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Chairman: Prof. Francesco Beguinot

PH.D. THESIS

Identification of predictive biomarkers for cell-based therapy in critical limb ischemia

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LIST OF SELECTED PUBLICATIONS (2018-2021)

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2. Santaniello A*, **Cimmino I***, Dipineto L, Agognon AL, Beguinot F, Formisano P, Fioretti A, Menna FL, Oriente F. *Zoonotic Risk of Encephalitozoon cuniculi in Animal-Assisted Interventions: Laboratory Strategies for the Diagnosis of Infections in Humans and Animals.* Int. J. Environ. Res. Public Health 2021, 18(17), 9333. doi:10.3390/ijerph18179333.
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3. Prisco F, De Biase D, Piegari G, Oriente F, **Cimmino I**, De Pasquale V, Costanzo M, Santoro P, Gizzarelli M, Papparella S, Paciello O. *Leishmania spp.-Infected Dogs Have Circulating Anti-Skeletal Muscle Autoantibodies Recognizing SERCA1*. Pathogens. 2021 Apr 12;10(4):463. doi: 10.3390/pathogens10040463.

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LIST OF ABBREVIATIONS

ABI: ankle-brachial index

β-AR: beta adrenergic receptor

bFGF: basic fibroblast growth factor

BM: bone marrow

CCL5: C-C motif Chemokine ligand 5/RANTES

CD: cluster of differentiation

CLI: critical limb ischemia

CTA: computed tomography angiography

DMEM: Dulbecco's modified Eagle's medium

eNOS: endothelial nitric oxide synthase

EPCs: endothelial progenitor cells

FACS: flow cytometry analysis

G-CSF: granulocyte-colony Stimulating Factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

hb-EGF: heparin-binding epidermal growth factor-like growth factor

IC: intermittent claudication

IFN-γ: interferon gamma

IL: interleukin

IP-10: interferon gamma inducible Protein 10

MCP-1: monocyte Chemoattractant Protein-1

MIP-1 α: macrophage Inflammatory Protein-1alpha

MIP-1β: macrophage Inflammatory Protein-1 beta

MRA: magnetic resonance angiography

MSCs: Mesenchymal stem cells

NO: nitric oxide

PAD: peripheral arterial disease

PB: peripheral blood

PBMC: peripheral blood mononuclear cell

PDGF: Platelet derived Growth Factor

PTA: percutaneous Transluminal Angioplasty

TASC: trans-Atlantic Inter-Society Consensus

TBI: toe-brachial index

tcpO2: transcutaneous oxygen dioxide tensions

TNC: total nucleated cells fraction

TNF-α: tumor necrosis factor alpha

TT: treadmill test

VEGF: vascular endothelial growth factor

ABSTRACT

Critical limb ischemia (CLI) is a pathological condition characterized by chronic ischemic at-rest pain, intermittent claudication, ulcers, or gangrene in one or both legs related to peripheral artery disease. Smokers, people affected by atherosclerosis, diabetes mellitus, hypertension and hyperhomocysteinemia have a high risk for CLI and prevalence of CLI is continuously rising in most countries as consequence of the several risk factors. Patients with CLI may develop irreversible ischemic damage to the leg or foot if they do not receive appropriate treatment and this may lead to the need for amputation. Actually, the main goal of critical limb ischemia treatment is to preserve the limb by re-establishing blood flow to the affected area. After surgical and/or endovascular treatment, approximately 50-60% of patients benefit, while the remaining will undergo primary amputation or conservative medical therapy. Biologic therapies, which include gene therapy and cellular therapy, offer the potential to promote wound healing and prevent amputation in patients who otherwise have poor options for revascularization.

In this study, I focused my attention on the application of bone marrow cellular and soluble components in order to identify prognostic biomarkers in patients undergoing stem cells therapy. To this aim, 40 patients were enrolled in the study. For each patient, bone marrow (BM) samples were collected before and after cell concentration with Sepax-2 instrument. In parallel, peripheral blood (PB) samples were collected at baseline (T0). BM samples showed a statistically significant increase of EPC fraction compared to PB samples. Among nucleate cells from BM concentrate EPCs and MSCs were isolated in presence of specific cell-culture conditions. Pro-angiogenic cytokines and chemokines were found at higher concentrations in BM compared to PB. Among the released factors, IL-9 and PDGF concentration, both in serum and in BM, positively correlated with improvement (T1-T0) of treadmill test (ΔTT) and transcutaneous partial pressure of oxygen (ΔtcpO2), respectively. Thus, this study confirmed the useful application of cell therapy in improving pain and revascularization in patients

affected by CLI and promoted soluble factors, such as IL-9 and/or PDGF, as prognostic factors in the management of the pathology.

1. BACKGROUND

1.1 Critical limb ischemia

Critical limb ischemia (CLI) is an advanced manifestation of peripheral arterial disease (PAD), characterized by the presence of rest pain, intermittent claudication (IC) and trophic lesions, such as ulcers and/or gangrene, due to a severe compromise of blood flow to the affected extremity. Unfortunately, to date, the most probable outcome of CLI, in the absence of significant hemodynamic improvements, is represented by a high rate of mortality and amputation (1) (Figure 1).

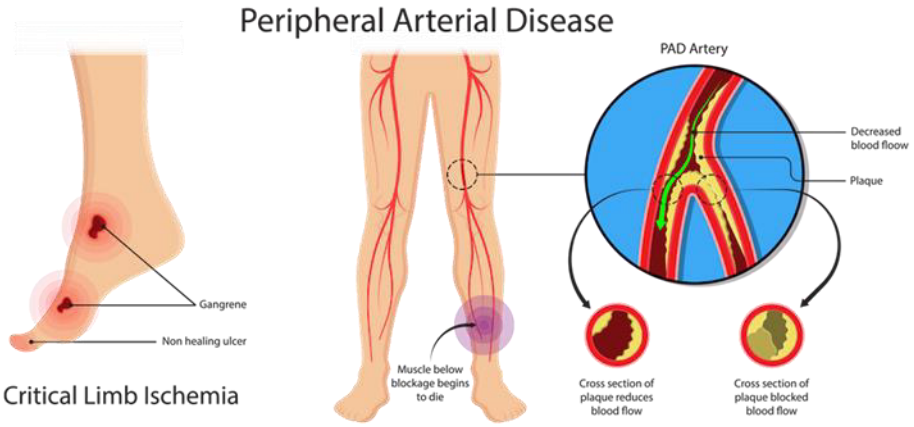

Figure 1. Critical Limb Ischemia, the advantage stage of PAD

The incidence of CLI is largely underestimated, since the data of severe arteriopathy affecting lower limbs are based on extrapolations deriving from the evolution of intermittent claudication (2-14). In general, the incidence of CLI calculated on the natural evolution of IC estimates an annual incidence of 1% in the 5 years following the diagnosis of IC (15). The Framingham study demonstrated an increase in the prevalence of the disease from 0.4 per 1000 males aged 35-45yrs to 6 per 1000 males aged >65yrs (16). In the United States, the incidence of CLI is estimated to be 3500 cases per 1 million (1), while

