGICAL AND ENDOVASCULAR THERAPY

A central role in the treatment of PAD is obviously played by invasive therapies whose goal is to ameliorate the blood flow to extremities, solving the stenosis of the vessel or overcoming the obstructed segment of artery with direct revascularization. Invasive procedures are nowadays the gold standard treatment of Peripheral Artery Disease, as they produce very good results, but, unfortunately, cannot always be performed, due to anatomical or clinical conditions. Decision making regarding revascularization is based on symptoms considering also the natural history of the patient's condition. For this reason, the treatment strategies could differ for patient with intermittent claudication compared to those with CLI, because the risk of limb loss is significantly higher in the second group. A crucial point is also whether or not a patient will experience a significant benefit from a technically successful procedure.[82] Graft patency or technical success not always directly correlate with clinical success, in fact 10% of patients who undergo surgical bypass to treat CLI do not show significant clinical improvements, despite the patency of the bypass graft at one year past the intervention.[90] In order to give a more patient-centered point of view, a working group of the Society of Vascular Surgery (SVS) elaborated the objective performance goals, guidelines developed specifically for comparative evaluations of treatments of CLI, with endpoints that include freedom from major amputation and re-intervention.[91,92] Patients suffering from CLI are condemned to a course of prolonged recovery, multiple re-operations and hospital admissions.[93] In these patients, clinically and technically oriented outcomes not always represent

successful results in the treatment. The quality of life (QoL) is also very important: the long-term graft and arterial patency are very important to achieve results in term of QoL, wound healing and relief of chronic ischemic pain.[82] Revascularization is not appropriate in every case, but there is clear evidence that QoL improves with revascularization treatments, in patients affected by PAD with intermittent claudication.[94,95,96,97] One of the largest studies performed to evaluate QoL in patients with CLI who underwent infrainguinal bypass is the PRoject of Ex-vivo Vein graft ENgineering via Transfection (PREVENT III) trial.[98] This and other studies demonstrated significant improvement in QoL after bypass intervention and a direct correlation between QoL and graft patency.[96,97,98,99,100,101] A large number of patients need more than six months to heal [102] so long term graft and arterial patency is fundamental for good outcomes. Furthermore, patients who saved their limbs and have a reduction of pain usually could be discharged from hospitalization, their wound care could be simplified and the QoL may improve for them and their families.

Invasive therapies of direct revascularization could be divided in two large categories: open surgical interventions and endovascular procedures.

Open surgical intervention is performed since the 1950s, has good results at long-term follow-up. Revascularization could be obtained with two main techniques: EndArterectomy (EA) and ByPass (BP). In the first case the blood flow to distal artery is restored removing the atherosclerotic plaque responsible of the stenosis or the obstruction of the vessel; usually endarterectomy is used in case of not too long atherosclerotic lesions. In some cases, it could be necessary to enlarge the artery after endarterctomy, performing an angioplasty using a patch (synthetic or autologous). The bypass procedure consists on using a tube graft that is attached to the artery with anastomosis proximally and

distally to the obstruction, in order to bridge the occluded segment of the artery. The graft prosthesis could be synthetic, usually made of polytetrafluoroethylene (PTFE) or polyester (Polyethylene terephthalate – PET: Dacron, Terylene or Lavsan), or autologous (generally the great saphenous vein, in situ or reversed) and could bridge also long segments of obstructed artery (e.g. below-knee femoropopliteal bypasses). Usually the use of an autologous graft is the first choice in presence of infected ulcers. Popliteal below-knee bypasses have good clinical results with rates from 50% to 74% of primary or secondary patency at five years.[103]

Compared to the open surgical revascularization, the **endovascular procedure** is a more recent technique and has rapidly developed in the last decades. These procedures mainly consist of dilatation of the stenosis (Percutaneous Transluminal Angioplasty - PTA) or recanalization of obstructed arteries from the inside of the vessel with an endovascular approach using a remote vascular access, usually percutaneal without direct exposure of the artery. The stenosis commonly is dilated using specific balloons inserted in the artery; some of the balloons can elute drugs and in some cases a stent could be positioned in order to reduce the risk of restenosis. The recanalization is performed with many different techniques using different tools (catheters for crossing techniques) and methods (e.g. sub-intimal or endo-luminal technique, excisional, rotational or excimer LASER debulking techniques, cutting-balloon technique, crioplasty technique, et cetera) and consists of reopening an occluded artery, overtaking the obstructed segment, dilating it with a balloon, and sometimes positioning a stent (bare metal stent or covered stent).[104,105,106] The stent could be made of different materials, the most common are stainless steel, cobalt-chromium alloy, platinum chromium alloy and nichel-titanium alloy (Nitinol - Nichel Titanium Naval Ordinance Laboratory) and could be covered with a fabric (covered stent) in case of need to repair vascular wall lesions (e.g. local dissections). Balloons and stents could be coated with drugs, named respectively DEB (Drug Eluting

Balloon) or DCB (Drug Coated Balloon) and DES (Drug Eluting Stent), and slowly release the anti-cell proliferation drug (e.g. Sirolimus and Paclitaxel) in order to reduce the risk of restenosis.[107,108,109,110,111] For recanalization of long segments of vessel some authors suggest the use of a stent-graft in the attempt to duplicate open prosthetic femoropopliteal bypass and reduce in-stent restenosis risk.[112] In case of treatment of plaques at high risk of embolization and of patients with poor runoff or undergoing thrombolytic therapy is suggested the use of embolic protection devices, in order to reduce the risk of microembolism.[113,114,115] Endovascular procedures are less invasive compared to traditional open surgery, but, to be performed, they require the use of X-rays and, almost always, the administration of a iodinated contrast medium (in some cases replaced by CO_2), which poses the risk of allergic reactions and above all is nephrotoxic, so that not always its use is safe (in particular in patients with renal failure, disease often associated to PAD). Clinical results of angioplasty have been studied since the first years of this century [116] and could be considered good even in CLI patients with occluded infrapopliteal arteries; in particular Ingle et al. reported a technical success rate of 86% and a limb salvage rate of 94% at three years follow-up for subintimal technique,[117] whereas Tartari et al. reported an 83% success rate and an 84% 24-month limb salvage rate, moreover major amputation was significantly correlated to angioplasty technical failure and long segment occlusions.[118]

The endovascular technique could be used in alternative to open surgery, but in many cases the two approaches are complementary [82] in the so-called hybrid therapeutic solutions that are becoming more and more frequent in vascular surgery.

1.6.3 TREATMENT RECOMMENDATIONS

According to the TASC II, the recommendation for treatment are endovascular procedure for type A and open surgery for type D lesions.[1]

For intermediate types of lesion (B and C), evidence is not sufficient to definitely prefer one treatment over the other. Hence, these lesions could be treated using either endovascular or surgical approach, and the choice should be made considering the clinical scenario. Some evidences suggest that endovascular reconstruction may be beneficial in patients with extensive disease (e.g. type C or D of TASC) at imminent risk of limb loss but not candidate for surgical reconstruction.[119,120,121,122]

According to 2016 AHA/ACC (American Heart Association/American College of Cardiology) guidelines on the management of patients with lower extremity peripheral artery disease, the main recommendations for CLI treatment are indicated in the Tables 4, 5, 6 and 7.[55]

COR	LOE	Recommendation
I	B-NR	In patients with CLI, revascularization should be performed when possible to minimize tissue loss.
I	C-EO	An evaluation for revascularization options should be performed by an interdisciplinary care team before amputation in the patient with CLI.

Table 4 – Recommendations for revascularization for CLI. COR: Class of Recommendation, LOE: Level of Evidence. From Gerhard-Herman et al..[55]

COR	LOE	Recommendation
I	B-R	Endovascular procedures are recommended to establish in-line blood flow to the foot in patients with nonhealing wounds or gangrene.
IIa	C-LD	A staged approach to endovascular procedures is reasonable in patients with ischemic rest pain.
IIa	B-R	Evaluation of lesion characteristics can be useful in selecting the endovascular approach for CLI.
IIb	B-NR	Use of angiosome-directed endovascular therapy may be reasonable for patients with CLI and nonhealing wounds or gangrene.

Table 5 – Recommendations for endovascular revascularization for CLI. COR: Class of Recommendation, LOE: Level of Evidence. From Gerhard-Herman et al..[55]

COR	LOE	Recommendation
I	A	When surgery is performed for CLI, bypass to the popliteal or infrapopliteal arteries (e.g. tibial, pedal) should be constructed with suitable autogenous vein.
I	C-LD	Surgical procedures are recommended to establish in-line blood flow to the foot in patients with nonhealing wounds or gangrene.
IIa	B-NR	In patients with CLI for whom endovascular revascularization has failed and a suitable autogenous vein is not available, prosthetic material can be effective for bypass to the below-knee popliteal and tibial arteries.
IIa	C-LD	A staged approach to surgical procedures is reasonable in patients with ischemic rest pain.

Table 6 – Recommendations for surgical revascularization for CLI. COR: Class of Recommendation, LOE: Level of Evidence. From Gerhard-Herman et al..[525]

COR	LOE	Recommendation
I	B-NR	An interdisciplinary care team should evaluate and provide comprehensive care for patients with CLI and tissue lost to achieve complete wound healing and a functional foot.
I	C-LD	In patients with CLI, wound care after revascularization should be performed with the goal of complete wound healing.
IIb	B-NR	In patients with CLI, intermitted pneumatic compression (arterial pump) devices may be considered to augment wound healing and/or ameliorate severe ischemic rest pain.
IIb	C-LD	In patients with CLI, the effectiveness of hyperbaric oxygen therapy for wound healing is unknown.
III: No Benefit	B-R	Prostanoids are not indicated in patients with CLI.

Table 7 – Recommendations for wound healing therapies for CLI. COR: Class of Recommendation, LOE: Level of Evidence. From Gerhard-Herman et al..[55]

1.7 TISSUES REGENERATION

The tissue regeneration process consists principally of two phases: in the first phase inflammation mediators and neurotransmitters are released; this is followed by a second phase in which the precursors of progenitors cells, called on the site of the lesion by the mediators, generate new vessels. This angiogenic process usually lasts from 4 to 8 weeks, with a time variability related also to the extension of the ischemic damage. The angiogenesis creates new collateral vessels increasing the tissue perfusion, the oxygen and nutrients provision to the cells helping the healing of ulcers, reducing the ischemia related symptoms improving the pain control. All these conditions maybe ameliorate clinical conditions and quality of life of the patients, possibly reducing the risk of infections and gangrene, and perhaps helping to increase the possibilities of limb salvage.

1.8 ANGIOGENESIS AND VASCULOGENESIS

The term angiogenesis means the creation of new vessels and capillaries starting from pre-existing vessels, thanks to migration and proliferation of endothelial cells. Angiogenesis usually occurs during embryogenesis, but the term vasculogenesis (or so-called postnatal angiogenesis) describes the *de novo* development of new vessels from endothelial progenitor cells with the eventual transformation to mature endothelial cells, vascular smooth muscle cells and pericytes.[123]

Generation of new vessels starts from endothelial progenitor cells (EPC) of the bone marrow. During this process, these cells express superficial stem markers as CD133, VEGFR2 and CD34, and are able to differentiate in Endothelial Cells (EC) with co-expression of other superficial markers like CD31, von Villebrandt Factor (vWF) or CD144

(VE Cadherin). This event can occur in pathologic conditions, as for example myocardial infarction and CLI, situations in which has been observed a migration of endothelial progenitor cells from the bone marrow to the ischemic tissues.[124,125]

Direct injection of these cells in the damaged tissues could lead to a faster and a more effective generation of new vessels.[126,127] Experimental studies of vascular regeneration performed with animal models demonstrated that only the cells $CD34^+$ are present in the new generated vessels.[128]

In the past two decades, numerous small-scale trials using several different growth factors have demonstrated angiogenic potential in humans suffering from CLI. Among them are the Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF) families, and the transcription factor, Hypoxia Inducible Factor 1 (HIF-1).[123]

1.8.1 GRANULOCYTE COLONY STIMULATING FACTOR

Some studies demonstrated that the Granulocyte Colony Stimulating Factor (G-CSF) promotes mobilization of the stem cells from the bone marrow, including EPC, to join the damaged ischemic tissues and induce vasculogenesis, and locally increase the blood flow.[129,130] Other authors have shown that either the Bone Marrow MonoNuclear Cells (BM-MNC) or the Peripheral Blood MonoNuclear Cells (PB-MNC) that are obtained after post-mobilization apheresis and are induced with Granulocyte Colony Stimulating Factor (G-CSF) administration, are effective in the therapy of CLI. Patients receiving a number of $CD34^+$ cells higher than 1×10^6/Kg have a better outcome: the Amputation Free Survival (AFS) rate obtained is 61% in patients who received a number of PB-MNC superior to 1×10^6 cells/Kg, against an AFS of 37% for patients with number of injected cells inferior to that value.[131] A direct inoculation of EPC in the

site subject to ischemia results in a faster and more efficient creation of new vessels. It was also demonstrated an important role of stromal stem cells, the so-called Mesenchimal Stem Cells (MSC), in angiogenesis and vasculogenesis processes.[132] On these bases, the two sub-populations of Mesenchimal Stem Cells, the hematopoietic $CD34^+$ and the stromal $CD34^-$ cells may have a synergistic action on vasculogenic process. The stromal $CD34^-$ cells secrete pro-angiogenic factors as Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (b-FGF) and Angiopoietin 1, but have low expression of their receptors (VEGFR2, FGFR and Tie-2 respectively), that are instead highly expressed on the membrane of the hematopoietic $CD34^+$ cells.[133,134]

1.8.2 CELLS' COLLECTION METHODS

Two methods are mainly used to obtain stem cells:

1. ***direct bone marrow suction from iliac bone***;

2. ***white blood cells apheresis after mobilization of bone marrow hematopoietic precursors ($CD34^+$)***.

In both methods a concentration of cells is obtained after centrifugation of the sample: Bone Marrow MonoNuclear Cells (BM-MNC) in the first case and Peripheral Blood MonoNuclear Cells (PB-MNC) in the second. Each method has advantages and disadvantages.

The **bone marrow direct suction** has these advantages:

- high quantity of hematopoietic stem and mesenchymal cells;
- reduced number of red blood cells and lymphocytes;

and these disadvantages:

- surgical invasive procedure;

- general anesthesia needed;
- pain.

The **apheresis after mobilization** (Ph. 5) has these advantages:

- easy procedure;
- repeatable (multiple apheresis);

and these disadvantages:

- pre-treatment for stimulation (some days of hospitalization);
- possible need of Central Venous Catheter (CVC);
- can be obtained only hematopoietic cells.

Photo 5 – Apheresis system.

The percentage of cells $CD34^+$ contained in the bone marrow is 0.5-1%. The percentage of cells $CD34^+$ contained in peripheral blood is 0.05%, but after stimulation with G-CSF could be reached values of 0.5-3%.

1.9 HYPOXIA AND INFLAMMATION

Hypoxia and inflammation are some of the conditions that mainly lead to release and activation of pro-angiogenic factors. Inflammation is associated to ischemia in which the tissue degradation leads to the activation of macrophages, fibroblasts, monocytes, platelets and endothelial cells and to the consequential release of inflammation mediators as cytokines like interleukyns (IL-1β and IL-6) and Tumor Necrosis Factor (TNFα) that induce production of proteins as Serum Ceruloplasmin (Cr) and C-Reactive Protein (CRP). Ceruloplasmin belongs to the α2-glycoprotein fraction of plasma proteins. It is synthesized in the liver and has been known for long time as the acute phase reactant and protein involved in copper transport. Ceruloplasmin has both prooxidant and antioxidant properties and may be clinically relevant in chronic heart failure (HF). The Ceruloplasmin plasmatic concentration increases in case of chronic HF, necrosis, ischemia or after surgical interventions and one of its main known effects is to protect from the negative action of Reactive Oxygen Species (ROS).[135] In case of CLI, the dose and the action of Ceruloplasmin is directly related to the concentration of CRP. Another important aspect pertains to the chemoattractive activity of the injected cells to the site of tissue damage and the Chemokyne SFD-1 is one of the factors with high attractive action on the stem cells which express its receptor (CXCR4) on their surface.[136]

In the last years the use of autologous cells injections as a therapy of chronic ischemic pathologies, either chronic limb ischemia or ischemic cardiomyopathy,[137] has been proposed by different research groups.[138,139] Tateishi et al. in 2002 reported the first clinical evidence of improvements in rest pain and pain-free walking distance obtained with intramuscular injections of Bone Marrow MonoNuclear Cells (BM-MNC) in patients affected by CLI.[140] From this beginning, many other clinical studies have been proposed and conducted on autologous cell therapies,

including BM-MNC, Granulocyte Colony-Stimulating Factor (G-CSF)-Mobilized Peripheral Blood MonoNuclear Cells (MPB-MNC).[141,142] In the last years, the treatment with MPB-MNC or BM-MNC transplantation of patients suffering from CLI has become more and more diffused; data from the literature indicate that both procedural strategies resulted beneficial for patients in terms of reduction/abolition of rest pain, increasing of Ankle Brachial Index (ABI) and Transcutaneous Pressure of Oxygen ($TcPO_2$) values, skin temperature, pain-free walking distance and quality of life.[143,144,145]

Table 8 shows some of the most interesting studies concerning the treatment of PAD (and CLI) with autologous cells therapy.