another interesting study reported an annual incidence of CLI in Italy which reaches approximately 26.700 individuals in the whole Italian population (almost 57.000.000 people) (17). However, these numbers are expected to increase in the next years, if no action is quickly taken, as consequence of the several risk factors. Indeed, risk factors of CLI are different and include atherosclerosis, cigarette smoking, diabetes mellitus, hypertension and hyperhomocysteinemia. Atherosclerosis accounts for more than 90% of cases of PAD (18). The chances of developing PAD in smokers compared to non-smokers is doubled and the severity of the disease is directly related to the number of smoked cigarettes and the length of time they smoke (19). In addition, smoking triples the risk of worsening arterial disease and doubles the risk of amputation (20, 21). In subjects with diabetes mellitus, CLI shows a triple frequency compared to the corresponding non-diabetic arterial disease and has a 15 times higher amputation rate (19, 21). Interestingly, hypertension is associated with CLI although weaker than the coronary artery disease (5, 16, 22, 23). Among the most recent risk factors of CLI, elevated homocysteine levels have been associated with a 2-3 times greater risk of developing PAD and, approximately 30-40% of patients with CLI have high levels of homocysteine (24-29). Hyperhomocysteinemia also appears to increase the risk of PAD progression (27, 30). However, it still not clear whether the treatment of hyperhomocysteinemia may improve ischemic events and contribute to the regression of arterial disease (31). Finally, nutrition also plays an important role in preventing CLI. Several studies suggest a probable protective effect exerted by the Mediterranean diet and the antioxidant action of red wines on the evolution of obliterating arteriopathy of the lower limbs (32-35).

The typical symptom of the CLI at rest is a constant burning sensation or numbness in the ankle or foot in the absence of activity. Elevating the extremity exacerbates symptoms while placing the limb in a dependent position provides relief. Intermittent claudication, a cramping pain in the muscles of the lower limbs downstream of the vascular lesion occurs during exercise, such as walking,

and is relieved by a short period of rest (1). This symptom is due to the discrepancy between the oxygen demand and oxygen supply by the muscle and has a negative impact on various aspects of quality of life (36). Progression of CLI induces the onset of pain even at rest, in particular during the hours of night sleep, where the supine position exacerbates an already compromised hemodynamic situation. In a minority of patients, the clinical picture can evolve towards critical ischemia, with the possible appearance of trophic lesions and gangrene due to severe skin hypoxia. This condition may require in the most serious forms the surgical revascularization or amputation of the affected limb (37). Among patients with claudication followed for 5 years, about 75% remain stable, 20% develop an aggravation of claudication and 5% have critical ischemia of the lower limbs. Contrary to what is generally believed, the risk of amputation in patients with claudication is about 1% per year (38). Amputation is much more frequent once resting pain symptoms or tissue ulcerations become manifest.

For adequate decision on medical treatment setting, it is important to classify CLI. The most widespread classifications of the CLI were proposed by Fontaine (39) and Rutherford (40). The first distinguishes four stages: 1st: asymptomatic PAD; 2nd: intermittent claudication; 3rd: pain at rest; 4th: skin lesions and gangrene. Stage 2, in turn, is divided into stage 2 A and stage 2 B, characterized by an autonomy of movement (absolute distance of claudication, ACD) respectively greater or less than 200 meters. Rutherford's classification indicates 3 degrees and 6 categories (grade 0, category 0: silent arterial disease; grade 1, category 1-2-3: mild-moderate-severe claudication; grade 2, category 4: ischemic pain at rest; grade 3, category 5 -6: partial loss of tissue - extensive loss of tissue) (Table 1).

Fontaine et al.		Rutherford et al.		
Stage	Clinical presentation	Grade	Category	Clinical presentation
I	Asymptomatic	0	0	Asymptomatic
IIa	Mild claudication	I	1	Mild claudication
IIb	Moderate to severe claudication	I	2	Moderate claudication
III	Ischemic pain at rest	I	3	Severe claudication
IV	Gangrene or ulceration	II	4	Ischemic pain at rest
		III	5	Minor tissue lesion
		III	6	Major tissue lesion

Table 1. Fontaine and Rutherford peripheral arterial disease classifications

Actually, Society for Vascular Surgery issued new classification named WIfI system which evaluate the foot lesion comprehensively by three factors; Wound (W), Ischemia (I), and foot Infection (fI). Guidelines for peripheral arterial disease recommend use of WIfI classification system. This classification attributes a 4-grade scale to each letter or parameter, running from 0 to 3, where 0 represents absent, 1 mild, 2 moderate, and 3 severe. Severe but localized infection is defined as grade 2, whereas systemic infection is defined as grade 3 (41).

Diagnosis of CLI is usually based on the anamnesis and physical examination, that allow to identify signs and symptoms of this diseases. However, to achieve the correct diagnosis it is necessary to perform several other clinical tests. For this purpose, non-invasive techniques are used including the measurement of the Ankle-Brachial Index (ABI), consisting in the systolic pressure at the ankle, divided by the systolic pressure at the arm, that represents the ratio of ankle-to-brachial systolic blood pressure. Normal values measured at rest are between 1.30 and 0.91; values between 0.90 and 0.40 indicate a mild to moderate degree, values below 0.40 indicate a severe level of illness. Values over 1.30 are an

indication of incompressibility of the vessels probably due to calcinosis (22, 42). The ABI can be falsely high in the presence of stiffened ankle arteries related to medial artery calcification, a condition mostly observed in patients with diabetes or chronic kidney disease. In this case, it is recommended to measure the toe-brachial index (TBI), the ratio of the toe-to-brachial systolic blood pressure, because medial calcification rarely affects digital arteries. In general, a TBI ≤0.70 is accepted as diagnostic for PAD (42). When required, especially in anticipation of surgery, the execution of other more sophisticated radiological imaging techniques such as computed tomography angiography (CTA), magnetic resonance angiography (MRA), or the more invasive angiography may be indicated. For evaluating the indication of revascularization in patients with PAD, both CTA and MRA are considered as appropriate imaging tests (43). The sensitivity and specificity of multidetector CTA compared with angiography is ≈90% for detecting PAD. CTA uses iodinated contrast and ionizing radiation to visualize pathology from the aorta to the lower extremity. The scan times take a few seconds, but diagnosis can be difficult in small tibial vessels with calcification and multiple occlusions.

The sensitivity and specificity of MRA in detecting PAD with stenosis >50% is the same as CTA, 90% to 100% (44). MRA has several advantages in diagnosing PAD over CTA. Indeed, MRA requires no radiation and calcium does not interfere with the diagnosis. Another advantage of MRA is that it allows for hemodynamic measurements.

Further investigations that can be carried out are the evaluation of the microcirculation through the monitoring of the transcutaneous oxygen and carbon dioxide tensions (TcpO2 and TcpCO2) (45). This technique is a non-invasive method for patients who need continuous monitoring of oxygen and carbon dioxide with minimal blood draws and can quantify the level of local muscle oxygenation in the arteriopathic patient during the execution of an incremental protocol on the treadmill and for the study of oxygen consumption at rest (46-48).

Diagnostic angiography, the gold standard for preoperative imaging, requires less contrast than CTA, provides optimal resolution for distinguishing distal lesions, and facilitates immediate endovascular treatment. However, catheter-based angiography is invasive and not without complications (1).