Table 8 – Cell-based therapy – Human trials, redrawn from Losordo et al..[146] ABI: Ankle-brachial index, TBI: toe-brachial index, $TcPO_2$: transcutaneous pressure of oxygen, BM: bone marrow, BM-MNC: bone marrow-derived mononuclear cells, BM-MNCs/MSCs: bone marrow-derived mononuclear/mesenchymal stem cells, CLI: critical limb ischemia, EPCs: endothelial progenitor cells, IA: intra-arterial, IM: intramuscular, SC: subcutaneous, NS: non significant, PAD: peripheral arterial disease, PGE_1: prostaglandin E_1, QoL: quality of life, rhGM-CSF: recombinant human granulocyte-macrophage colony-stimulanting factor.

Trial (Phase)	Therapeutic Intervention	Study Group	Results
EPCS			
van Royen (2005) START (STimulation of ARTeriogenesis) trial (II)	SC rhGM-CSF Control: placebo	40 patients (20 rhGM-CSF, 20 placebo)	Increased microcirculatory flow in treatment group No improvement in ABI or exercise tolerance over placebo group
Kawamoto (2009) EPOCH-CLI (I/IIa)	IM EPCs ($CD34^+$) Control: none	17 patients (6 low dose, 8 middle dose, 3 high dose)	Improvements in pain, TBI, $TcPO_2$, total/pain-free walking distance, ulcer healing
Burt (2010) (I)	IM EPCs ($CD133^+$) Control: none	9 patients	Improvements in amputation rate, QoL, trend toward improvement in pain and exercise capacity (NS). No improvement in ABI
BM-MNC/MSC			
Huang (2005) (I)	PB-MNCs Control: IV PGE_1	28 patients (14 PB-MNCs, 14 PGE_1)	Improvements in limb pain, ulcer healing, ABI, TBI, laser Doppler blood perfusion Angiographic evidence of new vessel development
Powell (2011) RESTORE-CLI (II)	IM BM-MNCs Control: placebo	46 patients (32 BM-MNCs, 14 placebo)	Improvements in time to treatment failure, amputation-free survival Trend toward improved wound healing (NS)
Walter (2011) PROVASA (II)	IA BM-MNCs Control: placebo	40 patients (19 BM-MNCs, 21 placebo), then both groups received BM-MNCs at 3 months	Improvements in ulcer healing and rest pain, associated with repeated administration and BM-MNC number/functionality No significant improvement ABI, limb salvage, amputation-free survival
Idei (2011)	IM BM-MNCs Control: placebo	97 patients BM-MNCs: 25 atherosclerotic PAD, 26 Buerger's disease Placebo: 30 atherosclerotic PAD, 16 Buerger's disease	In Buerger's disease, improvements in ABI and $TcPO_2$ at 1 month and 3 years In PAD, improvements in ABI, $TcPO_2$ at 1 month, with gradual return to baseline
Lasala (2012) (II)	IM BM-MNCs/MSCs Control: placebo	26 patients (BM-MNCs/MSCs in more ischemic leg, placebo in less ischemic leg)	Improvements in exercise tolerance, ABI; improved perfusion evaluated with technetium Tc 99m tetrofosmin scintigraphy
Losordo (2012) (I)	IM $CD34^+$ cells high/low dose Control: placebo	28 patients ($CD34^+$ cells high/low dose, placebo)	Increased amputation-free survival, further increase in high-dose group

In the literature, several studies seem to demonstrate the efficacy of treatment of critical limb ischemia with direct inoculation of autologue cells.[147,148] Summarizing, there are some crucial points, learned from the literature, to consider in a research study like this:

A. Only cells $CD34^+$ are involved in generation of new vessels.[128]

B. Patients receiving a number of cells $CD34^+$ superior to 1×10^6/kg have a better outcome considering ***Overall Survival (OS)*** and ***Amputation Free Survival (AFS)***.[131]

C. A fundamental role of Mesenchimal Stem Cells (MSC) was demonstrated in the stimulation of angiogenic and vasculogenic effects.[132]

D. We could suppose a complementary action between two bone marrow cells populations (hematopoietic $CD34^+$ and stromal $CD34^-$) in the process of generation of new vessels. [133, 134]

2 AIM OF THE STUDY

The aim of this research project is to evaluate the feasibility and the outcome of inoculation of mononuclear peripheral cells at high content of stem cells $CD34^+$ (at least 2 x 10^6/kg of bodyweight) as treatment of critical limb ischemia in order to reduce the risk of major amputation of the inferior limbs.

3 MATERIALS AND METHODS

Every year about 300 patients suffering of PAD at various levels are evaluated in the Vascular Surgery Unit of S. Croce e Carle Hospital of Cuneo (Italy), either in vascular laboratories or in emergency rooms: about 140 patients/year are at Fontaine stage II, 100/year are at stage III and 70/year are at stage IV. To all symptomatic subjects is proposed a medical therapy with statins and antiplatelet drugs (see Introduction section). About 150 endovascular and 80 surgical procedures (in about 10% of the cases, combined) are performed on these PAD patients every year. About 1/3 of these PAD patients are CLI cases, treated with endovascular procedures in 55% of cases and surgical interventions in 25% (about 15% are submitted to both kind of procedures, combined or staged); more or less 20% of CLI patients every year are technically or clinically unsuitable for none of these procedures, so they are treated just with medical therapy (Tab. 9).

PAD CASES PER YEAR	300
GENDER M/F	80/20
Fontaine stage: II/III/IV	140/100/70
Medical treatment	300
Endovascular treatment	150
Surgical treatment	80
CLI cases	33%
Endovascular treatment	55% of CLI
Surgical treatment	25% of CLI
Unsuitable for treatment	20% of CLI

Table 9 – Vascular Unit year volume of PAD and CLI cases.

3.1 PATIENTS' SELECTION

The selection of the patients to include in this study project has been carried out evaluating all consecutive patients affected by PAD, at the stage of Critical Limb Ischemia, treated in the Vascular Surgery Unit of S. Croce e Carle Hospital of Cuneo (Italy) from November 2014 to September 2017. To every subject diagnosed with Critical Limb Ischemia during the clinical daily vascular activity and suitable to be recruited according to inclusion and exclusion criteria (see below) was proposed to participate in the present study. All the patients who agreed to be part of the study had been recruited consecutively, until the required sample size had been achieved. In order to be included in the protocol of implantation of Mobilized Peripheral Blood MonoNuclear Cells (MPB-MNC) each patient has to match all the following INCLUSION CRITERIA:

- patients unresponsive after at least 6 weeks of pharmacological therapy or unsatisfactory clinical course after revascularization with bypass surgery or Percutaneous Transluminal Angioplasty (PTA);
- no further possibility of medical, surgical or endovascular therapy;
- more than 18 years old and ability of understanding characteristics and purposes of the protocol;
- signature of the informed consent.

Patients are excluded from the recruitment in case they meet even one of these EXCLUSION CRITERIA:

- life expectancy shorter than 6 months;
- CLI with widespread necrosis requiring prompt amputation;
- chronic renal failure requiring dialysis;
- oncohemathologic pathologies or concomitant not neoplastic dysfunctions of the bone marrow;
- unbalanced diabetes mellitus (Hb A1C > 8%);
- if considered unsuitable for anesthesia procedure due to severe hepatopathy or respiratory dysfunctions;

- severe ischemic heart disease (Ejection Fraction - EF < 30%) or history of Acute Myocardial Infarction (AMI) within 6 months before the scheduled beginning of the procedure;
- ECOG – Performance Status Grade > 3 (http://ecog.dfci.harvard.edu/general/perf_stat.html).

ECOG Performance Status [149]

ECOG Performance Status (ECOG PS) is a set of criteria and scales used by physicians to establish the progression of a disease in a patient, trying to understand how the disease limits his daily living abilities and defining a prognosis (Tab. 10).

Grade	ECOG
0	Totally active, able to carry on all pre-disease performance without restriction.
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work.
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Deceased.

Table 10 – ECOG Performance Status scale. From Oken et. al..[149]

Figure 4 shows the flow chart of the recruitment phase of the study which involves Vascular Surgeon and Hematologist.

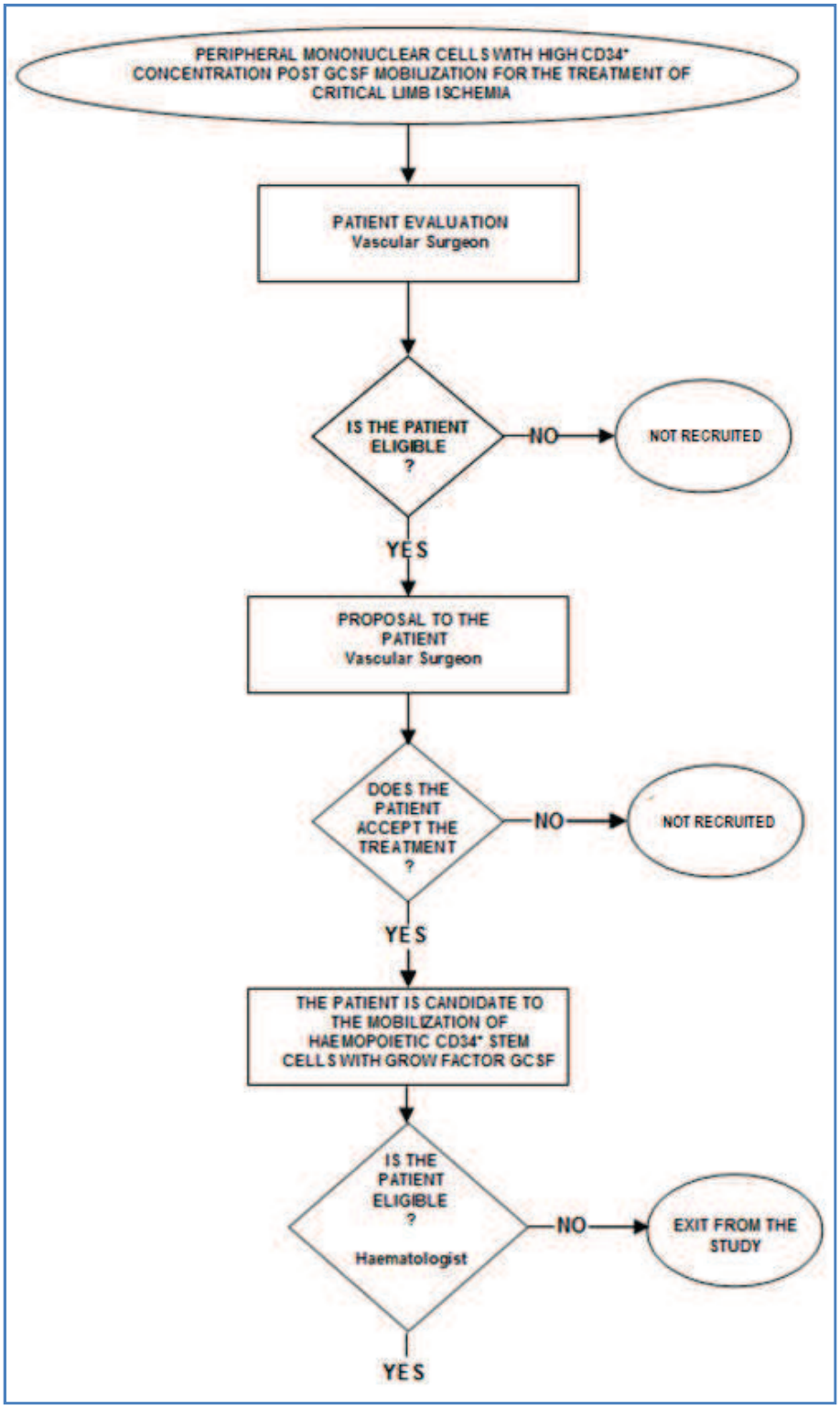


Figure 4 – Flow chart of the recruitment phase.

3.2 DATA COLLECTON

The initial steps after recruitment phase are:

1. Collection of demographic data: date of birth, sex.
2. Collection of clinical data: smoking habit, primary diseases, morbidities, presence of lower limb gangrene.
3. Collection of medical and therapeutic history: pharmacological therapy, previous lower limb amputations, surgical and endovascular previous treatments.
4. Clinical and instrumental examination: VAS score, ABI, $TcPO_2$, Fontaine stage, Wagner grade (in case of ulcers).
5. Each patient is then submitted to an angiographic exam before starting the protocol to have an objective iconographic comparison base.

The patients are divided into two sub-groups in relation to their age with a cut-off at 60 years old. This division is made to evaluate if there are any significant differences in mobilization capability after stimulation related to the age of the patient. The value of 60 years has been chosen, instead of a cut-off at higher age, to clearly separate young patients from the older ones (considering that PAD usually not affects very young people), with the goal of emphasizing as much as possible the possible differences in bone marrow functionality and reactivity to stimulation.

The collected data are inserted in a clinical personal form used for both initial evaluation and follow-up.

Other data collected are: number of $CD34^+$ cells, number of endothelial progenitors cells (EPC).

In another personal form are collected the technical treatment information: dose of G-CSF, apheresis and implantation details.

3.3 CLINICAL CHOICES' INDIPENDENCE

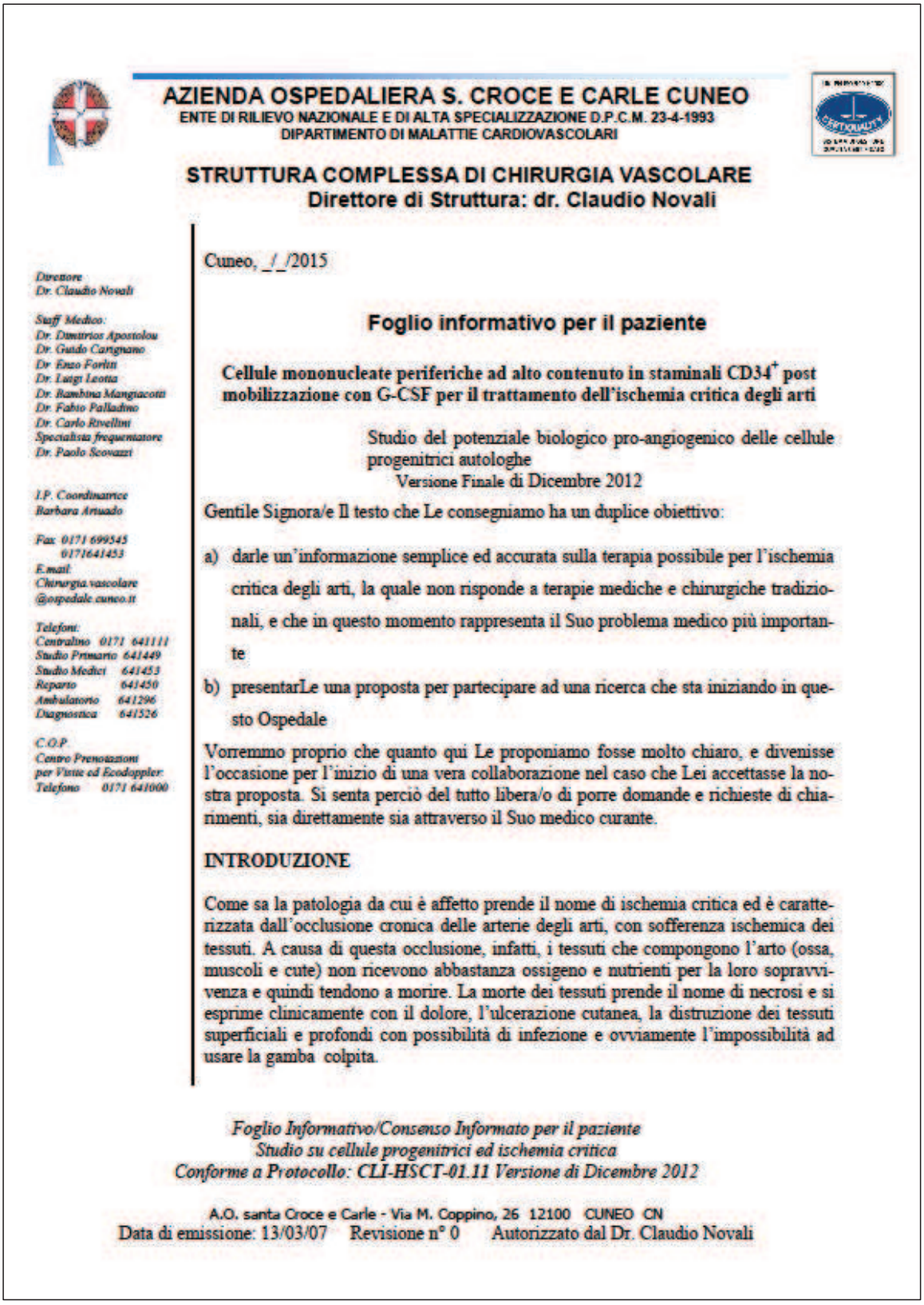
The surgeon's independence on clinical choices is preserved not to have influences on the Amputation Free Survival rates: the number of $CD34^+$ cells of every single patient is unknown to the vascular surgeon until the end of the follow-up period (6 months), so that any decision about major or minor amputations' execution could be taken only on clinical bases without any interference associated with the knowledge of the laboratory values considered in this study to reach the end points.

3.4 ETHICAL APPROVAL

This protocol has been previously approved by the ethical review committee of the S. Croce e Carle Hospital (Cuneo – Italy) where the patients are collected, recruited, informed, treated and followed.

3.5 INFORMED CONSENT

A detailed informed consent form is administered to each patient (Fig. 5) and to his General Practitioner (Fig. 6), is written in Italian language and must be signed before completing the recruitment of the patient. Moreover, an informed form on the treatment of personal sensitive data is given to each patient and an authorization for the clinical use of the data obtained is requested before starting the program.



AZIENDA OSPEDALIERA S. CROCE E CARLE CUNEO
ENTE DI RILIEVO NAZIONALE E DI ALTA SPECIALIZZAZIONE D.P.C.M. 23-4-1993
DIPARTIMENTO DI MALATTIE CARDIOVASCOLARI

STRUTTURA COMPLESSA DI CHIRURGIA VASCOLARE
Direttore di Struttura: dr. Claudio Novali

Direttore
Dr. Claudio Novali

Staff Medico:
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Dr. Guido Carignano
Dr. Enzo Forlin
Dr. Luigi Leotta
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C.O.P.
Centro Prenotazioni
per Visite ed Ecodoppler:
Telefono 0171 641000

Cuneo, _/_/2015

Foglio informativo per il paziente

Cellule mononucleate periferiche ad alto contenuto in staminali CD34$^+$ post mobilizzazione con G-CSF per il trattamento dell'ischemia critica degli arti

Studio del potenziale biologico pro-angiogenico delle cellule progenitrici autologhe
Versione Finale di Dicembre 2012

Gentile Signora/e Il testo che Le consegniamo ha un duplice obiettivo:

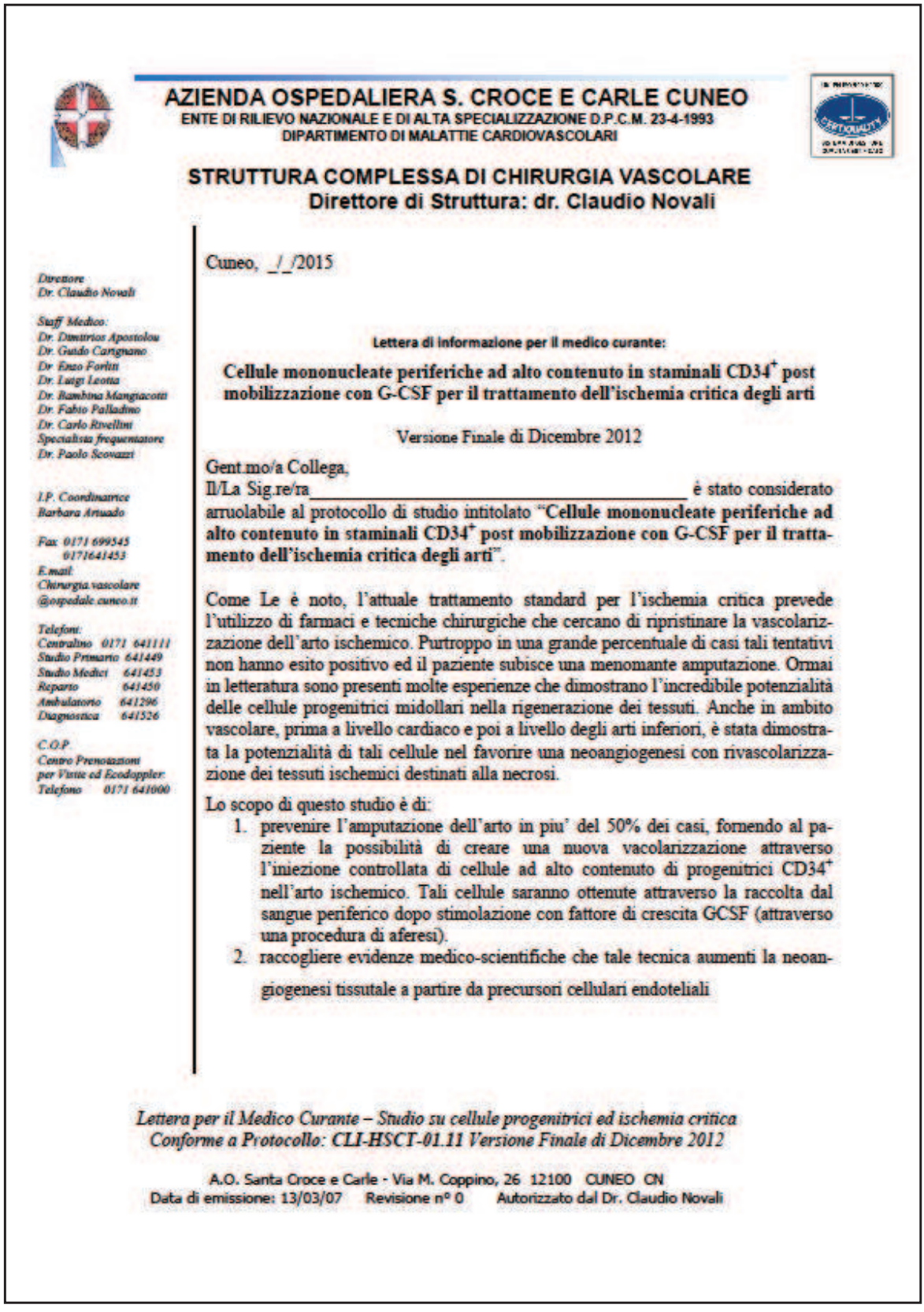
a) darle un'informazione semplice ed accurata sulla terapia possibile per l'ischemia critica degli arti, la quale non risponde a terapie mediche e chirurgiche tradizionali, e che in questo momento rappresenta il Suo problema medico più importante
b) presentarLe una proposta per partecipare ad una ricerca che sta iniziando in questo Ospedale

Vorremmo proprio che quanto qui Le proponiamo fosse molto chiaro, e divenisse l'occasione per l'inizio di una vera collaborazione nel caso che Lei accettasse la nostra proposta. Si senta perciò del tutto libera/o di porre domande e richieste di chiarimenti, sia direttamente sia attraverso il Suo medico curante.

INTRODUZIONE

Come sa la patologia da cui è affetto prende il nome di ischemia critica ed è caratterizzata dall'occlusione cronica delle arterie degli arti, con sofferenza ischemica dei tessuti. A causa di questa occlusione, infatti, i tessuti che compongono l'arto (ossa, muscoli e cute) non ricevono abbastanza ossigeno e nutrienti per la loro sopravvivenza e quindi tendono a morire. La morte dei tessuti prende il nome di necrosi e si esprime clinicamente con il dolore, l'ulcerazione cutanea, la distruzione dei tessuti superficiali e profondi con possibilità di infezione e ovviamente l'impossibilità ad usare la gamba colpita.

Figure 5 – The Patient's informed consent form.



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Telefono 0171 641000

Cuneo, _/_/2015

Lettera di informazione per il medico curante:

Cellule mononucleate periferiche ad alto contenuto in staminali CD34$^+$ post mobilizzazione con G-CSF per il trattamento dell'ischemia critica degli arti

Versione Finale di Dicembre 2012

Gent.mo/a Collega,
Il/La Sig.re/ra_____ è stato considerato arruolabile al protocollo di studio intitolato "**Cellule mononucleate periferiche ad alto contenuto in staminali CD34$^+$ post mobilizzazione con G-CSF per il trattamento dell'ischemia critica degli arti**".

Come Le è noto, l'attuale trattamento standard per l'ischemia critica prevede l'utilizzo di farmaci e tecniche chirurgiche che cercano di ripristinare la vascolarizzazione dell'arto ischemico. Purtroppo in una grande percentuale di casi tali tentativi non hanno esito positivo ed il paziente subisce una menomante amputazione. Ormai in letteratura sono presenti molte esperienze che dimostrano l'incredibile potenzialità delle cellule progenitrici midollari nella rigenerazione dei tessuti. Anche in ambito vascolare, prima a livello cardiaco e poi a livello degli arti inferiori, è stata dimostrata la potenzialità di tali cellule nel favorire una neoangiogenesi con rivascolarizzazione dei tessuti ischemici destinati alla necrosi.

Lo scopo di questo studio è di:

1. prevenire l'amputazione dell'arto in piu' del 50% dei casi, fornendo al paziente la possibilità di creare una nuova vacolarizzazione attraverso l'iniezione controllata di cellule ad alto contenuto di progenitrici CD34$^+$ nell'arto ischemico. Tali cellule saranno ottenute attraverso la raccolta dal sangue periferico dopo stimolazione con fattore di crescita GCSF (attraverso una procedura di aferesi).
2. raccogliere evidenze medico-scientifiche che tale tecnica aumenti la neoangiogenesi tissutale a partire da precursori cellulari endoteliali

Lettera per il Medico Curante – Studio su cellule progenitrici ed ischemia critica
Conforme a Protocollo: CLI-HSCT-01.11 Versione Finale di Dicembre 2012

A.O. Santa Croce e Carle - Via M. Coppino, 26 12100 CUNEO CN
Data di emissione: 13/03/07 Revisione n° 0 Autorizzato dal Dr. Claudio Novali

Figure 6 – The General Practitioner's informed consent form.

3.6 PROCEDURE

3.6.1 BONE MARROW STEM CELLS' MOBILIZATION

The aim of the protocol was set up on harvesting at least 2 x 10^6 $CD34^+$ cells/kg of bodyweight of the patient (see the Discussion section). In order to reach these values, to each patient are subcutaneously administered 10 mg/kg/day of Granulocyte Colony-Stimulating Factor (G-CSF) (Filgrastim® - 34 millions U/ml) for 5 consecutive days in order to mobilize $CD34^+$ haematopoietic bone marrow stem cells to the peripheral blood. A complete blood cells count was performed every administration day. The number of $CD34^+$ cells was counted at the end of the 4^{th} day and, in case of $CD34^+$ cell count lower than 20/µl, the protocol was stopped for that patient (from now on defined "Low Mobilizer"). With a $CD34^+$ cells count higher than 20/µl, the patient was recognised as a "Mobilizer" and the procedure was scheduled to be performed the following day.

The cut-off value of 20 cells/µl is obtained considering that the minimum blood volume processed during apheresis (see below) is 100 ml/kg, so to obtain a minimum number of 2 x 10^6 $CD34^+$ cells/kg, the minimum useful concentration of $CD34^+$cells is 20/µl (20000/ml or 2 x 10^4/ml):

2 x 10^4 cells/ml x 100 ml/kg = 200 x 10^4cells/kg = ***2 x 10^6 cells/kg***

3.6.2 CELLS' SAMPLE COLLECTION

In this protocol, it was decided to use only the apheresis technique in order to standardize the withdrawal procedure. Moreover, this choice has been made also for the reduced invasiveness of this method, evaluating all advantages and disadvantages of both methods and considering the elevated mean age of patients generally suffering of CLI.

The apheresis procedure is performed at day 5 of G-CSF administration using a blood cell separator Cobe Spectra with Auto-PBSC software (COBE Spectra, Gabbros BCT, Lakewood, CO, USA). The amount of blood volume processed was 100–200 ml/kg of bodyweight. During this procedure the patients were regularly monitored with blood-pressure measurement and electrocardiography trace to exclude complications such as vagal reflex, citrate intoxication, arrhythmia, symptoms of cardiac ischemia, bleeding or hematoma. The goal of the protocol was set up on harvesting at least 2 x 10^6 $CD34^+$ cells/kg of bodyweight of the patient. The blood collection bag was immediately processed with centrifugation in a D level room of the Transplant Laboratory of Hematology Unit in order to obtain almost 60 ml of cell suspension concentrate including mononuclear cells.

The number of $CD34^+$ cells in the cell suspension liquid including mononuclear cells is calculated by the Cytometric Section of Laboratory Analysis Unit, according to the methods of the International Society for Cellular Therapy using flow cytometry. Moreover, the number of Endothelial Progenitor Cells (EPC) is evaluated, and in particular the number of $CD34^+$ $CD133^+$ cells and of $CD34^+$ $CD133^+$ $CD309^+$ cells.

3.6.3 CELLS' IMPLANTATION

Within 2 hours from the apheresis, all harvested cells were implanted into the muscle of the ischemic lower limb without any further modification. The injection sites were determined on the base of the angiogram study executed at the screening step, can be different between patients, and are usually marked on the skin of the limb. The total volume is distributed into 60 to 120 single injections in the lower limb under general or spinal anesthesia. Cells were implanted intramuscularly in zones of arterial obstruction or vascular poorness, as previously identified by angiography (in areas including the gastrocnemial muscle,

anterior tibial muscle, planta pedis and toes) and in regions surrounding ulcers or gangrene lesions (Ph. 6, 7, 8).

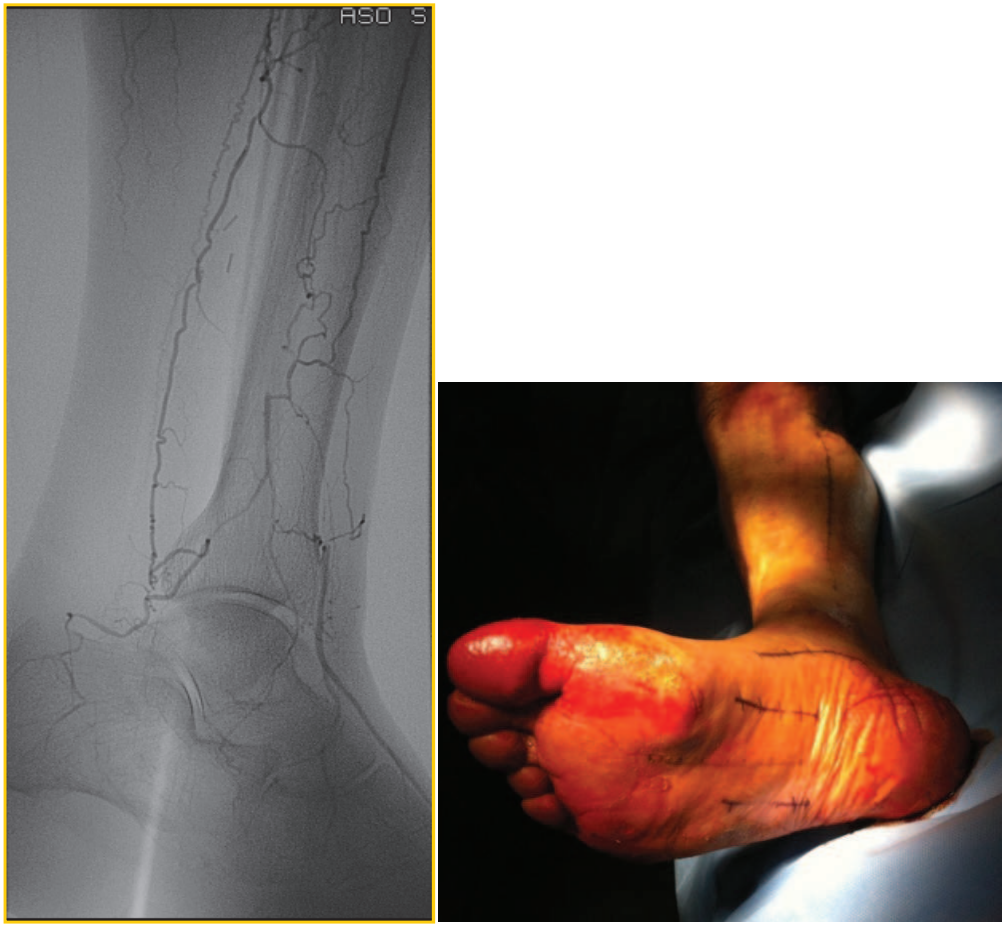

Photo 6 – Angiographic study (L) and skin markers for injection sites (R).