The prognosis of patients affected by critical ischemia of the lower limbs is particularly severe due to the high mortality and morbidity rate, already evident within a few months of follow-up from the diagnosis of CLI. Indeed, both the European Consensus and subsequently by the Trans-Atlantic Inter-Society Consensus (TASC) indicated that about 20-25% of the patients have a risk of major amputations and mortality already within one year of diagnosis (20, 21, 49).

In addition, an Italian multicenter study of 1.560 patients with no option CLI showed a rate of amputations greater than 12% within one year of diagnosis, while the mortality rate was 13% within six months, 22% within 1 year and 31.6% within two years (49).

Therapeutic treatments of a CLI patient depends on the severity of the disease. They should predict the progression of the atherosclerotic process, prevent morbidity and mortality from cardiovascular events, improve walking performance and quality of life and in multiple severe stages also suggest the best choice between the different surgical revascularization techniques. The reduction of the atherosclerotic and the cardiovascular risks should include the elimination of cigarette smoking and the monitoring of glycemia, blood pressure and dyslipidemia (37). Among the drugs specifically indicated for PAD, it seems recommended the only the use of antiplatelet agents, in particular aspirin and alternatively clopidogrel. Indeed, some studies performed on claudicant patients showed that these drugs were able to slow down the progression of the atherosclerotic process and to reduce the risk of heart attack and stroke (50, 51). Other drugs, such as alpha blockers, calcium channel blockers, papaverine seems to be not particularly useful (52). In some studies, Cilostazol, a phosphodiesterase III inhibitor with vasodilator and anticoagulant activity, has

shown benefits in PAD (53, 54). L-propionyl-carnitine, in addition to being well tolerated, has shown a positive effect on functional capacity and protection from ischemic damage (55-59).

The mainstay of CLI treatment in patients with salvageable limbs is revascularization to improve distal perfusion. Revascularization options include open surgery, endovascular treatment, or "hybrid" combinations of both approaches. Open techniques for revascularization comprise arterial bypass and endarterectomy while endovascular therapy incorporates angioplasty, stenting, and/or atherectomy. The optimal approach in patients who are candidates for multiple revascularization options is controversial (1).

Revascularization includes several techniques such as dilation and recanalization of an artery (Percutaneous Transluminal Angioplasty or PTA) or traditional surgical techniques such as thromboendarterectomy or by-pass of blocked arteries. The use of surgery is justified in the more advanced stages of the disease, while in the intermediate stages is to be considered for situations of symptomatic failure or failure of global rehabilitation and pharmacotherapy.

Despite the rapid development of interventional techniques, therapeutic options for patients with CLI remain limited: approximately 40% of patients with CLI are not eligible for surgical or endovascular revascularization (patients with chronic critical limb ischemia with no revascularization option) (60). Thus, new diagnostic and therapeutic options for revascularization are of the utmost importance.

Gene therapy offers a potential efficacious therapy for patients with CLI, especially given that in the >1000 individuals treated with gene therapy for therapeutic angiogenesis in phase I and II trials, adverse events have been similar between treatment and control groups. Endothelial progenitor cells (EPC) or mesenchymal stem cells (MSC) derived from bone marrow or peripheral blood are another subset of emerging therapies implicated in the regeneration of injured endothelium and neoangiogensis after tissue ischemia and thus have been identified as a new potential therapeutic target in CLI (61) (Figure 2).

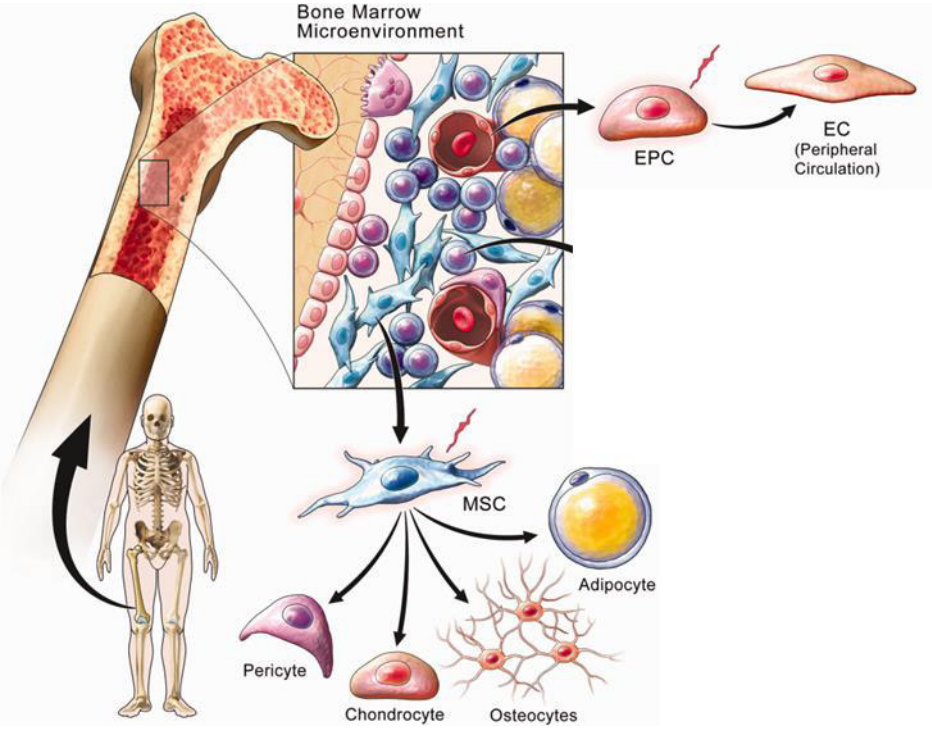

Figure 2. Pro-vascular progenitor cells in patients with critical limb ischemia – ***modified by Concise Review: Cell Therapy for Critical Limb Ischemia: An Integrated Review of Preclinical and Clinical Studies - Qadura M. et al.- 2018***

1.2 Endothelial progenitor cells (EPCs)

Until a few years ago it was thought that the vascularization of ischemic tissues in adults was restricted to the migration and proliferation of mature endothelial cells, a process known as "angiogenesis" (62). However, a population of mononuclear cells (MC), including endothelial progenitor cells (EPCs), has been identificated in both bone marrow and peripheral blood. The first descriptions of EPCs were by Asahara et al.in 1997 (63) who showed that, even in adults, bone-marrow-derived hematopoietic progenitor cells (PCs) can give rise to endothelial cells (ECs) that contribute to active neovascularization in ischemic tissues. These cells were termed EPCs, and since their discovery intense effort has been focused on defining the role of EPCs in the regeneration of injured endothelium, neovascularization of ischemic tissue and cancer angiogenesis. Other evidence has suggested that particular stimuli (e.g., growth factors) or pathological

conditions (e.g., ischemia) are able to mobilize EPCs, which are cells circulating in small quantities in the blood stream and are capable of implanting themselves in physiological and pathological neovascularization sites or in damaged tissues and then, to differentiate into endothelial cells (62). This process is known as "vasculogenesis" (64, 65) (Figure 3).