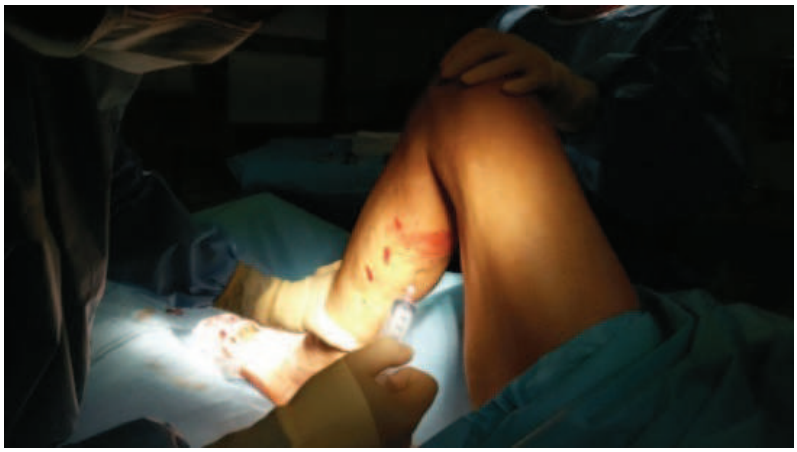

Photo 7 – Inoculation procedure in gastrocnemius muscle.

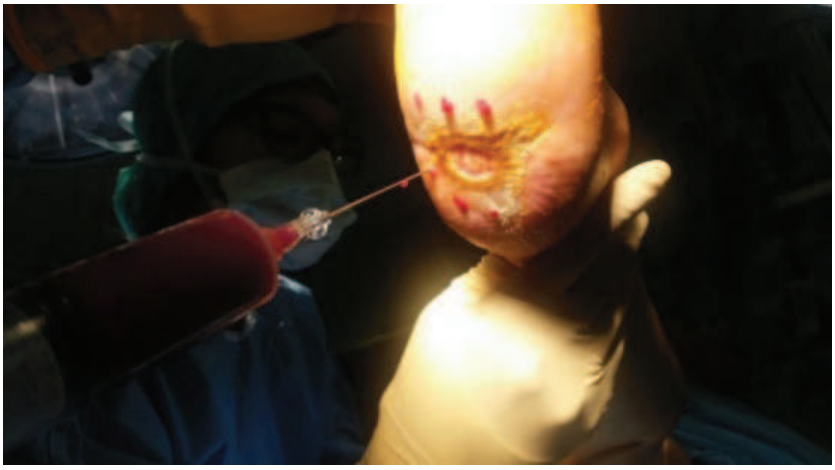

Photo 8 – Inoculation procedure: perilesional injections.

In patients under oral anticoagulant therapy the assumption has always been suspended during the days of administration of G-CSF, substituted by body weight-related dose of low molecular weight heparin (enoxaparin sodium) twice a day until the evening before the day of intramuscular injections. Heparin administration is then restarted the evening after the treatment (complete suspension of anticoagulants for 24 hours) and few days later is finally restarted the oral anticoagulant medication, in absence of hematomas or other contraindications.

In figure 6 is shown the flow chart summarizing the principal steps of the protocol for the “Mobilizer” subjects after the G-CSF administration period and until the discharge of the patient. “Low mobilizer” subjects were excluded from the protocol just before these steps (Fig. 7).

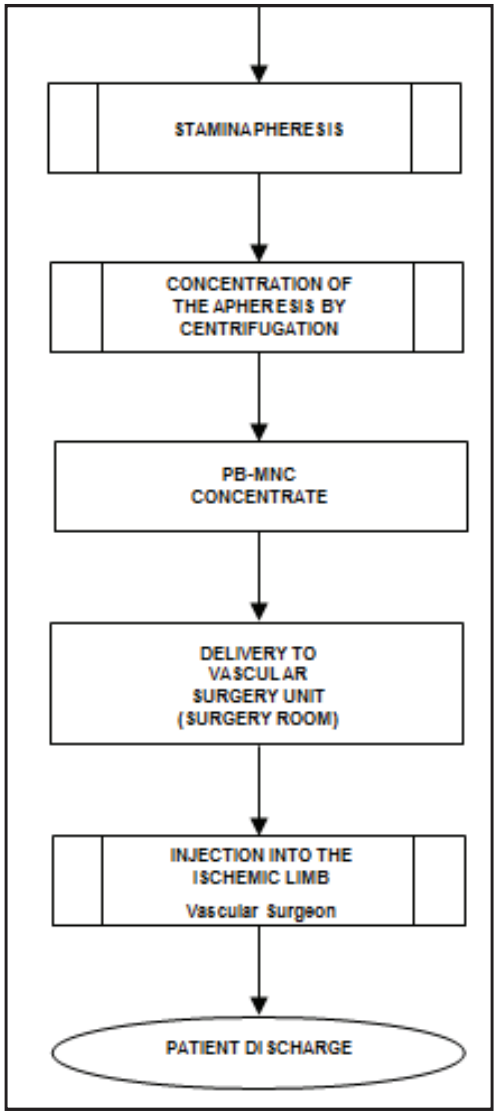

Figure 7 – Flow chart of treatment steps after G-CSF stimulation.

3.7 FOLLOW-UP

Every month, for six consecutive months after the procedure, each patient is evaluated in order to follow the clinical outcomes of the treatment (Fontaine Classification Stage, Visual Analog Score, Wagner Classification Score, Treadmill Test, $TcPO_2$ values, ABI/TBI); other clinical data collected are: the Overall Survival time (OS), the Amputation

Free Survival (AFS), eventual causes of death, adverse events and serious adverse events.

At six months of follow-up a new angiographic exam is performed to be compared to the pre-procedure angiography.

The total population of "Mobilizer" patients, who have been treated with the transplantation of cells, will be divided into 2 subgroups: the "Responder" group consisting of patients with good response to the treatment (lack of major amputation during the follow-up period) and the "Non Responder" group in which are included the patients undergone to major limb amputation during the follow-up period despite the completion of the procedure.

3.7.1 ADVERSE EVENTS

In this protocol study, the complications and adverse events are identified and cataloged following the indications of the "E6 Good Clinical Practice: Consolidated Guidance" [150] of U.S. Food and Drug Administration whose definitions are the following:

- An ADVERSE EVENT is defined as any inconvenient medical occurrence associated with the use of a drug in humans, whether or not considered drug related. An adverse event (also referred to as an adverse experience) can be any unfavorable and unintended sign (e.g. an abnormal laboratory finding), symptom or disease temporarily associated with the use of a drug, without any judgment about causality or relationship to the drug.

- A SERIOUS ADVERSE EVENT is any adverse event or suspected adverse reaction that, in the view of either the investigator or sponsor, it results in any of the following outcomes:
  - inpatient hospitalization or prolongation of existing hospitalization;

- a life-threatening adverse event;
- a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions or a congenital anomaly/birth defect;
- death.

➢ A **"Life Threatening Adverse Drug Experience"** is defined as any adverse experience that places the subject, in the view of the investigator, at immediate risk of death from the reaction as it occurred or it is suspected that the use or continued use of the product would result in the patient's death.

3.8 STATISTICAL ANALYSIS

This is a Phase II study planned with the Simon's two-stage Minimax Design.[151] Two-stage designs have been widely used in phase II clinical trials. Such designs are desirable because they allow to decide whether a treatment is effective or not after the accumulation of the data at the end of each stage. Optimal fixed two-stage designs, where the sample size at each stage is fixed in advance, were proposed by Simon when the primary outcome is a binary response.[152,153,154] The failure level (p0) of not enough activity of the treatment was set up to 50% of AFS (0.5) at 6 months from transplantation, as reported in previous studies.[128] The level of good response to treatment (p1), corresponding to the level of activity expected from the injection of at least 2 x 10^6 $CD34^+$ cells/kg, was set to 70% (0.7). The corresponding sample size needed for this result was 61 patients. The first stage of Simon's two-stage Minimax Design was fixed at 24 patients to be treated with at least 14 of them with a good response (no amputation after 6 months of follow-up): this first target was successfully reached (see the Results section), so the protocol

could proceed to the second phase until the complete recruitment of the expected full sample.

We used three time-to-event variables to evaluate the long-term clinical outcomes and to identify significant prognostic factors:

1 - Overall Survival (OS): the number of days from the day of MPB-MNC implantation to day of death;

2 - Time-To-Amputation (TTA): the number of days from the day of MPB-MNC implantation to the day of lower limb amputation;

3 - Amputation Free Survival (AFS): the number of days from the day of MPB-MNC implantation to the day of lower limb amputation or death, whichever occurred first.

In this study, “lower limb amputation” refers to major amputation (over ankle, indifferently below or above the knee). Survival curves were plotted using the Kaplan–Meier method. Differences in OS and AFS were estimated with log-rank test. Cox regression model was used to identify prognostic factors associated with OS and AFS. Correlations were evaluated with linear regression (R test). The T-test was used for continuous data analysis. Data management and statistical analyses were performed using NCSS statistical software (Kaysville, Utah, USA).[152,153] P-values < 0.05 were considered statistically significant.

4 RESULTS

4.1 PATIENTS' RECRUITMENT

Between November 2014 and September 2017 one hundred and ten (110) patients, suffering of Critical Limb Ischemia (CLI) with no more surgical or endovascular option, were screened to be included in the treatment protocol. According to inclusion/exclusion criteria, forty (40) of them were excluded and seventy (70) patients were recruited for the study: 56 males and 14 females with a median age of 67 years. Table 11 shows the baseline characteristics of recruited patients: 77% were older than 60 years, 46% suffer of diabetes mellitus; Fontaine stage was III in 30 and IV in 40 of them.

Total Patients ("Low Mobilizer")	70 (8)
Gender	20%/80% (F/M)
Mean Age (Range) Age < 60: Group A Age ≥ 60: Group B	67 years (33-89) 16/70 (22.86%) 54/70 (77.14%)
Comorbidities and risk factors Hypertension CAD Hystory of stroke Arrhythmia Diabetes mellitus Hyperlipidemia Active smoke History of Oncologic pathologies	 58/70 (82.86%) 43/70 (61.43%) 19/70 (27.14%) 13/70 (18.57%) 32/70 (45.71%) 18/70 (25.71%) 9/70 (12.86%) 2/70 (2.86%)
Buerger's Disease	6/70 (8.57%)
CLI Fontaine stage III CLI Fontaine stage IV	30/70 (42.86%) 40/70 (57.14%)
Previous revascularization (surgical or endovascular)	34/70 (48.57%)

Table 11 – Baseline recruited patients' characteristics.

Two patients with history of cancer were not excluded from the study, as they suffered of thyroid tumor at low invasiveness, treated almost 15 years before with total thyroidectomy and declared completely free of recurrence at the long-term follow-up.

The follow-up period terminated in January 2018.

4.1.1 PATIENTS' MEDICATION

All recruited patients (70) took antiplatelet or anticoagulant therapy due to PAD or comorbidities and took antalgic drugs due to rest pain and ulcers presence.

4.1.1.1 Antiplatelet/anticoagulant therapy

Between the 62 "Mobilizer" patients (see below – Paragraph 4.4) antiplatelet medication was taken as single drug therapy (ASA 100 mg/day) by 37 patients (59.67%) and as double drug therapy (ASA 100 mg/day + Clopidogrel 75 mg/day) by 12 patients (19.35%).

Oral anticoagulant therapy (Warfarin) was taken by the remaining 13 patients (20.96%) suffering of arrhythmia. This therapy in all cases has been temporarily suspended (as described in the Materials and Method section).

After the procedure and during the whole follow-up interval, all antiplatelet and anticoagulant drugs has been continued (or restarted) at the same dosage taken before the recruitment.

4.1.1.2 Antalgic therapy

Before starting the treatment all the 62 "Mobilizer" patients (see below – Paragraph 4.4) took analgesic therapy: every patient took Paracetamol (2 or 3 g per day); in addition to Paracetamol, 45 patients (72.58%) took Oxycodone and Naloxone combination drug (10/5 mg once or twice per

day) and 14 patients (22.58%) took controlled release Tramadol (150 or 200 mg per day) of which 7 (11.29%) took it in association also with Oxycodone/Naloxone combination (triple antalgic therapy).

Antalgic drugs consumption was not object of the study and the corresponding data has not been included in the statistical analysis. At the clinical evaluation during the follow-up, in some treated subjects was noticed a reduction of the assumption of antalgic drugs. After the 6 months period of follow-up Paracetamol use resulted reduced in 11 patients (17.74%) and 5 patients (8.06%) stopped its chronic daily use. 11 patients on 50 (22.00%) reduced the daily dosage of Oxycodone/Naloxone and 3 on 14 (21.42%) completely suspended the assumption of Tramadol, continuing to use Paracetamol. The 7 patients under triple antalgic therapy underwent major amputation during the follow-up period (Non Responder, see below – Paragraph 4.7). The 5 patients who completely suspended chronic continuative use of Paracetamol are all at Fontaine stage III. 14 of 25 patients who reduced antalgic drug assumption were at Fontaine stage III (14/30 – 46.67%) and 11 at Fontaine stage IV (11/40 – 27.50%). This reduction of dosage in many cases was progressive and noticed in different periods of the follow-up, consequently was not possible to define in which precise moment after the treatment it occurred, moreover the variability of dosage and drugs used is due to the fact that, in most cases, the antalgic therapy was set before the patient came to our observation.

4.1.2 PATIENTS' PRE-TREATMENT CHARACTERISTICS

4.1.2.1 ECOG Performance Status

The median value of recruited patients' ECOG PS was 1.1; in particular 14 patients had score 0, 37 had score 1, and 19 score 2. Being just used as exclusion criteria in recruiting phase and not being object of the study, ECOG PS values have not been included in the statistical

analysis and no new evaluation was performed at follow-up. The same attitude has been followed for other exclusion criteria (e.g. EF and Hb A1C values).

4.1.2.2 Hemodynamics values (ABI/TBI)

Globally average of ABI measurement values in recruited patients was 0.34; in Fontaine stage III patients the average value was 0.37 and in Fontaine stage IV group the average value was 0.32.

Global, Fontaine stage III, and Fontaine stage IV TBI measurement values were respectively 0.28, 0.30, and 0.26.

4.1.2.3 Ulcers characteristics

Ulcers size (total area in case of multiple ulcers) was inferior to 5 square centimeters (cm^2) in 31 cases (77.50%), between 5 and 25 cm^2 in 6 cases (15.00%), and superior to 25 cm^2 in 3 cases (7.50%).

In 29 cases (72.50%) the ulcer was single, in 7 cases (17.50%) there were 2 ulcers and in the remaining 4 cases (10.00%) the ulcers were multiple (usually three or four): in one of these patients the number of ulcers was 6 (small ulcers).

Localization of ulcers was forefoot or toes in 31 cases, ankle or distal leg in 13 cases and middle leg in one case (a very extensive single ulcer of the distal 2/3 of the leg).

In 5 cases of double or multiple ulcers there was a double localization (forefoot/toe and ankle), for this reason the total number of localization sites exceeds the number of Fontaine stage IV patients (40).