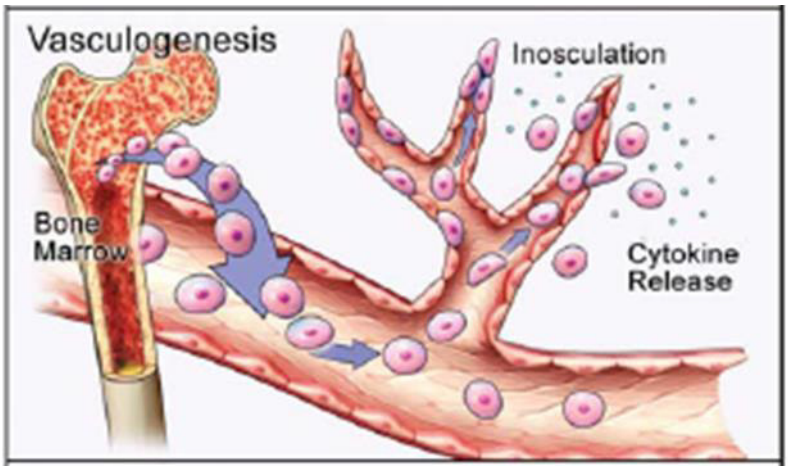

Figure 3. Vasculogenesis, one of the major mechanisms of blood vessel regeneration – ***modified by Concise Review: Cell Therapy for Critical Limb Ischemia: An Integrated Review of Preclinical and Clinical Studies - Qadura M. et al.- 2018***

Studies, conducted both in animals and in humans, have shown that EPCs are able to improve the function of ischemic tissues, both by inducing and modulating vasculogenesis and angiogenesis, in areas with reduced oxygen supply, and by stimulating the re-endothelialization of damaged blood vessels (66). EPCs express some markers including CD34, vascular endothelial growth factor receptor-2 (VEGF-R2) and CD133 (67-69). Upon release, EPCs begin to lose the expression of CD133, and to acquire the expression of markers characterizing endothelial cells, in particular, CD31, which plays an important role in the interaction between adjacent endothelial cells, VE-Cadherin, involved in the organization of new vessels and vWF, a multimeric protein that mediates platelet adhesion closer to endothelial damage.

The release of EPCs into the circulation from the bone marrow is regulated by a large variety of molecules such as the endothelial nitric oxide synthase (eNOS) (70). Tissue ischemia stimulates the production of factors such as VEGF, which by acting on its KDR receptor, activates the phosphorylation of eNOS via Akt. The production of nitric oxide (NO) is essential for the activation of MMP-9, a key enzyme, for the mobilization of stem and progenitor cells, including the haemangioblast (70, 71). eNOS-mediated NO production is also under the control of the adrenergic system, which through the α2 and β adrenergic receptors (βARs) modulates the release of NO, causing endothelial vasodilation. Several evidence has indicated that CLI risk factors play a negative role on EPC. Indeed, smoking is the major independent predictor for the reduction of EPC levels, while a reduced migration is mainly related to high blood pressure. Hyperglycemia significantly affects the function of EPCs, reducing their survival and altering their functioning (72, 73).

1.3 Mesenchymal stem cells (MSCs)

Likewise, apart from EPCs, mesenchymal stem cells (MSCs) also represent an important group of cells used in vascular regeneration.

MSCs are stromal cells with the ability of self-renewal and differentiation into various cell types such as chondrocytes, osteoblasts, adipocytes, and myoblasts (74). These cells were discovered more than 40 years ago by Friedenstein A.J. et al., which described a population of bone marrow cells similar in appearance to fibroblasts, capable *in vitro* of differentiating into typical cells of the mesenchymal line (75) through the use of specific media (76). In next years, several studies have demonstrated the ability of these cells to differentiate both *in vitro* than *in vivo*, into neuronal cells (77), hepatocytes (78), skeletal muscle cells (79), cardiac cells (80). Human mesenchymal stem cells were initially isolated from the bone marrow (hMSCs) and subsequently identified in other tissues as well, such as muscle, connective tissue, adipose tissue and various fetal tissues. Amniotic fluid and placenta are also rich in MSCs (81, 82).

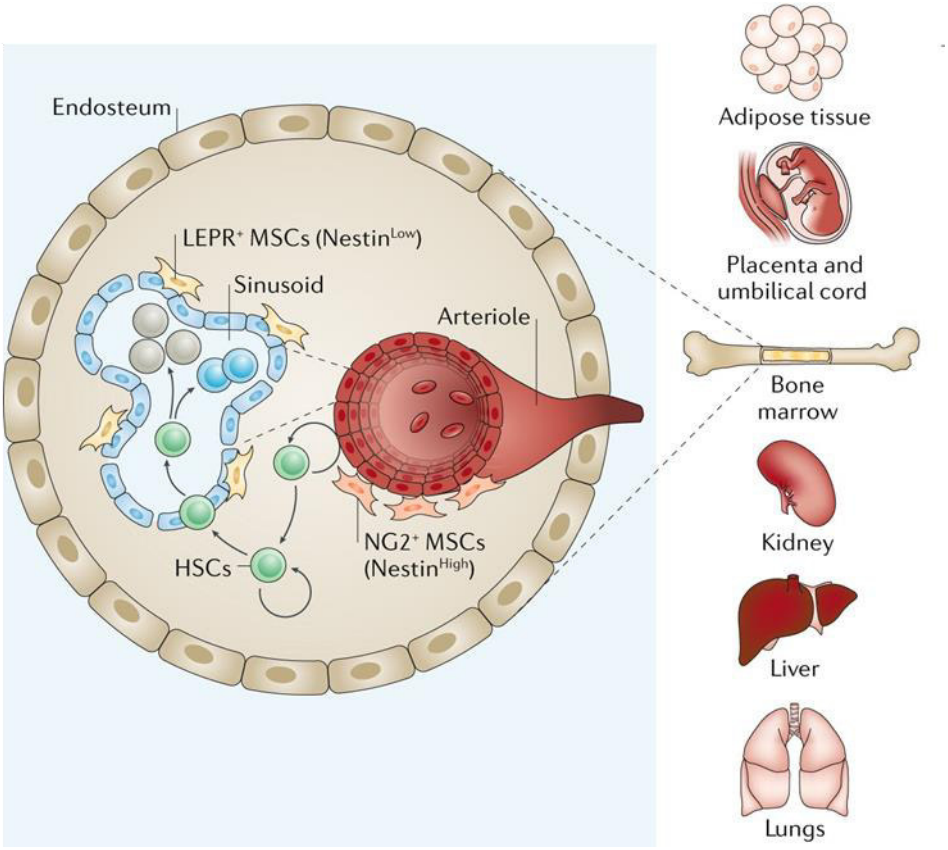

Figure 4. Isolation and characterization of MSCs – *modified by Immunoregulatory mechanisms of mesenchymal stem and stromal cells in inflammatory diseases – Shi Y et al. – 2018*

Adult MSCs isolated from bone marrow aspirates are characterized by a heterogeneous morphology and represent the ideal candidates for the development of future cellular and gene therapies for several reasons: 1) they are easily obtainable from adult tissues and therefore their use it does not present ethical problems, unlike embryonic stem cells; 2) they show poor immunogenicity as they possess a superficial phenotype poorly recognized by T cells (absence of MHC II) (83) with consequent poor rejection reaction in autologous and allogeneic transplants; 3) they are able to migrate to damaged tissues and inflammation sites; 4) they can be rapidly expanded in vitro and grown over several passages, without losing the phenotype or differentiation capacity. In addition, MSCs placed in culture are easily identifiable as they form colonies of adherent cells characterized by the typical fibroblastoid morphology.

MSCs can be induced to differentiate through the use of specific media (76) or several tissue factors, such as basic fibroblast growth factor (bFGF) or heparin-binding epidermal growth factor-like growth factor (hb-EGF), which seem able not only to increase their proliferation, but also to interfere with the differentiation capacity, keeping them in the state of multipotentiality (84).

Unfortunately, to date, it is difficult to determine with certainty the role of these multipotent stem cells *in vivo*, a role that cannot be predicted from *in vitro* culture.

For this reason, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC. First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (74).

The therapeutic potential of MSCs transplantation for the treatment of ischemic conditions such as coronary artery disease, peripheral arterial disease, and stroke has been explored in animal models and early-phase clinical trials (85). A substantial database documents the safety profile of MSC administration to humans in a large number of disease states. The mechanism of the therapeutic effect of MSC transplantation in ischemic disease has been postulated to be due to paracrine, immunomodulatory, and differentiation effects (85). In particular, MSCs are reported to promote angiogenesis because of their capacity to induce ECs proliferation, migration, and tube formation, while decreasing apoptosis and fibrosis. Furthermore, MSCs support neoangiogenesis, releasing soluble factors that contribute to stimulate angiogenesis. These cells are thought to improve hind limb ischemia by secreting cytokines that regulate macrophage differentiation to M2, an anti-inflammatory phenotype (86).

Despite promising preclinical studies in animal models, transplantation of bone marrow-derived cell populations in patients with CLI has shown partial benefit

preventing limb amputation (87). Thus, careful preclinical evaluation of emerging concepts and technologies are critical for the expedited development of cell therapy trials for CLI.

2. AIM OF THE STUDY

Critical limb ischemia (CLI) is the most advanced form of peripheral arterial disease and has serious clinical implications such as rest pain, intermittent claudication, and trophic lesions. Smokers, as well as patients with type 2 diabetes and cardiovascular diseases, are the most affected and have a high risk of amputation. Therapy can be pharmacological, but when the used drugs fail, it is required a surgical approach. In particular, the goals of surgical revascularization are to provide an adequate flow in the periphery in order to alleviate ischemic pain at rest or promote the healing of trophic lesions. However, the treatment and management for CLI patients must not only aim to rescue the limb through a peripheral revascularization intervention, but also to optimize medical therapy and to promote tissue repair and regeneration.

Recent studies have highlighted the role of endothelial progenitor cells (EPC), or mesenchymal stem cells (MSC) derived from bone marrow or peripheral blood in restoring tissue response. In particular, CLI patients not amenable to surgical or endovascular revascularization have shown a clear improvement of the disease after treatment with cellular concentrates extracted from autologous bone marrow, as indicated by the increase in blood flow compared to placebo-treated controls. autonomy of travel, increase of the tissue oxygen saturation and significant reduction of pain. Furthermore, amputation has been avoided in 75% of cases.

In this scenario, the main purpose of this study has been to identify possible predictive biomarkers released by stem cells derived from bone marrow for the therapy of no option critical ischemia in patients who have no other therapeutic options.

3. MATERIALS AND METHODS

3.1 Study population

The study population included 40 patients from Vascular Surgery of Casa di Cura Villa dei Fiori - Acerra, 20 of whom affected by no option CLI. The inclusion criteria included: diagnosis of critical limbs ischemia in which the infiltration of bone marrow concentrate is expected; age> 18 years; ability to understand the treatment and acceptance of informed consent; patients in whom at least one of the following diagnostic criteria is found:

- occlusion of the ankle artery with absolute pressure <50 mmHg or ABI <0.4.
- toe pressure <40 mmHg.
- Initial non-hemodynamic macroangiopathic lesions (stage I)
- Intermittent claudication (stage II)
- arterial disease with pain at rest (stage III)
- vasculopathy with the presence of trophic lesions and / or gangrene (stage VI)
- tcpO2 <35% in ambient air.

The patient enrolled in therapy with the following drugs must show at least one month of stability of the relative reference hematochemical parameters: Plavix / aspirin, TAO, statins, anti-hypertensive; Hct> = 28%, GB <= 14000 / mmc, Plt> = 50000 / mmc, INR <= 1.6 or PTT <1.5 x control (to avoid bleeding) NB patients on Coumadin therapy must have an INR <1.6 at the moment enrollment. The exclusion criteria were: life expectancy <6 months for other diseases; history of hematological diseases (Non-Hodgkin's Lymphomas, Hodgkin's Lymphomas, Myelodysplastic Syndromes) which prevent bone marrow transplantation; chronic renal failure during the dialysis treatment; malignant neoplasms or changes in the following tests; PSA (age > 45 years); chest x-ray (age > 50 years); mammography (age > 40 years); PAP test (age > 21 years); decompensated Diabetes Mellitus (glycosylated hemoglobin > 10%); high anesthetic risk (ASA class IV, V); ischemic injury that puts the patient's life at risk and requires immediate amputation; complete occlusion of the iliac axis not

treated surgically or endovascularly, ipsilateral to the side to be treated; extensive necrosis of the lower limb to be treated or other conditions that make limb amputation inevitable; clinically active infection treated with antibiotics within 7 days of enrollment; treatment with immunosuppressive drugs (including Prednisone > 5 mg / day); women who are pregnant or breastfeeding. Personal, anamnestic, and biochemical data will be collected at the admission in hospital (T0) and after 30 days (T1) (glycaemia, creatininemia, alanine-amino-transferase, total and HDL cholesterolemia, triglyceridemia, total protidemia, ferritinemia, C-Reactive protein). Measurements of height, weight and abdominal circumference will be taken. In all subjects, a peripheral blood sample and an aliquot of bone marrow aspirate will be collected before and after cell separation with SEPAX-2 instrument. The bone marrow samples (2 mL of pre-concentration bone marrow aspirate and 2 mL of post-concentration bone marrow aspirate) have been used for:

- Determination of the percentage of mesenchymal stem cells (MSCs) and endothelial progenitors (EPCs) by flow cytometry analysis (FACS) of specific surface antigens.
- The isolation and *in vitro* culture of MSC and EPC cells population.
- The determination of a panel of cytokines, chemokines, and growth factors by ELISA in Multiplex.

The study was approved by the local Ethics Committee. All procedures performed in the study were in accordance with the ethical standards of the institutional or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and conformed to the Declaration of Helsinki on human research. All patients included in the study gave written informed consent after receiving an accurate explanation of the study protocol and of the potential risks related to the procedures adopted by the study.

3.2 Specimen collection

Bone marrow samples were collected during surgery employing Sepax-2 technology. The Sepax® system equipment, combined with the kits for each application protocol, is a fully automated cell separation technology, with the CE ed approval by the American FDA. The Sepax cell separator is a medical device that processes blood or other biological matrix, and its own components through a closed system, therefore sterile, safe, and operator independent. The Sepax operation is based on centrifugation, density gradient separation and extraction for optical detection through the use of the latest generation of high-quality sensors frequency. The equipment also has the unique ability to automatically process small volume cells products. Sepax® technology is patented by Biosafe SA, protected by patents EUR 0912250 and US 6123655, and GMP-compatible. The application software included in the device have been developed specifically to manage processes of volume reduction and automated cell separation, ensuring their total safety. The Sepax system works in combination with dedicated, exclusive disposable separation kits Biosafe SA production. The Sepax disposable kit, an integral part of the Sepax system, offers a system closed and sterile circuit in which the cellular product is separated into its various components.