4.2 FIRST PHASE

This is a Phase II study planned with the Simon's two-stage Minimax Design. The calculated total required sample size (first stage + second stage) is 61 patients. The pre-specified number of patients to treat in the first stage of the study was 24 (see above in Materials and Methods section).

The first 24 patients have been treated in the first 6 months of the study, so the follow-up period of this stage was completed in the first year.

In the first phase the limb salvage (AFS) rate obtained after 6 months of follow-up between the treated patients was 16/24 (66.67%). The first phase of this multistage study has been successfully completed, considering that the cut-off for proceeding from the first to the second phase was the limb salvage in at least 14 of the first 24 procedures.

4.3 SECOND PHASE

After the completion of the first phase and avoided the termination of the protocol for lack of efficacy, in the following second phase the recruitment proceeded and the treatment was performed on other 38 patients for a total of 62 procedures.

4.4 $CD34^+$ CELLS' MOBILIZATION

On the total amount of 70 patients, during the bone marrow stimulation, 8 patients did not reach the minimum level of concentration of 20 $CD34^+$ cells/µl on day 5 of G-CSF administration ("Low Mobilizer") and were excluded from the protocol. Among the remaining 62 correctly mobilizing patients ("Mobilizer"), the median harvest of cells was 4 x 10^6

CD34+/Kg (Range: 1.4-8.9). In particular, the median harvest of cells was 4.9 x 10^6 CD34+/Kg among 16 patients younger than 60 years old and of 3.3 among the remaining 46 older patients (p = 0.004) (Fig. 8). Harvest was better in male patients (4.2 vs 2.3, p = 0.02) and was not significantly influenced by the Fontaine stage or presence of diabetes.

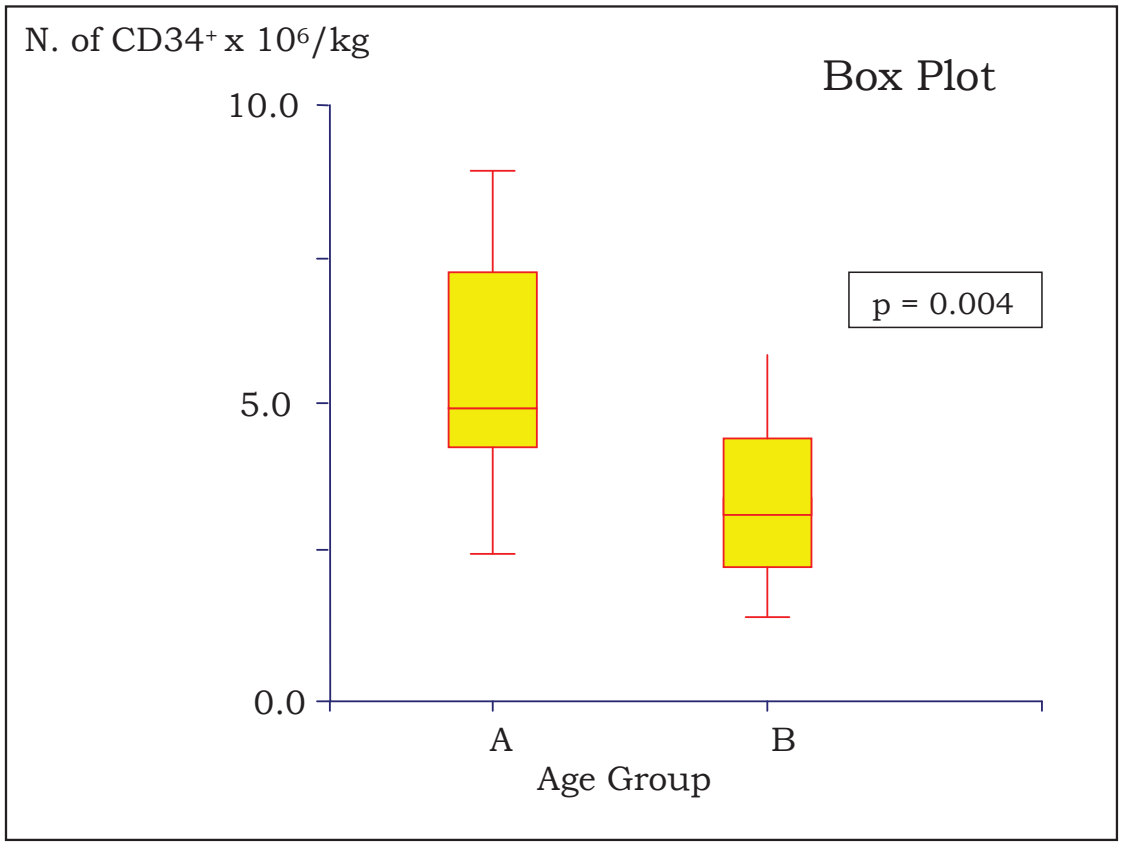

Figure 8 – Mobilization capability of CD 34+ cells for age group.

4.5 OTHER EPC CELLS' MOBILIZATION

Considering the entire population of 62 Mobilizer patients, subdivided in relation to the expression of CD34, CD133 and CD309 receptors, the findings are a median harvest of CD34+ CD133+ cells and CD34+ CD133+ CD309+ cells respectively of 2.62 x 10^6 CD34+ CD133+/Kg (Range: 1.07-6.51) and of 1.18 x 10^3 CD34+ CD133+ CD309+/Kg (Range: 0-16.70). A

median harvest value of 3.69 x 10^6 $CD34^+$ $CD133^+$/Kg was obtained among 16 patients younger than 60 years old and of 2.41 x 10^6 $CD34^+$ $CD133^+$/Kg among the remaining 46 older patients (p = 0.005) (Tab. 12). Median harvest of $CD34^+$ $CD133^+$ $CD309^+$ cells was not influenced by age.

	Number of $CD34^+$ $CD133^+$ cells/kg
Total (62 pts)	2.62 x 10^6
Group age < 60 (16 pts)	3.69 x 10^6
Group age ≥ 60 (46 pts)	2.41 x 10^6

Table 12 – Number of $CD34^+$ $CD133^+$ cells/kg after mobilization in total population and divided by age groups.

4.6 ADVERSE EVENTS

Total adverse events occurred in the period of G-CSF administration were: bone pain 16/70 (22.86%), angina pectoris 4/70 (5.71%), headache 4/70 (5.71%), nausea 2/70 2.86%), insomnia 8/70 (11.43%) and fatigue 2/70 (2.86%) (Tab. 13). No cases of fever occurred.

The four patients with the serious adverse event of angina pectoris complain about chest pain and had a moderate increase of cardiac enzymes: two cases occurred in day 4 and 5 of bone marrow stimulation in patients in which the count of $CD34^+$ cells leads to classify them as Low Mobilizer subjects, so the procedure was stopped and they were excluded from apheresis and transplantation (as scheduled by the protocol); other two cases happened during the night of the first and second day of stimulation with G-CSF, but the symptoms spontaneously regressed after few hours. These episodes did not influence the prosecution of the treatment, with no further cardiac problems during the hospitalization or the follow-up.

During or after the procedure of cells' injection, the unique adverse event was constituted by intramuscular or subcutaneous hematoma on the site of injection, as local complication of the procedure (Ph. 9). It occurred in 4 cases (5.71%), all of them at the beginning of the protocol and resolved spontaneously within few days without any further action or therapy. One case occurred in a patient under oral anticoagulant therapy, regularly suspended in the days before the treatment (as explained above in Materials and Methods section).

Adverse event	**N. (%)**
Bone pain	16/70 (22.86%)
Insomnia	8/70 (11.43%)
Angina pectoris	4/70 (5.71%)
Headache	4/70 (5.71%)
Nausea	2/70 (2.86%)
Hematoma	4/70 (5.71%)

Table 13 – Adverse events.

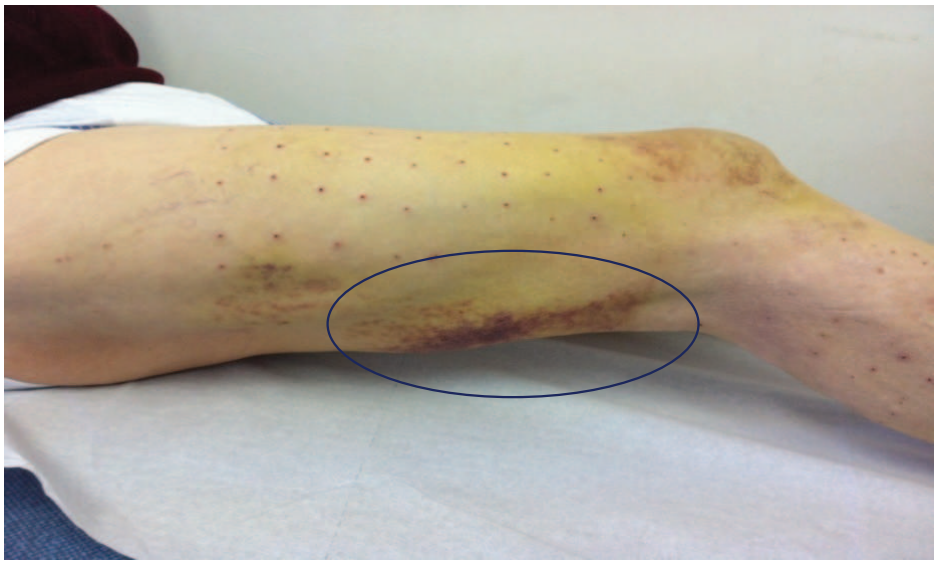

Photo 9 – Post-procedure hematoma.

4.7 CLINICAL EVALUATION

The median follow-up between the 62 treated patients ("Mobilizer") was of 28 months (range 4-38 months), the observed Overall Survival (OS) was 97%. Four (4) patients died (Tab. 14) respectively after 4, 5, 13 and 14 months from transplant, in all cases for heart failure of ischemic origin; none of them was one of the two patients who suffered of angina pectoris during the G-CSF stimulation period (see above – Paragraph 4.6). All the deceased patients had previously undergone a major amputation during the follow-up period (Non Responder patients, see below). Two-years OS was 100% in Responder patients group and 71% in Non Responder patients group (p = 0.006) (Fig. 9).

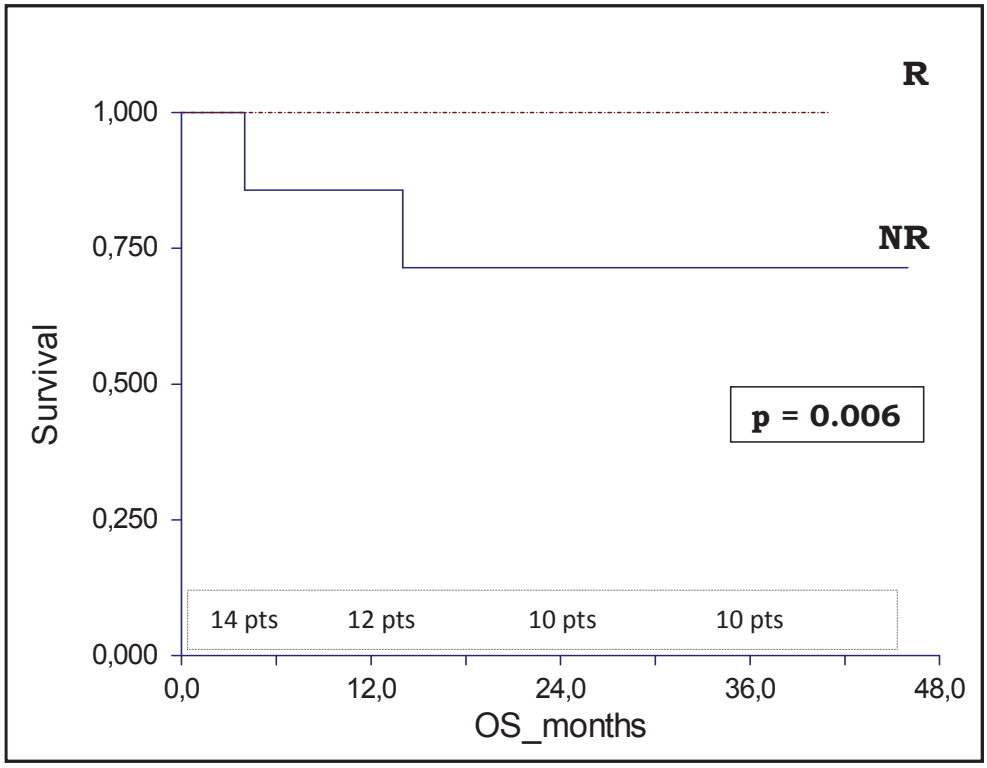

Figure 9 – Overall Survival (OS) Kaplan-Meier curve: (R) Responder group, (NR) Non Responder group. In the box at the bottom is expressed the progressive number of patients NR at risk at each time point. The R group is constantly composed by 48 patients.

Globally 14 subjects (Non Responder) on 62 patients (Tab. 14) underwent a major amputation with a median Time-To-Amputation (TTA) of 3 months. Amputation Free Survival (AFS) was 81% at 6 months and 77% at 1 and 2 years (Fig. 10). In the Non Responder group, two (2) were Buerger's patients (both amputations of leg, below-the-knee) with an AFS at one year for Buerger's patients of 66.67% (4/6).

An important finding to notice is that AFS was not significantly influenced by the number of $CD34^+$ cells, $CD34^+$ $CD133^+$ cells and $CD34^+$ $CD133^+$ $CD309^+$ cells transplanted ($p = 0.05$).

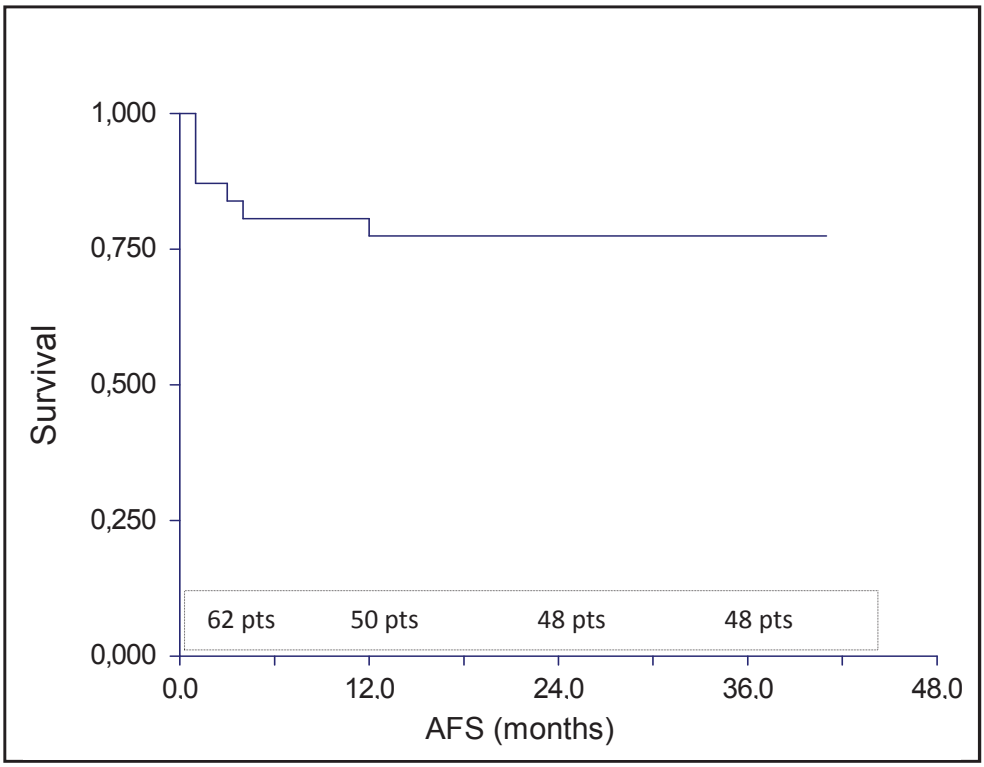

Figure 10 – Kaplan-Meier curve by Time-to-Amputation (TTA). In the box at the bottom is expressed the progressive number of patients (pts) at risk of death for each time point.

Moreover, at long term follow-up, no significant correlation was seen with age (Fig. 11) and Fontaine stage (Fig. 12) as well as diabetes mellitus (Fig. 13), dyslipidemia and Buerger's Disease.

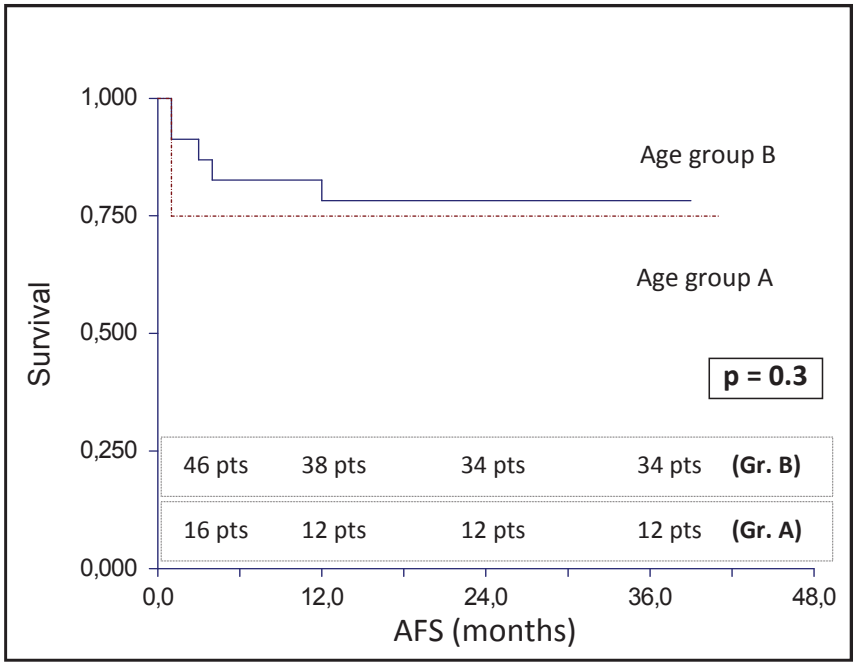

Figure 11 – Kaplan-Meier curve by Time-To-Amputation (TTA) by Age groups. In the boxes at the bottom are expressed the progressive numbers of patients (pts) at risk of death for each time point.

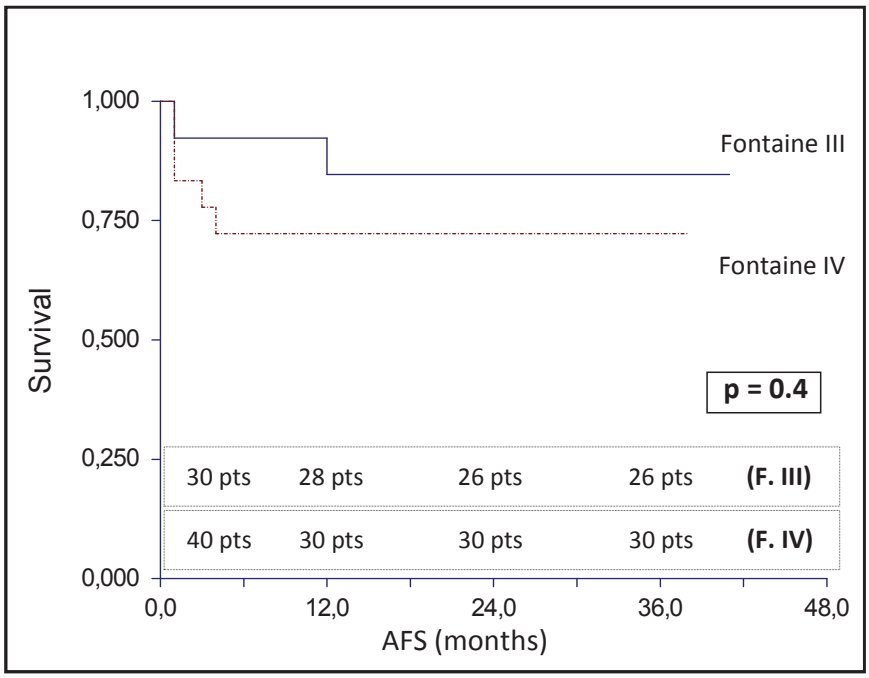

Figure 12 – Kaplan-Meier curve by Time-To-Amputation (TTA) by Fontaine stage. In the boxes at the bottom are expressed the progressive numbers of patients (pts) at risk of death for each time point.

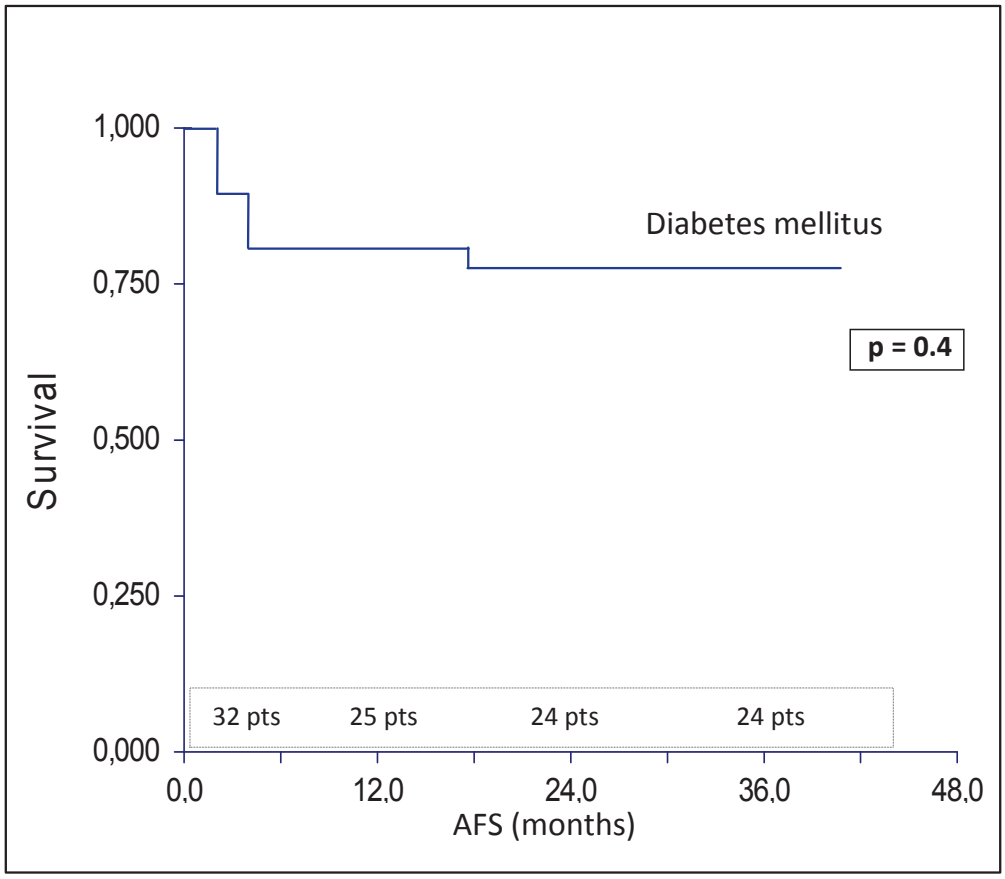

Figure 13 – Kaplan-Meier curve by Time-To-Amputation (TTA) for diabetes mellitus. In the box at the bottom is expressed the progressive number of patients (pts) at risk of death for each time point.

Minor amputations are not object of the study and are not included in the statistical analysis. However, during the 6-month follow-up period have been performed 7 minor amputations (Tab. 14) on patients of the Responder group (14.58%): 5 toes amputations and 2 forefoot amputations. Five (5) of the minor amputations have been effectuated on diabetic patients (3 toes and 2 forefeet) and two of them on Buerger's patients (1 of toes and 1 forefoot). All minor amputations of PAD patients regarded subjects included in the Fontaine stage IV group, with necrotic lesions already present at the moment of inclusion in the study.

	Responder (R)	Non Responder (NR)	**TOTAL**
Number of patients	46	14	**62**
Death	0	4	**4**
Survivors	46	10	**56**
Major amputations	0	14	**14**
*Minor amputations**	*7*	*0*	*7*
*Survivors without any amputation**	*39*	*0*	*39*

Table 14 – Final deaths and amputations report. *Not included in statistical analysis.

An interesting clinical result was obtained about pain, in fact the transplanted patients showed a significant reduction of pain after the procedure: median Visual Analog Scale (VAS) score decreased from a value of 6.2 before transplant to 3.8 at 1 month after transplantation ($p < 0.0001$ for differences between pre and 1 month post) and to 1.5 at 6 months ($p = 0.01$ for differences between 1 and 6 months post) (Fig. 14).

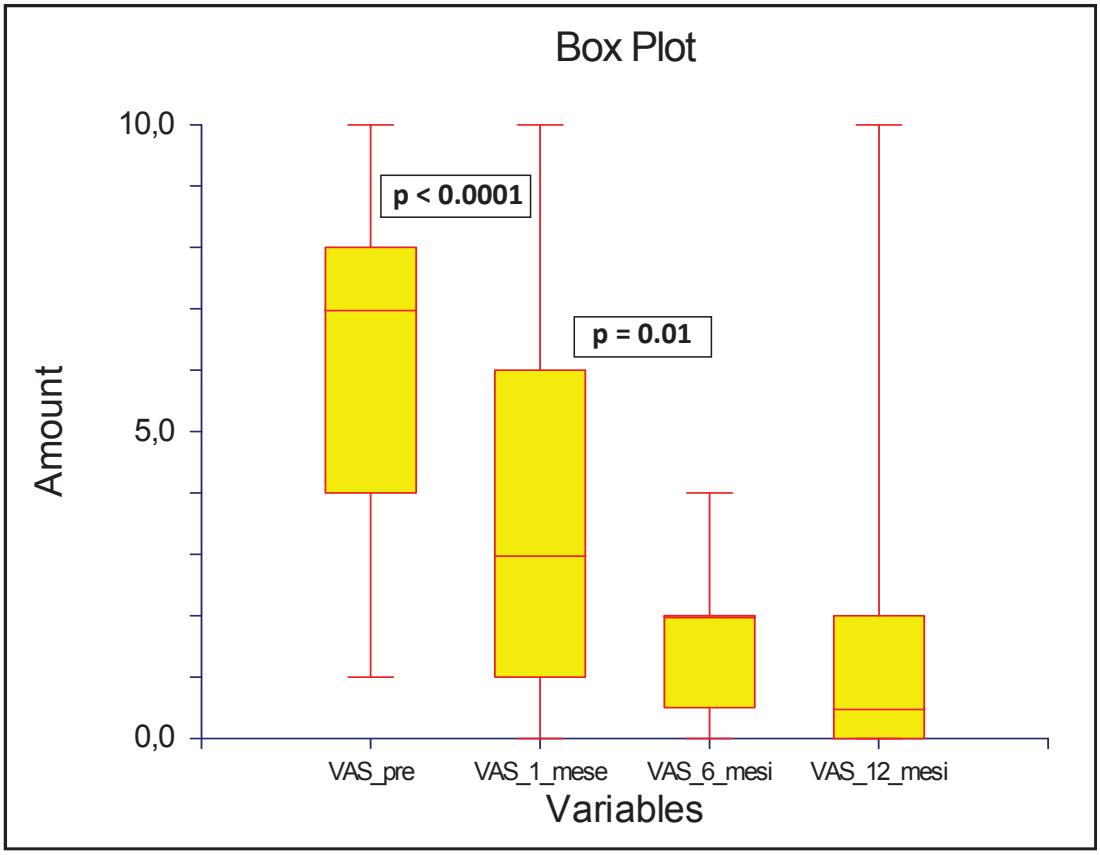

Figure 14 – VAS median values evolution during follow-up.

In some cases we observed an effect on pain, happened during the G-CSF stimulation phase: seven (7) patients referred a relief in index leg pain (median lowering of VAS scale of 2.3 points) from the second or the third day of bone marrow stimulation.

Pain reduction occurred in both groups of patients (Fontaine stage III and Fontaine stage IV) and there was no significant difference between the two groups ($p = 0.1$) with discrepancy in the median Visual Analog Scale (VAS) score values of 0.6 points at 1 months and 0.8 points at 6 months of follow-up (lower values for Fontaine stage III group).

Concerning ulcers, it has been observed a reduction of the severity of the ulcers' grade: the median Wagner score was 1.7 before transplant (range 0-4), 0.2 at 6-month (range 0-2, $p < 0.001$) and 0.1 at 12-month (range 0-2, $p < 0.001$) follow-up (Fig. 15). Nine (9) ulcers reached a

complete healing at the 6-month follow-up, condition maintained for the following 6 months. The three patients with very extensive ulcers (> 25 cm^2) were all in the Non responder group.

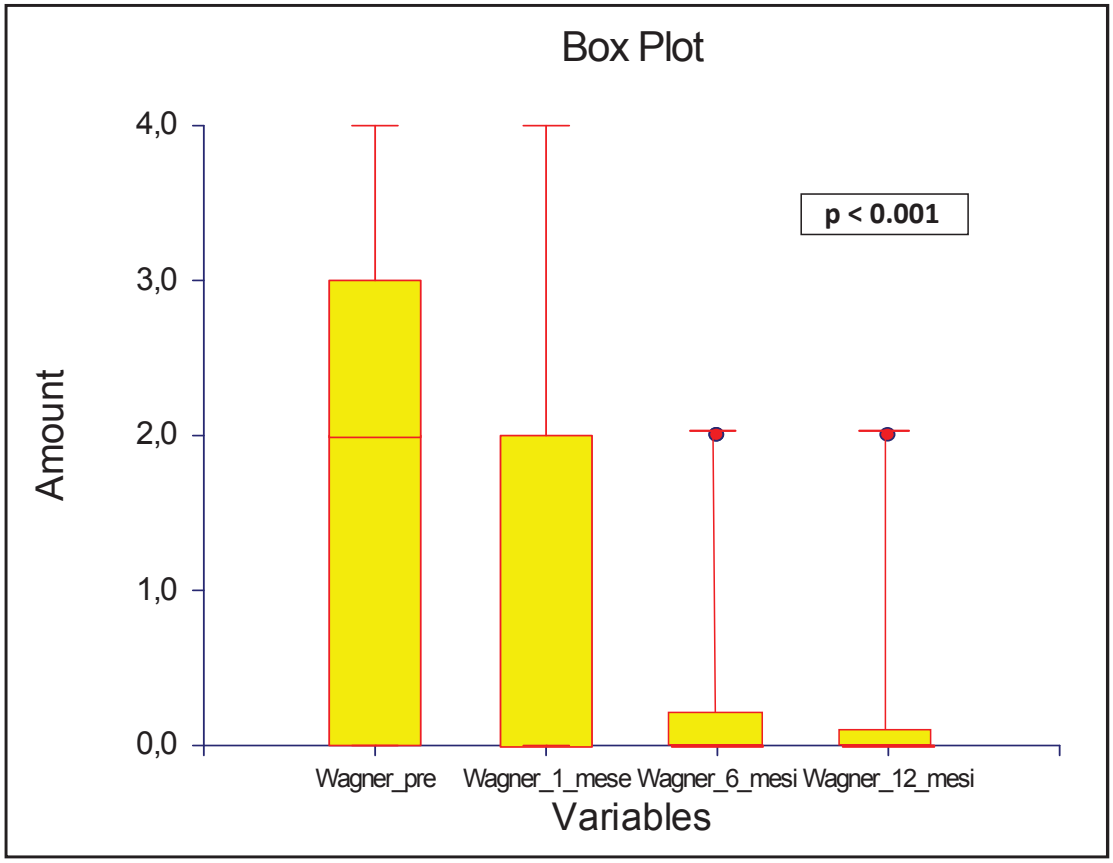

Figure 15 – Wagner median values evolution during follow-up.

Furthermore there was a trend towards an increase of $TcPO_2$ values from an average of 35 at the recruiting level to an average of 40 at 6-month follow-up measurements ($p = 0.07$).

No statistically significant modifications were instead seen in ABI/TBI values.

ABI measurement globally passed from an average value of 0.35 at the moment of the recruitment to an average value of 0.44 at the 6-month follow-up ($p > 0.06$). In particular in Fontaine stage III group the average

value increased from 0.37 to 0.49 ($p > 0.06$), in Fontaine stage IV group the average value increased from 0.32 to 0.41 ($p > 0.06$).

TBI measurement globally passed from an average value of 0.28 at the moment of the recruitment to an average value of 0.33 at the 6-month follow-up ($p > 0.07$). In particular in Fontaine stage III group the average value increased from 0.30 to 0.35 ($p > 0.08$), in Fontaine stage IV group the average value increased from 0.26 to 0.31 ($p > 0.07$).