The software protocols represent an integral part of the Sepax system. The ReadyCell protocol is designed to isolate the fraction of total nucleated cells (TNC, Total Nucleated Cells) from bone marrow blood (30ml to 220ml) in a small final volume of 7-20 ml, with a processing time of about 20 minutes. The procedure involves the withdrawal of the medullary blood; the connection of the bag to the disposable kit (CS-470.1); the installation of the kit on the Sepax equipment; the automatic procedure with the priming and filtration phase; and finally, the cells are collected in a final vial present in the disposable kit. The disposable kit CS-470.1 is designed to process the bone marrow, with the aim of obtaining TNC fraction with a high cell concentration, placing the product final in a vial.

3.3 Cell isolation and growth

1X lysing solution was added to each bone marrow red blood samples that were immediately vortexed. Each tube was incubated at room temperature, protected from light, for 15 minutes. The peripheral blood mononuclear cell (PBMC) fraction containing stem cells was obtained by centrifugation at 200 g for 5 min. After having carefully aspirated the supernatant, 1X PBS containing 1% heat-inactivated fetal bovine serum and 0,1% sodium azide (PBS-FBS) was added to each sample that was next centrifugated at 200 g for 5 min. Then, supernatant was carefully aspirated, and the pellet was resuspended in 1mL of serum-free medium supplemented with 0.25% bovine serum albumin and counted. For growth evaluation, cells were counted with the TC10TM automated cell counter (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol and divided equally in two aliquots that were plated in T-25 flasks in regular Dulbecco's modified Eagle's medium (DMEM)-F12 (1:1) supplemented with 20% fetal bovine serum, 2-mM glutamine, 100-unit/ml penicillin, and 100-unit/ml streptomycin for MSC isolation (Lonza Group Ltd, Basel, Switzerland) and EGM-2 Endothelial Medium BulletKit supplemented with 2% fetal bovine serum and specific growth factor (human Epidermal growth factor hEGF, vascular endothelial growth factor VEGF, R3-Insulin-like growth factor R3-IGF-1, Ascorbic acid, Hydrocortisone, human Fibroblast growth factor-beta hFGF-β, Heparin) for EPCs isolation (Lonza Group Ltd, Basel, Switzerland).

3.4 Flow cytometry analysis

Percentage and expression of endothelial and mesenchymal markers on isolated cells were analyzed by flow cytometry. Total BM and PB samples were suspended in 100µl phosphate-buffered saline (PBS) for analysis. Cells were incubated with PE-conjugated anti-CD73 antibody, FITC-conjugated anti-CD90 antibody, APC-conjugated anti-CD34 antibody, PE-conjugated anti-CD133 antibody and PE Vio770-conjugated anti-CD45 antibody as well as dye/isotype-

matched antibodies (all from BD Biosciences, San Jose, CA, USA) in the dark for 20 min at 4C. Afterward, unbound antibodies were washed out and the samples were centrifuged at 200 g for 5 minutes. Then, the supernatant was carefully aspirated, the pellet was resuspended in 400µl PBS and each sample processed by a BD LSR Fortessa (BD Biosciences, San Jose, CA, USA) and analyzed using BD FACS Diva software.

3.5 Determination of cytokines, chemokines, and growth factors.

Samples obtained after 10.000 g for 10 minutes centrifugation were stored at -80°C until assay was performed. Samples were screened for the concentration of interleukin (IL)-1ra, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, basic Fibroblast Growth Factor (FGF), Eotaxin, Granulocyte- Colony Stimulating Factor (G-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Interferon-γ (IFN-γ), Interferon-γ inducible Protein 10 (IP-10), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 (MIP-1) α, MIP-1β, C-C motif Chemokine ligand 5 (CCL5)/RANTES, TNF-α, Platelet derived Growth Factor (PDGF-BB) and Vascular Endothelial Growth Factor (VEGF) by using the Bioplex multiplex Human Cytokine, Chemokine and Growth factor kit (cat. n. M500KCAF0Y, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol, as previously described [16]. The magnetic Bead–Based Assay was performed on a Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA). (88)

3.6 Statistical analysis

Statistical Analysis were performed using both R software platform (http://www.R-project.org/) and GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, Ca).

3.6.1 Normality Test

The D'Agostino-Pearson normality test was performed to check whether continuous variables followed the normal (or gaussian) distributions model. This test firstly computes skewness and kurtosis of the given distribution, to quantify how much it is far from the gaussian distribution in shape and skewness. Then, it computes how much the single values diverge from the expected values of the gaussian distribution, computing a single p-value for the sum of these discrepancies (89).

3.6.2 Two-variables confrontation

According to the results obtained from the normality test, to check the differences among two continuous variables, t-test for normal distributions and Mann Whitney U test for non-normal distributions were performed.

T-Test

The t-test (or Student's test) is frequently used to statistically compare the equality of the means of two normal populations (unpaired t-test) or of the same population (paired t-test) respect to a given parameter. The t-test is based on two fundamental hypotheses: the first hypothesis assumes that the data relating to the two populations follow a Gaussian statistic, while the second hypothesis assumes that the two populations must have the same variance.

Called X1 and X2 the representative vectors of the data relating to the two populations, the hypotheses to be tested are: $H_0: \mu_1 = \mu_2$ and $H_1: \mu_1 \neq \mu_2$

Where the null hypothesis H0 states that the averages of the data relating to the two populations are equal, while the hypothesis H1 disproves this hypothesis, verifying the two averages differ (90).

Mann Whitney U Test

The null hypothesis in the Mann-Whitney test (MW) is that the two samples are drawn from a single population, and therefore for this reason their probability distributions are equal. The alternative hypothesis is that one of the samples is larger in a stochastic way. This requires that the two samples be statistically independent and that the observations are at least continuous or discrete ordinals. The assumptions of the MW test are:

- The two samples under study by the test are mutually independent and the observations within each sample are independent.
- The observations are comparable (for example, for any two observations, it is possible to establish whether they are equal or whether on the contrary, which of the two is greater).

This test is used to make comparisons on the medians of two subgroups of observations, and therefore constitutes the non-parametric counterpart of the independent-sampled t-test. (91)

Wilcoxon test

The Wilcoxon signed-rank test is a non-parametric statistical hypothesis test used either to test the location of a set of samples or to compare the locations of two populations using a set of matched samples. When applied to test the location of a set of samples, it serves the same purpose as the one-sample Student's t-test. On a set of matched samples, it is a paired difference test like the paired Student's t-test (also known as the "t-test for matched pairs" or "t-test for dependent samples"). Unlike Student's t-test, the Wilcoxon signed-rank test does not assume that the differences between paired samples are normally distributed (92).

3.6.3 Comparison of more than two variables

Comparisons between more than two variables were made using the Tukey corrected ANOVA test or the Kruskal-Wallis test.