4.8 ANGIOGRAPHIC FINDINGS

As programmed, at 6-month of follow-up, we performed an angiographic exam to the index leg, to be compared with the pre-treatment angiogram. The two exams were performed with the same scheme: injection of 10 ml of iodinated contrast medium (Iomeprol: Iomeron300 - Bracco, Milano, Italy) in femoral artery at 5 ml/s of infusion rate through a 4 French catheter.

In the majority of cases it is hard to clearly identify the presence of new collateral vessels by comparison of pre- and post-treatment exams. However, in four patients, it was observed a new vessel formation with new capillary arteries in the area of stem cells injection in the calf region in two cases and in the anterior region of the ankle, near the pedal artery, in the other two (Ph. 10 and 11 below; Ph. 12 and 13 in Discussion section).

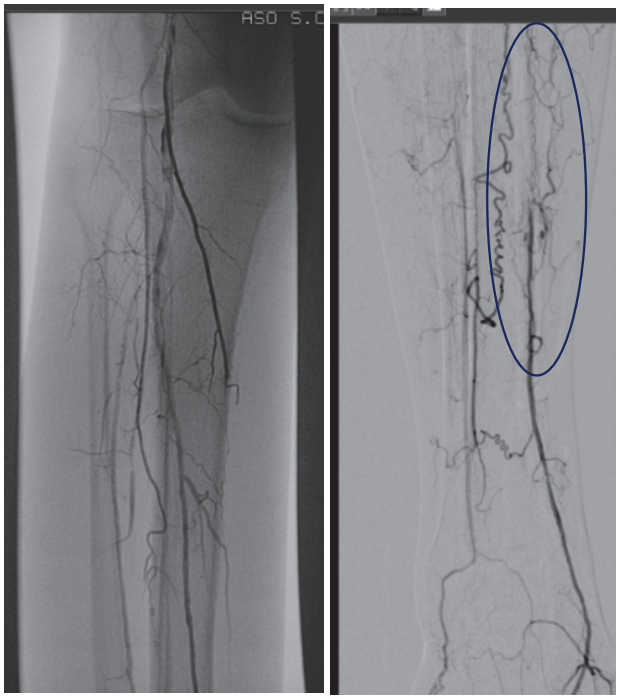

Photo 10 – Angiography pre (L) and post (R) treatment with neo generated collateral vessel.

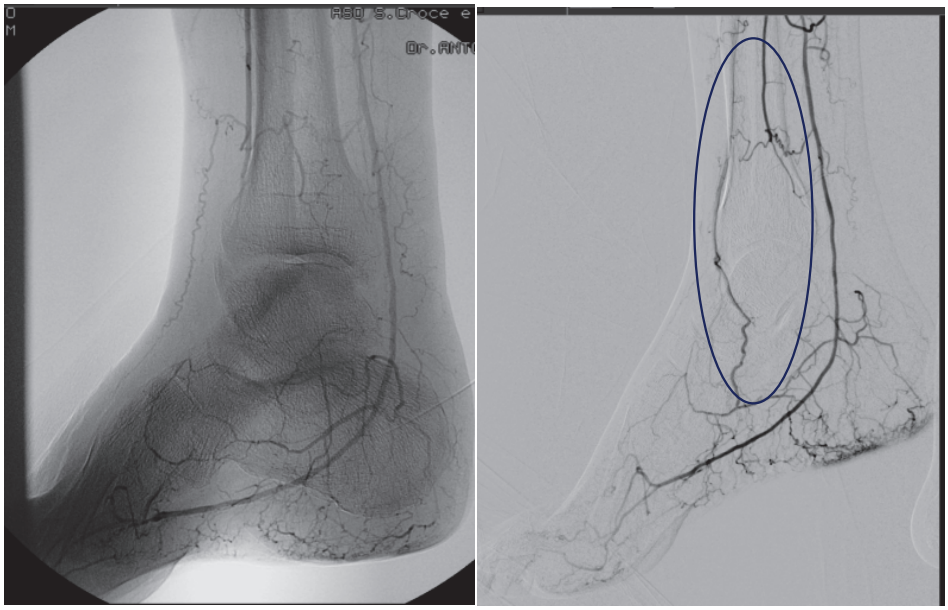

Photo 11 – Angiography pre (L) and post (R) treatment with neo generated collateral foot vessel.

In other 9 cases have been identified vessels which apparently show a more regular wall with a more contrasted lumen in the last angiography,

although these modifications are not easy to objectify and to show with static iconography.

4.9 LABORATORY FINDINGS

MPB-MNC blood cells count after G-CSF stimulation showed an evident increment of cells (Tab. 15). Although no correlation had been found between cells' counts and follow-up clinical parameters ($p > 0.07$ in all subgroups analisys).

	MPB-MNC x 10^9/kg
All Patients	
Median Value	47.68
Range	17.26 – 155.55
Group Age A (< 60)	
Median Value	47.88
Range	31.03 – 73.70
Group Age B (≥ 60)	
Median Value	45.62
Range	25.40 – 155.55
Group R	
Median Value	50.01
Range	31.03 – 73.70
Group NR	
Median Value	40.50
Range	31.36 – 155.55
Group Fontaine III	
Median Value	51.68
Range	31.03 – 73.70
Group Fontaine IV	
Median Value	43.50
Range	20.66 – 155.55

Table 15 – MPB-MNC count after G-CSF stimulation.

5 DISCUSSION

Starting from promising results of other studies [125,128,132] the main goal of this clinical research project, begun in November 2014, is to ameliorate the Amputation Free Survival (AFS) rate in patients affected by CLI; in particular the main endpoint is to outdo, at the 6-month follow-up, the average value of 50% of limb salvation obtained in other previous clinical reports.[131]

Nowadays is universally accepted that the Gold Standard treatment for severe PAD and CLI is direct revascularization, either surgical or endovascular. Despite the continuous progresses in medical, surgical and endovascular therapies, stilltoday up to 30% of patients is not eligible for these therapies.[157]

The clinical decision to include in the study patients suffering of Buerger's disease was made in order to exclude a number of patients as smaller as possible from the potential benefits of the treatment. Being impossible, for ethical reasons connected to the authorization obtained by ethical committee, to perform the treatment out of the protocol and on patients not included in the study, these 6 patients have been included. According to Idei et al.,[158] patients with Buerger's disease have a better outcome compared to those with PAD. Including these patients in the study, even if that group constitutes less than 10% of the population (6/62), should have partly affected the results. Nevertheless, the findings of the study, even without statistical significance ($p = 0.1$), do not confirm a better response of Buerger's patients with an amputation rate of 33.33% (AFS 66.67%) between those patients, so their presence should not have positively influenced the overall AFS result.

Considering how the study was structured, the recruitment was based on the consecutive sampling technique, without any possibility to "choose the best patient" who have a best prognosis or who can more likely reach the best results for the study. The selection was randomized on the base of the influx of patients from the clinician's or ultrasound's laboratory or from the emergency room. The only clinical selection was based on the presence of CLI and on inclusion/exclusion criteria. In other words, patients have been screened in a rigid consecutive way.

Speaking about HR Quality of Life (HRQOL), according to Flowers et al., this worsens with worsening of ECOG Performance Status and was worse among patients with fatigue, especially in physical/functioning domains, pain/discomfort, and mobility. These results indicate that baseline ECOG PS and physician-rated fatigue are rapid assessments that predict robust measures of HRQOL.[159] ECOG PS is a scale widely used in oncohematology. This protocol has been chosen among other methods of evaluation of HR Quality of Life because the group of clinicians of the study is heterogeneous, composed of vascular surgeons as well as hematologists, radiologists, pain physicians, and clinical biologists, all involved during different stages of the protocol. It was decided to use the best-known and most commonly used method by all clinicians, to have a more uniform evaluation. The use of this widely known method is one of the specific suggestions proposed by the ethical committee before approving the study protocol.

The improvement of QoL score related to cells' therapy is still debated. Peeters Weem et al. evaluated the quality of life after treatment with autologous bone-marrow derived cells in patients with severe CLI without other therapeutic options. The improvement in QoL at 6 months persisted in both arms at a median follow-up of 35 months. The long-term QoL did not differ between the BM-MNC and placebo group. Patients with and without a major amputation had similar QoL scores. The increased QoL

in patients with no-option severe limb ischemia persisted until 3 years after inclusion, but did not differ between the BM-MNC and placebo arms or between patients with and without a major amputation.[160] In this study the ECOG PS has been used, as mentioned in Materials and Methods section, in the recruitment phase, but QoL was not object of the study and was not included in the statistical analysis.

The proposed procedure, in relation to this experience, seems to be safe even in elderly patients. Angina pectoris occurrence is described, as well as major cardiac events, as a consequence of mononuclear cells therapy [148] and is one of the events related to the procedure, maybe induced by neutrophilia and hypercoagulability observed after G-CSF stimulation.[161] In fact, the increasing of WBC or MNC circulating in the blood, as occurs during G-CSF stimulation, could probably generate a hypercoagulability state [162] and an increase in platelet aggregation,[163] thus increasing the risks of angina pectoris and arterial acute thrombosis in these patients, already at high risk of cardiovascular disease.[164,165] In this experience the serious adverse events, related to the mobilization or transplantation procedures, were the mentioned four (4) cases of angina pectoris reported in the Adverse events paragraph of Results section (4.5); angina pectoris did not result in myocardial infarction or other major cardiac events and symptoms spontaneously regressed after few hours. In two cases these episodes did not influence the prosecution of the treatment, with no further cardiac problems during the hospitalization or the follow-up; the other two cases occurred in Low Mobilizer patients for whom the protocol has been interrupted and they did not develop any further cardiac problem in the following months.

The risk of genesis of tumors after G-CFS stimulation has to be considered. This point has been discussed for this study with the ethical committee and the exclusion criteria have been decided in order to reduce as much as possible even this risk. The only two patients not

excluded from the study, despite a clinical history of cancer, have been included because they suffered of thyroid tumor at low invasiveness, treated almost 15 years before with total thyroidectomy and declared completely free of recurrence at the long term follow-up. Liang et al., analysing data with a five-years follow-up period, concluded for the safeness of this procedure without incidence of malignancies or diagnoses of clinically significant proliferative retinopathy.[148]

In the present study, intramuscular or subcutaneous hematoma on the site of injection (Ph. 9 in the Adverse Events paragraph of Results section – 4.5) occurred in 5.71% of procedures, a value comparable to other studies;[166] all of them resolved spontaneously and developed at the beginning of the experience, when were used 14 Gauge (G) needles, maybe too big. A correction was brought using 18 G needles with no more cases of hematoma. Only one (25%) of the four cases of hematoma occurred in patient under previous anticoagulant oral therapy, regularly suspended before the hospitalization and with International Normalised Ratio (INR) normal values at the moment of the cells injections.

Wang et al. [139] analyzed the incidence of severe adverse events in some studies on autologous bone marrow cell therapy. Data shown in table 16 seem to confirm that this kind of treatment is relatively safe, coherently with the results of other meta-analyses.[167,168,169]

Study group	Follow-up	Adverse event		
		Death	Recurrence	Fever
Tateishi-Yuyama 2002		0/45		
Serkan Durdu 2006		0/28		
Koji Miyamoto 2006	684 ± 549 days	1/8	1/8	
Xinshijie 2006	3 – 24 months	5/42		
Yutaka Saito 2007		0/14		
Liugeling 2007	2 – 12 months	0/20		
Ganyu 2008	6 – 12 months	0/15		1/15
Wangguangyu 2009	6 months	0/83		
Chenbing 2009		0/40		
Amann 2009		0/51		
Wangwei 2010	12 months	0/56	3/56	
Yihai 2011		0/13		
Wangxiuhui 2011		0/69		

Table 16 – Clinical safety issues in the eligible trials. Adverse events are classified into three types: death, recurrence and fever, with the corresponding follow-up time. From Wang et al..[139]

The method chosen for the statistical analysis is the Simon's two-stage minimax design. Two-stage designs are commonly used in phase II clinical trials (especially in cancer clinical trials). Simon [151] in 1989 proposed two criteria (minimax and optimal) for selecting sample sizes and critical values for these two-stage designs. In a typical two-stage trail, a defined number of patients are enrolled in stage I. If the number of responses is fewer than or equal to a pre-specified number, the trial is terminated for lack of efficacy. Otherwise an additional number of patients are enrolled in stage II. The total number of patients is the sum of the patients enrolled in the two stages. If the cumulative number of responses is fewer than or equal to a pre-specified number, then lack of

efficacy is stated. Otherwise, it is concluded that the treatment is sufficiently effective for further investigation. There are different designs proposed for different studies. For a given expected p0, the minimax design has the smallest total number of patients to enroll.[170]

This statistical method has been chosen for this study in order to reduce as much as possible the number of patients treated before understanding if the treatment lacks of efficacy. The predefined number of patients to treat, with the minimax design method, is the smallest possible for this type of study and this condition allows stopping the treatment after a small number of cases if the responses obtained lead to the termination for lack of efficacy.[170] Considering that CLI is a pathology at high risk of limb loss which affects complex patients with high number of comorbidities and often suffering of high pain degree, the clinicians involved in the study and the ethical committee judged that this two-stage protocol in this case was a good option, even if usually used in oncology trials rather than in PAD trials. Phase II clinical trials are only able to detect a large treatment improvement, e.g. greater than 10%. To detect a small difference in treatment, e.g. less than 5%, is required a much larger sample size, which is not possible in Phase II studies due to the limited number of subjects eligible for the study inclusion. In this case the difference expected in the result is 20% (70% of limb salvage rate versus 50% of other mentioned studies), so this method in our opinion could be considered useful. The absence of a control group is due to the low number of patients potentially involved in the study. The sometimes rapid evolution of this pathology may generate a rapid worsening if untreated, characteristic that could be considered similar to the evolution of some oncologic pathologies. For these two reasons, in accordance with the indications of the local ethical committee, was decided to conduct an uncontrolled observational study.

An important controversy regarding the studies concerning this kind of therapy is that, whereas non-controlled studies have encouraging results concluding effectiveness,[171] as seems confirmed analysing data of this study, placebo controlled trials fail to show the same benefit of stem cells implantation.[172]

A criticism of the study is the absence of a control group of patients affected by CLI with the same clinical characteristics, who did not receive the treatment and who were treated with best medical therapy, whose clinical evolution could be related to the treated group. With this lack of control group, the possibility of spontaneous amelioration cannot be excluded, and clinicians that deal with PAD know that the response to the treatment could be different from patients in apparently similar clinical conditions.[173] Theoretically, the Low Mobilizer group of patient could have been used as control group, but the number of these patients was very low and with differences in the clinical conditions (Fontaine stage, VAS score et cetera), so that the two groups (Mobilizer and Low Mobilizer) are not enough homogeneous to be statistically compared.

Another limitation of this and similar studies is the exiguous number of potentially recruited patients due to the fact that luckily only 30% of them could not be treated with surgical or endovascular procedures (gold standard treatment) and the restrictive exclusion criteria reduce significantly the number of enrolled patients that usually suffers of many of the pathologies listed in the exclusion criteria. The results of these studies are promising bases for further larger and placebo-controlled trials.

With these assumptions and limitations of the study, the rate of AFS obtained in this study, at the 2-years follow-up, is 77% versus about 50% of most of the literature's published data: in our opinion, this may be considered an encouraging result in limb salvage possibilities. A reason of this good result should be sought in the dose of cells administered. In

fact, the protocol was set up by administering a minimum dose of 2 x 10^6/kg patient's body weight of $CD34^+$ cells, that is, about twice the number reported by other previous studies.[158,174,175,176] In doing so, our goal was to increase as much as possible the AFS ratio, with the assumption that may exist a positive relation between the number of cells locally deployed and the clinical result.

No correlation had been found between cell counts and follow-up clinical parameters. The reason of this finding is unclear, but maybe it could have been influenced by the low number of patients: cell count comparisons between the two groups, Responder and Non Responder, may not be significant because of the limited number of patients in each of the two groups. Moreover, the clinical result may be caused by several factors, currently not all clearly explainable, influencing at various levels the outcome.

Table 17 shows obtained data on cells' mobilization: median values of transplanted cells, values divided by age (groups A and B), by response to treatment (R and NR) and by grade of PAD (Fontaine stages III and IV).

In Table 17 is indicated the MPB-MNC count instead of the total of White Blood Cell (WBC) count because G-CSF mobilizes from the bone marrow prevalently lymphocytes and monocytes. A minimal quote of neutrophil granulocytes is also present at an immature stage in which they are mononuclear, and for this reason are counted together with the mononuclear cells in the cytofluorometric count performed with the machine used in our institution.

	MPB-MNC **x 10^9/kg**	**$CD34^+$** **x 10^6/kg**	**$CD34^+$ $CD133^+$** **x 10^6/kg**	**$CD34^+$ $CD133^+$ $CD309^+$** **/kg**
All Patients **Median Value** **Range**	47.68 17.26 – 155.55	4 1.41 – 8.9	2.62 1.33 – 6.51	1080 0 – 160500
Gr. Age A (< 60) **Median Value** **Range**	47.88 31.03 – 73.70	4.91 2.47 – 8.90	3.69 1.82 – 6.51	1700 0 – 6700
Gr. Age B (≥ 60) **Median Value** **Range**	45.62 25.40 – 155.55	3.33 1.41 – 5.81	2.41 1.33 – 3.99	890 0 – 160500
Group R **Median Value** **Range**	50.01 31.03 – 73.70	3.36 1.41 – 8.90	2.50 1.33 – 4.97	1.18 0 – 160500
Group NR **Median Value** **Range**	40.50 31.36 – 155.55	4.31 2.86 – 7.64	3.42 2.44 – 6.51	630 0 – 16700
Gr. Fontaine III **Median Value** **Range**	51.68 31.03 – 73.70	3.78 1.41 – 8.90	1.81 1.33 – 4.97	1310 0 – 4590
Gr. Fontaine IV **Median Value** **Range**	43.50 20.66 – 155.55	4.10 1.55 – 7.64	3.02 1.07 – 6.51	1080 0 – 160500

Table 17 – Median values of transplanted cells, divided in groups by age (groups A and B), response to treatment (R and NR), and grade of PAD (Fontaine stages III and IV).

Analysing the data, another finding is that patients with PAD at stage IV of Fontaine Classification have not a reduced capability of stem cells mobilization with respect to patients at a less advanced grade. In fact, in the two groups of Fontaine stage III and Fontaine stage IV, there are no significant differences of stem cells amount. The subdivision of cell counts by stratification of patients for Fontaine stage showed the same level of mobilization between the two groups, as summarized in Table 17. This state is not clearly explainable, in fact, although the clinical results

seem to be encouraging, the cytofluorometry count data did not correlate with them. Possibly, the number of cells mobilized is not a parameter who can alone have influence on the evolution of more advanced stages of CLI, especially when ulcers, gangrene and infection are present. In our opinion, our data, showing a good cells' mobilization even in these kinds of patients, could be considered a promising starting point for further studies.

Furthermore, an argument can be made on the relation between age and cells' mobilization. As we know, PAD affects mainly elderly patients and the results obtained demonstrate that adults over 60 years old are also able to mobilize $CD34^+$ hematopoietic cells from the bone marrow and, with adequate stimulation, very high numbers of $CD34^+$ cells/Kg of weight. Cells count results into two groups differentiated by patient's age is displayed in Table 17. Summarizing, the levels of cell mobilization are higher in younger patients of group A; in particular, the absolute numbers extrapolated without relation to body weight are, respectively for group A (< 60 years old) and group B (≥ 60 years old): 428.33 x 10^6 versus 253.29 x 10^6 for $CD34^+$ cells ($p < 0.001$) and 330.47 x 10^6 versus 190.83 x 10^6 for $CD34^+/CD133^+$ cells ($p < 0.001$). Despite the evident differences in values, we can notice that even elderly patients reached good levels of mobilization (Ranges: 90 – 447 x 10^6 of $CD34^+$ cells; 58 – 333 x 10^6 of $CD34^+/CD133^+$ cells; 0 – 1000000 x 10^6 of EPC cells).

The statistically significant reduction (range 0-2, $p < 0.001$) of the median Wagner score between the evaluation before transplant and the measurement at 6-month follow-up and the healing of a part of the ulcers after the cells treatment could be considered an encouraging outcome of this therapy. It is not easy to define what could be the involved mechanism, as well as is not possible to exclude that some of those ulcers should heal even without the treatment, since it is known that evolution and extension of ulcers, especially if of small dimensions in

diabetic patients, often do not follow a linear way, alternating periods of worsening to periods of amelioration or healing. Besides, localization, extension and number of ulcers are important in the healing mechanism. In our experience the more extensive ulcers are associated to a worse response to the treatment as well as all the three patients with wider ulcers underwent to major amputation. The variability of localization and number of ulcers is one of the points which make difficult to clearly define the results on ulcers, in fact some of the ulcers can heal and some other not in the same patient and the choice to use a classification to evaluate the grade of ulcers more than their dimensions and number was made to have an instrument easier to manage and confront.

The decision to inject the cells also in regions surrounding the ulcers or gangrene lesions has been taken in order to try to obtain the best results in ulcers healing and pain reduction, following the encouraging results of some authors. In particular González Sarasúa et al. obtained good results in the treatment of type IV pressure ulcers in patients with spinal cord injuries (SCI) using autologous BM-MNCs.[177] Moreover Lu et al. compared BM-MSCs, BM-MNCs, and saline to see which of the cell populations increased the rate of healing in diabetic ulcers, injecting subcutaneously cell suspensions of the different cell populations around the foot ulcers. Both of the cell treatments led to improve the blood circulation around the wounds, as well as increase the amount of pain-free time experienced by the patients.[178]

One of the more interesting clinical results is the reduction of pain, in some cases quite immediate after the procedure. Most of transplanted patients showed a significant improvement of pain. A rapid decrease of VAS values is observed during the first month post transplantation and another reduction occurs after 6 months (Fig. 14 in the Results section).

Pain relief sometimes is observed already in the first hours after the treatment, as showed in another review study.[171] In these cases is hard

to define if the reduction of pain is really due to the procedure or should be considered even a possible psychosomatic positive response in people aware that this procedure could be the last therapeutic chance before limb amputation. Besides, the result observed in the first month could be influenced by analgesic treatment, often still continued at high levels during the first months of follow-up. In our opinion, 6 months after the procedures these influences are very unlikely, so the pain relief could be considered real although is not possible to clearly relate this clinical finding to an effective beneficial response of tissues of the ischemic limb after the transplantation.

The reduction of analgesic medication consumption occurred in a large number of patients, in particular in the Fontaine stage III group, this reduction of dosage in many cases was progressive and noticed in different periods of the follow-up, consequently is hard to define in which precise moment after the treatment it occurred. Moreover the variability of dosage and drugs used is due to the fact that, in most cases, the antalgic therapy was set before the patient came to our observation. The explanation of the greater reduction of antalgic drug need in Fontaine stage III patients is unclear as not directly correlate with VAS obtained data on the two Fontaine Groups, but in presence of ulcers or gangrene the pain relief is usually more difficult. Maybe the VAS reduction in Fontaine stage IV patients, without a correspondent reduction of drug assumption, could be explained with the fact that, in many cases, these patients cannot reach an optimal pain control despite the use of high dosage of antalgic drugs, so a subjective reduction of pain could be supposed to mean that the same dosage of drug, after the procedure, has become sufficient to obtain a better pain control.

Obtaining pain relief, in our opinion, is fundamental, mostly in more advanced CLI stages, in fact in everyday clinical practice is well known that, in some cases, the decision to proceed to the amputation of the limb

is taken not only for the worsening of ulcers or perfusion indicators, but often in order to give pain relief to patients without any other further therapeutic option for pain management.

A curious effect in the G-CSF injection phase (5 days of bone marrow stimulation and $CD34^+$ mobilization) occurred in some cases: some patient reported an important relief in index leg pain (lowering of VAS) after the second or third day of bone marrow stimulation. This singular event has not a clear explanation, but it could be supposed to be promoted by the spontaneous homing in the damaged tissue of the EPC mobilized after bone marrow stimulation, as reported by other authors.[124,125,126,127,130,179]

It can be supposed that the generation of new vessels, even if is clearly identifiable at angiography only in a reduced number of cases (Ph. 12 and 13; see also above Ph. 10 and 11 in the Angiographic finding paragraph of in Results section – 4.8), and the qualitative vessel improvement found in other cases could be involved in the long-term pain relief and in the facilitation of the healing of the ulcers as well as in limb salvage thanks to an increased blood flow.

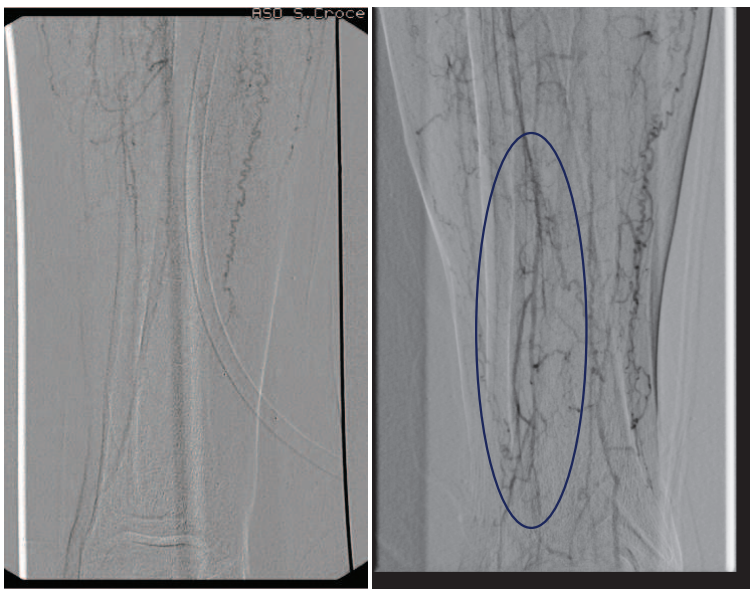

Photo 12 – Angiography pre (L) and post (R) treatment with neo generated vessel.

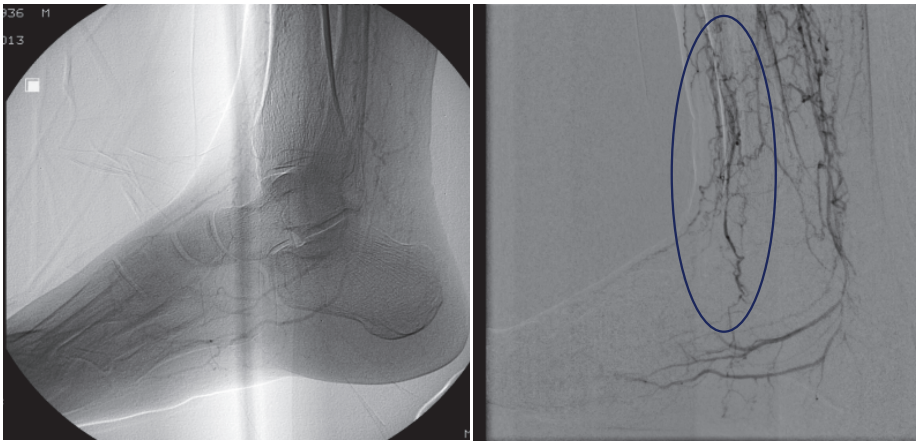

Photo 13 – Angiography pre (L) and post (R) treatment with neo generated ankle vessel.

The genesis of new collateral vessels is described in other studies and, in some cases, the improvement at the angiography is defined not only as the appearance of new vessels, but also as the increasing in length, intensity or apparent size of previously visible vessels.[180] In particular, this phenomenon is observed in patients who saved their legs: the vessels at the follow-up angiogram appeared wider and with more regular walls than in the first examination. Hence, even if the quantity of vessels did not change, it could also be important to consider the quality of the vessel of the limbs. An improvement of which could suggest an explanation for the clinical amelioration of patients that did not show any increase in the number of collateral vessels, supposing an improvement of distal flow after the stem cells treatment.

New vessel generation was observed in just a small number of patients in this study, therefore another reason for pain and clinical improvement should be searched in the variation of inflammatory response maybe generated by the homing of EPC in the damaged tissue.[126,127,130] This migration of EPC, even in the bone marrow stimulation period before the transplantation, could be involved to explain the singular effect, experienced by some patients, of relief of pain symptoms during the days of injection of the G-CSF (see above).

No statistically significant modifications were reported in ABI and TBI values. The average values of ABI and TBI calculated before the treatment seem to confirm that the selected patients might be considered a group of "milder" CLI and, from this point of view they can be considered a good sample for the study. The average values increased after the procedure in all groups of Responders but, as previously mentioned, these variations were not statistically significant. However the importance of an increase of these hemodynamic parameters as a predictive tool for PAD or CLI clinical improvement after the cells therapy is still today unclear and, about this matter, literature findings are controversial. In fact, in some studies, it is underlined an increase in ABI values after the procedures, but, according to other authors and review papers, there is no evidence of this occurrence even in case of good clinical results.[139,166]

Analysing the data obtained during this study, the results indicate that the intramuscular transplantation of a high number of $CD34^+$ cells/kg in patients suffering of CLI can be considered a safe procedure, as showed by other authors.[147]

As mentioned, the main goal of this protocol is to ameliorate the rate of inferior limb salvage obtained in other similar previous studies, possibly reaching a percentage near to 70%.

The obtained Amputation Free Survival (AFS) rate was 77.4% at 24 months of follow-up, so the principal goal was achieved. Moreover, according with complication rates, we can confirm that the protocol is safe and could be considered encouraging in effectiveness in the treatment of patients affected by CLI and untreatable by other means, with results equivalent or improved with respect to similar studies.[158,174,175,176]

The low AFS rates obtained in patients with advanced necrotic lesions or extensive gangrene, either with surgical/endovascular therapy or stem

cells therapy, and the promising results of this study suggest, once again, how is important to be prompt to treat CLI patients. The stem cells intramuscular transplantation could be a good choice in case of poor results or unsuitability of surgical/endovascular direct revascularization, but is essential to act promptly and avoid waiting too long before considering this kind of therapy, in order to avoid treating patients at a too advanced stage of arterial disease.

An issue not yet adequately understood and still to clarify is whether the stem cells stay on site after implantation or not. In fact, as we have seen, one of the goals of the procedure of transplantation is to inject the cells as close as possible to the site in which the generation of new vessels is desired, but is not possible to exclude that some of that cells are flushed from that site to other locations, especially if the injection occurs accidentally near a venous vessel. In further studies, the injected cells could be marked to quantify the percentage of them that are washed away or dislocated. If this phenomenon were confirmed in high percentage, it could be proposed to design scaffolds to reduce this problem and to help the cells to remain where they are more useful. Recently, some authors suggested the use of pulsed Focused UltraSounds (pFUS) to enhance homing of MSC intravenously infused in mice with induced CLI (ligature of iliac artery). The results obtained with proteomic analyses seem to confirm that pFUS upregulates local chemoattractans and increases the muscle tropism of MSC. Moreover, it has been observed that MSC express more Interleukyn 10 (IL-10) and Vascular Endothelial Growth Factor (VEGF) when the homing occurs in pFUS treated ischemic muscles compared to untreated CLI muscles.[181] These findings, if confirmed in humans with more studies, could be very interesting in order to associate the local intramuscular injection with concomitant or antecedent ultrasound treatment to enhance the efficacy of the procedure.

6 CONCLUSIONS

Obtained data confirm that these procedures are safe with relatively low rates of complications, considering the age and the comorbidities of the patients' population.

Stem cells therapy confirms to be promising for the effective treatment of "no-option" CLI patients with a favorable safety profile.

The inoculated dose of $CD34^+$ cells could have a central role in this treatment as higher doses of these cells (almost 2×10^6/Kg of bodyweight) seem to be associated with clinical benefit and better results. An important role in the mechanism could also be hypothesised for the supposed synergistic effect of a coordinated action with a mixture of active cells, cytokines and trophic factors in response to ischemia and inflammation.

On the other hand, the most advanced stage of arterial disease and limb ischemia are associated with worse clinical response to the cells' transplantation treatment.

These results are promising, but should be interpreted carefully, as the duration of follow-up is not sufficiently long. Further studies should be focused on the specific mechanisms by which these cells promote revascularization and tissue repair.

In our opinion, it remains the need of additional phase III, double-blinded, randomized and placebo-controlled trials on a larger patient population, to verify the efficacy and safety of data obtained in this and other studies.

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