ANOVA test

The analysis of variance (Analysis of Variance, ANOVA) is a method, developed by Fisher, which has become fundamental for the statistical interpretation of many biological data and is the basis of many experimental designs. The analysis of variance is used to test the differences between sample means and to this aim, the respective variances are considered.

The principle of this test is to establish whether two or more sample means can derive from populations that have the same parametric mean. When the means are only two it is indifferent to use this test or the t-test, while the ANOVA turns out to be necessary when the means are more than two, or when it is necessary to subdivide the grouping variable into several variables to eliminate sources of variation beyond to that produced by the factor whose effect is to be evaluated.

Even in the case of the analysis of variance, two hypotheses are made. Usually, the null hypothesis H0 tries to state that all the means of the different groups are equal to each other: $H_0: \mu_1 = \mu_2 = ... = \mu_K$.

On the contrary, however, the H1 hypothesis tries to disprove the H0 hypothesis, that is, to verify that there is at least one average different from the others (93).

Kruskal-Wallis

The Kruskal-Wallis test is a non-parametric method to verify the equality of the medians of different groups; is the non-parametric correspondent of the analysis of variance, in which the data are replaced by their rank, and is usually used when a normal distribution of the population cannot be assumed.

The ranks are then assigned to each data by considering jointly the data of the various groups, using rank 1 for the smallest observation and rank N for the largest observation (N = total number of observations). In the event of a tie, the average rank is assigned among those that the observations would have had had there been no draws (94).

Friedman test

The Friedman test is a non-parametric statistical test developed by Milton Friedman. Similar to the parametric repeated measures ANOVA, it is used to detect differences in treatments across multiple test attempts. The procedure involves ranking each row (or block) together, then considering the values of ranks by columns. The Friedman test is used for one-way repeated measures analysis of variance by ranks. In its use of ranks it is similar to the Kruskal–Wallis one-way analysis of variance by ranks (95).

3.6.4 Correlation between two variables

The correlations between two variables were obtained by carrying out the Pearson correlation test. This test, also known as the bivariate correlation test or r test, expresses a possible linearity relationship between said variables (96).

Given two statistical variables X and Y, Pearson's correlation test produces a correlation index, defined as the covariance of the two variables divided by the product of the standard deviations of the two variables:

$\rho XY = \sigma XY / \sigma X \sigma Y$

This coefficient always assumes values between -1 and 1.

In practice, various "types" of correlation are distinguished:

If $\rho XY > 0$, the variables X and Y are directly correlated, or positively correlated.

If $\rho XY = 0$, the variables X and Y are said to be intercorrelated.

If $\rho XY < 0$, the variables X and Y are said to be inversely correlated, or negatively correlated.

Furthermore, for the direct correlation (and similarly for the inverse one) we distinguish:

A weak correlation if $0 < |\rho XY| < 0.3$.

A moderate correlation if $0.3 < |\rho XY| < 0.7$.

A strong correlation if $|\rho XY| > 0.7$.

Simple Linear Regression

Linear regression analysis is a technique that allows you to analyze the linear relationship between a dependent variable (also called response variable) and one or more independent variables or predictors (quantitative or dichotomous).

In the analysis of simple or bivariate regression, we have only one independent variable, on which the dependent variable "regresses". The analysis of simple linear regression identifies the straight line that best interpolates the points defined by the joint distribution of the two variables and therefore allows to better predict the values of the dependent variable starting from those of the independent variable. If the relationship between X and Y were perfect, for each observation on X there would be one and only one observation on Y. In reality, the relationships between the variables are not perfect, so an error term (or residual) for each case (97).

The equation of the regression line is given by $y = \alpha + \beta x + \varepsilon$

With:

Y = dependent variable

α = intercept

β = angular coefficient

x = independent variable

ε = error

4. RESULTS

4.1 Patient Features

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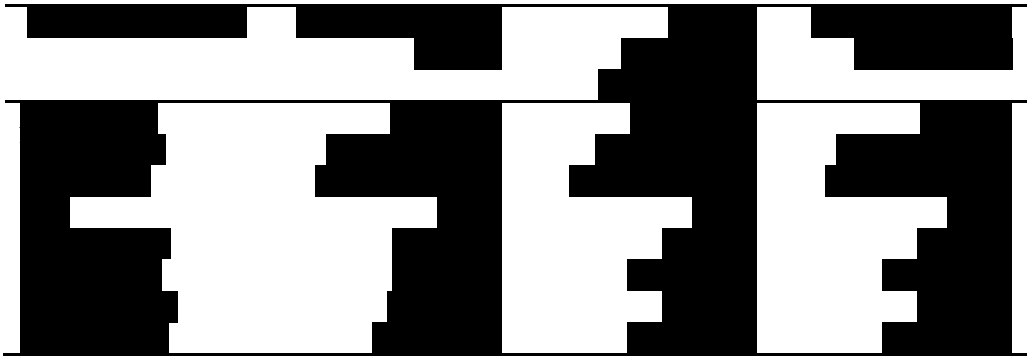

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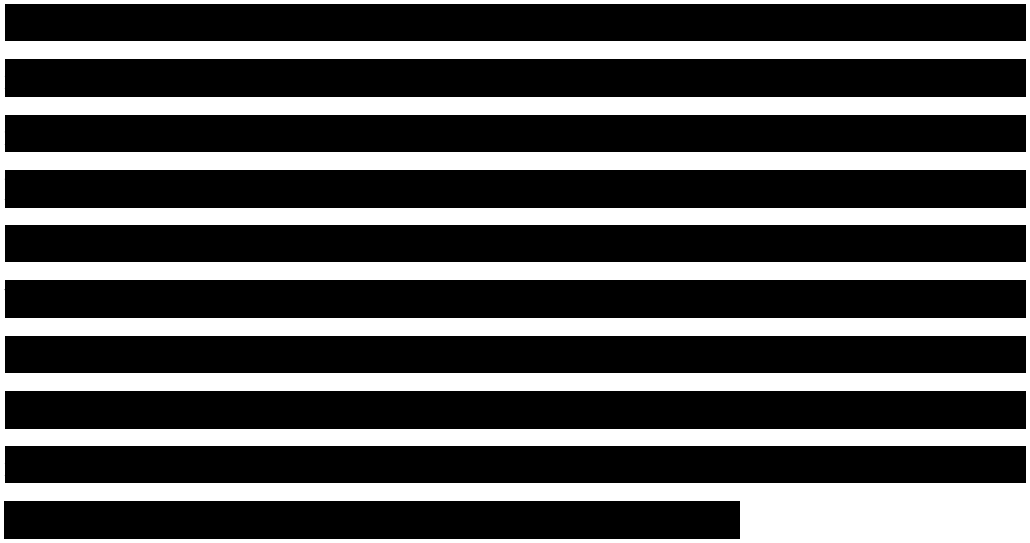

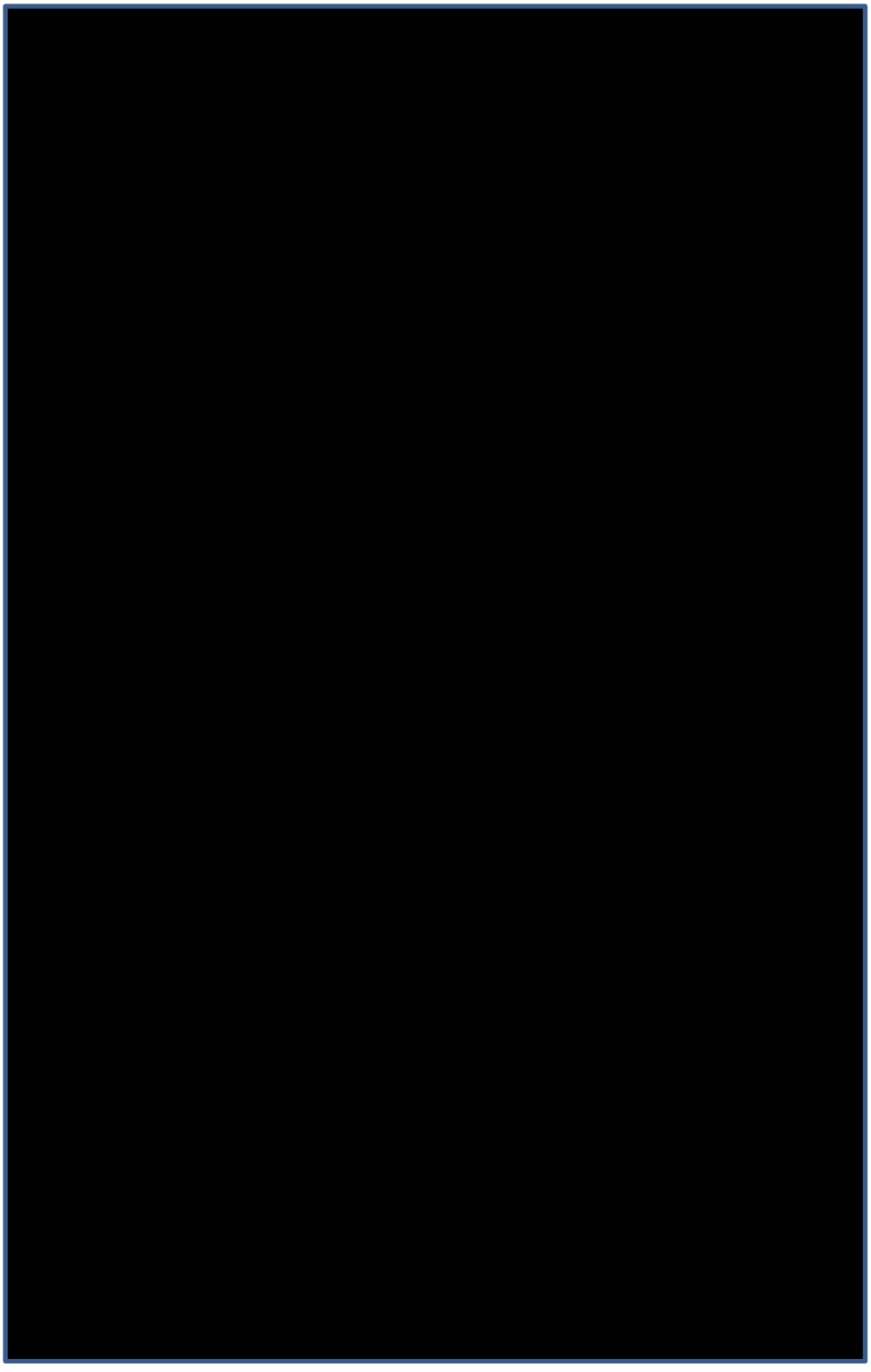

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5. DISCUSSION

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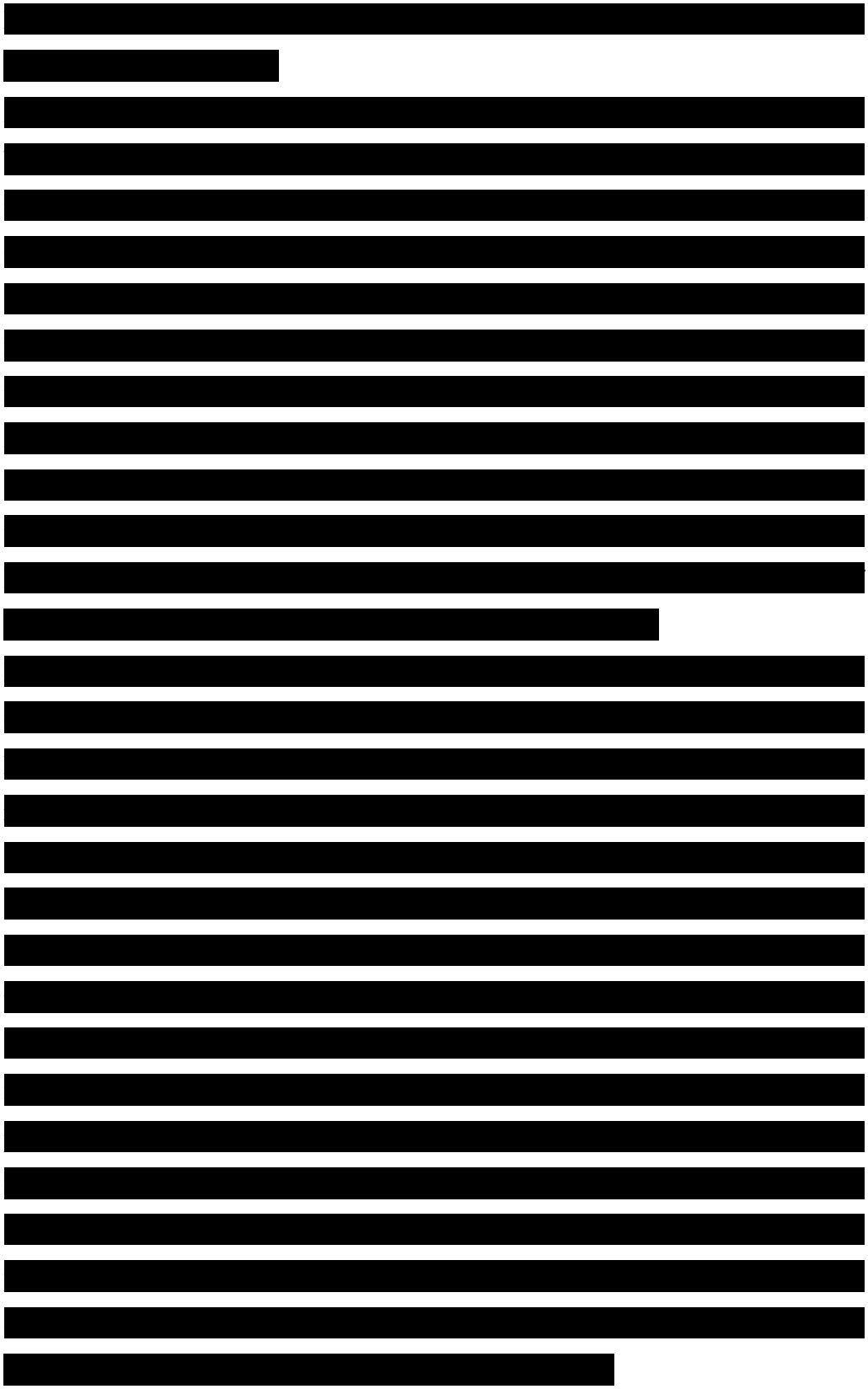

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